Nanograting structure promotes lamellipodia-based cell collective migration and wound healing

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***Abstract*— Wound healing is a dynamic and complex process of replacing missing or dead cell structures and tissue layers. The aim of this research is to discover biocompatible materials and drugs that can promote cell migration in the wound area and thus enhance desirable wound healing effects. In this paper, we report that PDMS nanogratings could accelerate the migration of epithelial cells along the grating axis, and the addition of Imatinib could further increase the epithelial cell wound healing speed to 1.6 times the speed of control cells. We also demonstrate that this migration is mediated by lamellipodia protrusion, and is Rac1-GTPase activity dependent. Lastly, we discuss the potential application and prospect of different nanostructured biomaterials for wound healing studies.**

I. Introduction

Wound healing is a current focus for both medical practice and research in tissue engineering. After injury, epithelial cells rapidly migrate to the vicinity of the wound to close the wound; in a subsequent process, fibroblasts move into the wound, and produce extracellular matrix (ECM) proteins (such as collagens) to re-establish the ECM layer, and form granulation tissue. Researchers are interested in the early event -- from a few hours to one or two days after the injury -- when epithelial cells move toward the wound to form a barrier between the wound and the environment, thereby preventing bacterial infection and interstitial fluid outflow. In the past, diverse efforts have been undertaken to promote wound healing effect: (i) discovery of drugs, growth factors, or cytokines to induce epithelial cell proliferation and migration [1-2]; (ii) development of biocompatible materials as a substrate for epithelial cell movement and wound closure [3-4]. Recently, with the development of microfabrication technology, a number of new materials with features at the micro- or nano-scale have been proposed to accelerate the migration of epithelial cells and thus promote the wound healing process [3-4]. Here we report that by using nanopatterned PDMS and a drug called "Gleevec"(Imatinib) we successfully stimulated the migration of epithelial cells towards the wound, and significantly improved the epithelial wound healing efficiency.

Research is supported by NIH UH2TR000505; and AOSpine Foundation.

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II. Material and Methods

A. Fabrication of nano-grating PDMS substrate

The nanostructures we designed had a width of 350nm, a pitch of 700nm, and depth of 280nm [5-6]. The fabrication of this nano-grating PDMS is shown schematically in Figure 1A. We first used electron beam lithography (EBL) to write on spin-coated poly(methylmethacrylate) (PMMA) thin film to produce a silicon wafer with the above features. This mold was then used to make PDMS nano-gratings. PDMS base and crosslinker (9:1 ratio) were mixed and poured onto the mold. After casting, PDMS was cured by heating it on a hot plate at 75°C for an hour. The PDMS was then cooled down and peeled off from the mold. The collagen coated PDMS nanogratings promoted cell attachment and migration. Figure 1B shows two cells migrating across the nanogratings. The nanostructure (350nm linewidth, 700nm pitch) on PDMS was confirmed with microscopy (Figure 1C).

B. PDMS surface coating

PDMS sheets were plasma treated and rinsed with 70% ethanol followed by autoclaved water. PDMS was then coated with 0.01N HCl + 300ug/ml collagen for 30min and rinsed twice with PBS (Ph7.4) before usage.

C. Cell culture

Both Madin-Darby Canine Kidney (MDCK) cells and NBT-II (Nara Bladder Tumor) cells from ATCC were cultured in DMEM/F12 (Invitrogen) with 10% FBS (GIBCO) and 100 unit of Penicillin/Streptomycin. Inhibitors: Abl family inhibitor: Imatinib (20μM) (Novartis, Switzerland), Rac1 inhibitor: RSC23766 (50μM) (Millipore).

D. Wound healing assay

Cells were cultured to confluence on PDMS substrates in 35 mm dishes. Cells were then rinsed with PBS and starved in low serum media (1.5 ml; 0.5% - 1% serum in DMEM) overnight. Using a sterile 200μl pipet tip, a wound was scratched through the confluent cell layer. PBS was used to rinse the cells and then replaced with 2.5 mL of media containing additives indicated in each group. The wound regions were imaged at 0, 6, 12 and 18 hours post wound formation.

E. Imaging

Differential Interference Contrast and phase contrast imaging were carried out on an Olympus IX81 inverted microscope equipped with a 10× objective. Images were captured using an air-cooled SensiCam QE CCD camera (Cooke Corp., Romulus, MI) driven by Metamorph (Molecular Devices/Meta Imaging, Downingtown, PA).

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Confocal imaging was performed with an inverted Olympus FV1200 equipped with a live cell chamber and a 40× silicone oil N.A. 1.25 objective. Cell protrusion was tracked with time-lapse images taken at 10-second intervals for 20 minutes.

F. Data Analysis

The cell migration was tracked using ImageJ "cell tracking" (using the ImageJ plugins: "Manual Tracking Plug-in" (http://rsbweb.nih.gov/ij/plugins/track/track.html)). The trajectories for each migrating cell were recorded and plotted by Origin. Cell migration speed was then calculated. The cell migration speed and directionality among different groups were then analyzed and compared with Origin 6.0 using a one-way ANOVA test with the Bonferroni post hoc test.

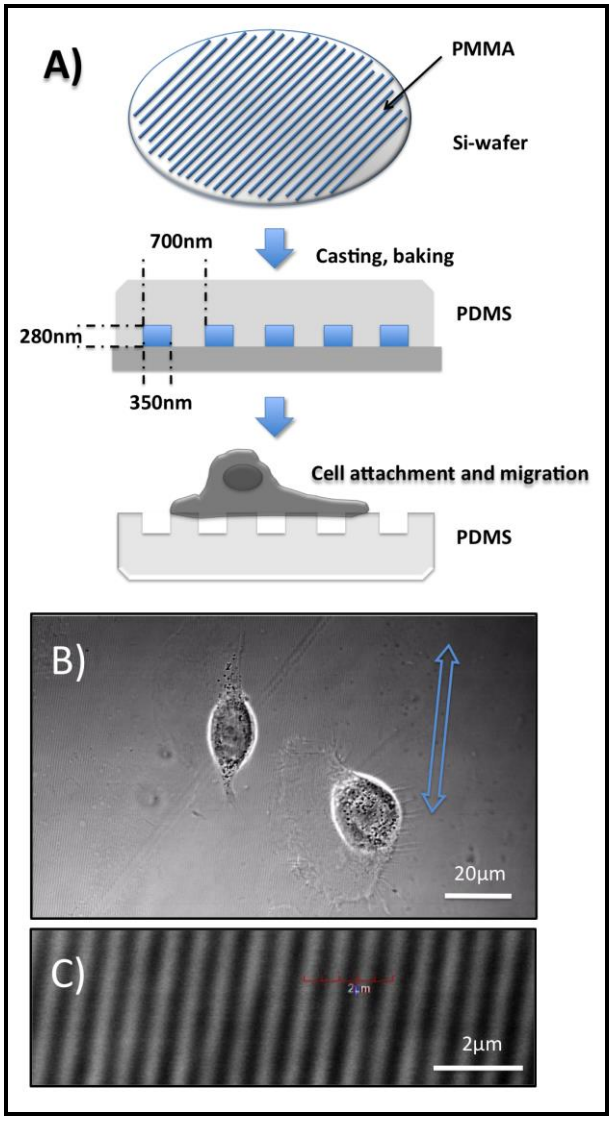

Figure 1. A) The fabrication of nanopatterned PDMS substrate. Electron beam lithography (EBL) was used to write on spin-coated poly(methylmethacrylate) (PMMA) thin film to produce a silicon wafer with grating features of 350nm width, 700nm pitch, and 280nm depth. This mold was then used to make PDMS nano-gratings. B) The collagen coated PDMS nanogratings promote cell attachment and migration. Two NBT-II cells are seen migrating on the nanogratings. NBT-II cells on the nanogratings form lamellipodia protrusions, and crawl across the nanogratings.

III. Results

To measure the effect of nanopatterned PDMS substrates on cell migration, we used an *in vitro* cell wound healing assay. MDCK cells were cultured on PDMS with or without nanograting structures. A wound that perpendicular to the nanograting direction was introduced to the confluent MDCK cell layer. .Wound healing assay results showed that MDCK cells on PDMS surfaces with parallel nanograting structures migrated faster towards the wound than the smooth PDMS control group (Figure 2A, 2B). After 12 hr, the wound on PDMS surfaces with nanogratings were almost fully healed by MDCK cells, while cells on control PDMS surface fully healed at ~18 hr. At 6, and 12 hr, the MDCK cells on nanogratings had closed the gap faster than those cultured on smooth PDMS surfaces. (Fig2A, 2B)

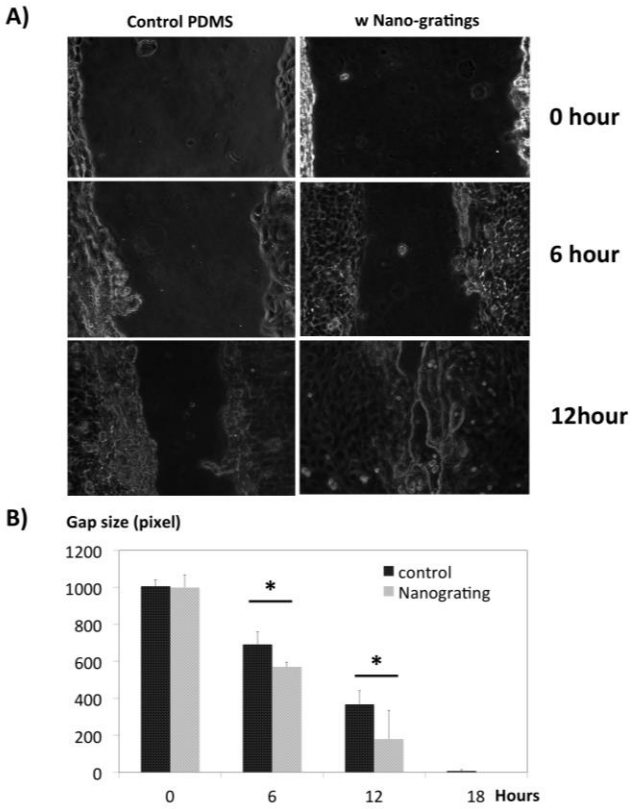

Figure 2. Wound healing assay shows that nanogratings, which are parallel to the wound edge, promote MDCK cell migration. A) The wound healing assay imaged at the 0-hour, 6-hour and 12-hour time points. B) The gap distance measured at different time points. MDCK cells on nanograting substrates migrate faster than cells on control PDMS surfaces at each time point. At the 18-hour time point, cells in both groups fully close the wound. * p-value is < 0.05 by student t-test (n=5).

MDCK cells constantly form protrusions during their wound healing process. However, because the cells were cultured on PDMS and imaged with a 10X objective using phase contrast mode, the detailed cell structures, i.e. cell leading edge protrusions, could not easily be observed. Using confocal microscope with a 40X silicon oil high working distance objective, we took videos to examine the leading edge of the MDCK cells that faced the wound. With this setup, a significant amount of cell protrusion could be

observed (Fig. 3A). The protrusions were very dynamic. Most of the protrusions exhibited sheet-like extensions -- often referred to as lamellipodia, and other protrusions exhibited finger-like extensions -- often referred to as filopodia. In Figure 3A we marked those cells with lamellipodia protrusions with "*". The majority of the MDCK cells facing the wound formed lamellipodia protrusions. We used kymographs to display and compare the lamellipodia protrusion in MDCK cells (Data not shown). By comparison, we found that MDCK cells on nanogratings had faster lamellipodia protrusions than the cells on control PDMS surfaces.

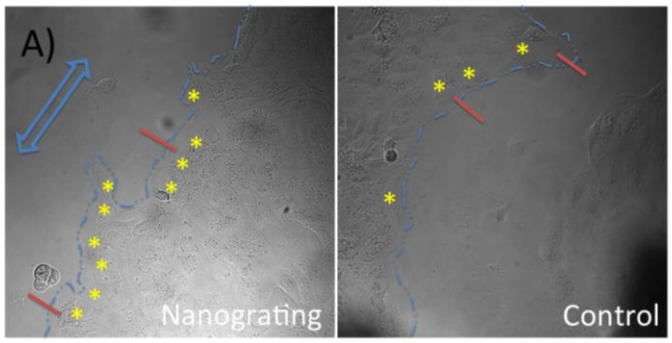

Figure 3. Measurement of lamellipodia protrusions at the leading edge of MDCK cells. A) The arrow shows the grating direction, and the dotted line indicates the rim of the cell sheet. Stars indicate the cells with lamellipodia structures. The red line is where we measured the kymograph. MDCK cells on nanogratings had faster lamellipodia protrusions than the cells on control PDMS surfaces (seen by the slope of the kymograph).

Lamellipodia protrusion is dynamically regulated by Rho family GTPases ---- mostly through Rac1 GTPases [7-8]. Inhibition of Rac1 activity may significantly inhibit cell lamellipodia protrusion and thus affect wound healing. To test this, we added Rac1 inhibitors (RSC23766, 50μM) to the culture medium and found that MDCK cell wound healing on nanopatterned substrates was significantly decreased (Figure 4). This indicates that Rac1 GTPase activity is important for the MDCK cell wound healing behavior.

We have previously reported that Imatinib (Novartis) could promote lamellipodia protrusion and cell migration [8]. Therefore, we suspect that Imatinib may positively affect the MDCK wound healing, since MDCK cell migration is also lamellipodia-dominated. To test this hypothesis, we added Imatinib at time 0 to MDCK cells on the nanogratings. At a concentration of 20μM, Imatinib stimulated the MDCK cells on nanogratings to close the wound at ~ 1 μm/min, which is about a 60% increase over the control group (Figure 4). There is no statistical significance between the first 6 hr vs the second 6 hr in all the groups.

In summary, the above results from the Rac 1 inhibitor and lamellipodia promoter, Imatinib, illustrate that the wound healing behavior (collective migration) of MDCK cells is mainly dictated by lamellipodia via Rac1 activity.

IV. Discussion

The cell wound healing assay has been widely used as a quantitative assay to study cell migration in tissue engineering. Here we used this assay and epithelial MDCK cells to test the nanostructure's effect on cell migration.

Lamellipodia protrusion and actin arc-shaped bundles (stress fibers) at the wound edge are two of the most important factors to induce wound healing [9-10]. In our study, we see both lamellipodia protrusion and actin arc bundles in MDCKs. Interestingly, we found that the more the lamellipodia protruded, the faster the wound was healed. This observation is consistent with previous research results, which reported that lamellipodia protrusions provide traction forces that pull the cells to move towards the wound [9-10].

Gleevec (Imatinib), an Abl family kinases inhibitor, could promote lamellipodia formation in an epithelial cell-derived tumor cell line (NBT-II) [8]. Collectively migrating MDCK cells have similar lamellipodia protrusion as NBT-II cells. Therefore, we treated MDCK cell-sheets with Imatinib, and found that it did positively affect MDCK cell-sheet lamellipodia formation and migration. Though the effects of Imatinib on other cell types remain to be determined, the results here suggested that Imatinib could have a positive effect on inducing lamellipodia formation in epithelial cells and maybe also in other cells, i.e. keratinocytes, if their wound healing is also lamellipodia dependent.

We also inhibited the lamellipodia formation, and found that the collective-epithelial cell migration speed decreased. Nobes et al. reported the function of small RhoGTPase in regulating cell protrusion and migration: Rac1 GTPase can promote lamellipodia protrusion. Our results validated their findings -- the presence of Rac1 inhibitors impaired the lamellipodia formation, and reduced the whole collective-MDCK cell layer's moving speed.

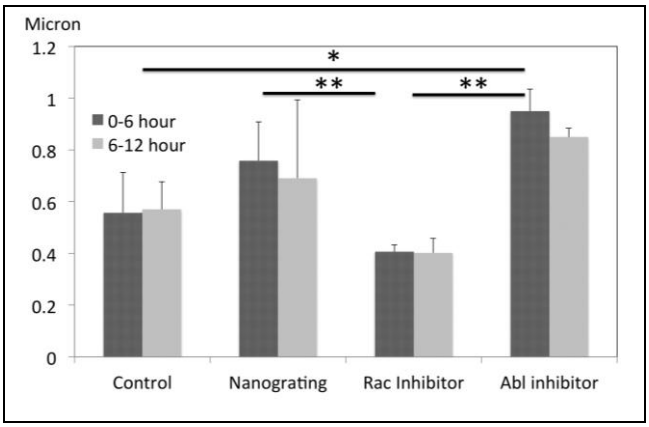

Figure 4. Both Rac 1 activity and lamellipodia structure are essential for MDCK wound healing. The promotion of lamellipodia further increased the speed of MDCK cell migration ---- at about 1.6 fold ---- compared to cells on control PDMS sheets. * p-value < 0.05 ** p-value < 0.01 by one-way ANOVA test with the Bonferroni post hoc test (n=5 for each experiment).

Nanograting structures may affect cell adhesion pattern. Cells on PDMS nanogratings have been reported to form elongated adhesions along the nanogratings [5-6]. The MDCK cells also form adhesions along the grating structure (data not shown). Parker et al. reported that pre-designed shape of substrates could induce changes in cell adhesion patterns, actin dynamics, and cell internal force distribution [11]. Therefore, we hypothesize that nanogratings structures may also affect the internal forces and actin dynamics (e.g. lamellipodia protrusion) in collective migrating MDCK cells, thus modulate their migration speed.

Crawling cells with constant lamellipodia protrusions usually have different force patterns than cells with filopodia protrusion [8, 12-13]. For instance, the majority of internal traction forces and adhesions in fish/amphibian keratinocytes, or in Imatinib treated NBT-II cells, are perpendicular to their migration direction. These cells do not follow the classic migration cycle -- protrusion at the front side and retraction at the backside. Alternatively, they constantly disassemble their cytoskeleton structures on their side and wing regions [8, 12-13]. Collective migrating MDCK cells also form lamellipodia protrusion at their leading edge, thus their internal force pattern could be similar to fish keratinocytes or NBT-II cells. As the orientation of the nanogratings is perpendicular to the cell-sheet migration direction, MDCK cells can easily form aligned/patterned adhesions, which may promote MDCK collective migration. In contrast, fibroblasts or human mesenchymal stem cells prefer to migrate along the nano-gratings; their migration speed decreases if they crawl across the nano-gratings [6, 14].

The above evidence suggests that certain migrating cells may require special patterned substrate to reach a fast migration speed. Therefore, topographical features of a substrate or membrane may be a design parameter to optimize wound healing.

V. Conclusion

In summary, here we reported that the use of PDMS nanogratings could accelerate the migration of epithelial cells toward an *in vitro* wound edge, and addition of Imatinib could further accelerate the wound healing speed up to 60% faster than control cells. We demonstrated that this migration is dominated by lamellipodia protrusion and is Rac1 dependent.

This study advances our knowledge in epithelial cell-nanotopography interactions. However, the behavior of other cell types (i.e. fibroblast, epithelium, neuron) on different nano-patterned materials (i.e. with features of corners, angles or lines with different width) could be very different and is still largely unknown. Therefore, further research is required to discover what configuration can promote and maximize certain cell's migration.

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