Stamp Wound Assay for Studying Coupled Cell Migration and Cell Debris Clearance

Jiyeon Lee,[†] Yu-Lin Wang,[‡] Fan Ren,[†] and Tanmay P. Lele*,[†]

[†]*Department of Chemical Engineering, University of Florida, Gainesville, Florida 32611, United States, and* [‡]*Institute of Nanoengineering and Microsystems, National Tsing Hua University, Hsinchu, Taiwan 30013*

A new method for studying wound healing under realistic conditions in vitro was developed. The method involves creating defined patterns of damaged cell debris with poly(dimethyl)siloxane (PDMS) stamping. This novel assay permitted the quantification of wound healing rates in the presence of cell debris. Experimental results with this assay suggest that cell migration in the presence of cell debris is a two step process requiring (1) non-muscle myosin II-dependent cell clearance followed by (2) cell migration into newly cleared wound areas. The novel stamp wound assay allows the study of coupled cell migration and debris clearance and is a more realistic wound healing assay in vitro.

Introduction

Wound healing is a complex process that is critical for preserving the integrity of multicellular organisms and tissue homeostasis.[1] The process involves the migration of cells of different types directed by chemotactic signals into the wound. Traditional in vitro models of wound healing[2−4] involve scratching a confluent cell monolayer with a microneedle or micropipet tip, and capturing the time-dependent closure of the cell-free wounded area with microscopy. Such studies have allowed the discovery of key signaling pathways that control the migration of cells during wound closure.[5−7] One limitation of the scratch wound assay is that it lacks precision for creating a controlled wound. Alternative assays to create wounds have been recently reported that use laser photoablation[8] or masks to prevent cell adhesion to defined "wound" areas.[9−11] While these techniques offer more reliable models to study wound healing, they all share the common feature that the wound area is devoid of any cells. However, wound healing in the body involves not only the migration of cells into the wound but also the simultaneous clearing of cell debris. The clearing of cell debris is performed by both professional phagocytes (such as macrophages) and nonprofessional phagocytes (such as epithelial cells).[12−16] Realistic assays that allow study of the coupled process of wound healing and phagocytosis of cell debris are therefore desirable.

Here, we report a new technique to make wounds on an epithelial cell monolayer using a stamping technique. The method involves the physical contact of a soft mold with raised features onto confluent epithelial cells. With this method, we successfully created well-defined wounds with dead cell debris in the wound area. Imaging over several hours showed that the cells migrate into the wound after first clearing the wound area of cell debris. The clearing process is remarkably efficient with no trace of debris detectable after clearance. The rate of wound closure in the presence of cell debris was found to be comparable to that in the absence of cell debris. Interestingly, the inhibition of non-muscle myosin II with blebbistatin slowed the healing of the wound in the presence of cell debris, but was unable to inhibit wound healing without dead cells.

Experimental Methods

Fabrication of Poly(dimethyl)siloxane (PDMS) Molds. A master mold was fabricated by a conventional photolithography method. Prepolymers of soft PDMS (Sylgard 184, Dow Corning, MI) were poured over the photoresist master mold and degassed

*To whom correspondence should be addressed. E-mail: tlele@che.ufl.edu. Telephone: (352) 392-0317. Fax: (352) 392-9513.

(1) Shaw, T. J.; Martin, P. Wound repair at a glance. *J. Cell Sci.* **2009**, *122*(Pt 18), 3209–3213.

(2) Pinco, K. A.; He, W.; Yang, J. T. alpha4beta1 integrin regulates lamellipodia protrusion via a focal complex/focal adhesion-independent mechanism. *Mol. Biol. Cell* **2002**, *13*(9), 3203–3217.

(3) Yarrow, J. C.; Perlman, Z. E.; Westwood, N. J.; Mitchison, T. J. A high-throughput cell migration assay using scratch wound healing, a comparison of image-based readout methods. *BMC Biotechnol.* **2004**, *4*, 21.

(4) Wang, E.; Zhao, M.; Forrester, J. V.; McCaig, C. D. Electric fields and MAP kinase signaling can regulate early wound healing in lens epithelium. *Invest. Ophthalmol. Visual Sci.* **2003**, *44*(1), 244–249.

(5) Rodriguez, L. G.; Wu, X.; Guan, J. L. Wound-healing assay. *Methods Mol. Biol.* **2005**, *294*, 23–29.

(6) Lo, C. M.; Keese, C. R.; Giaever, I. Monitoring motion of confluent cells in tissue culture. *Exp. Cell Res.* **1993**, *204*(1), 102–109.

(7) van der Meer, A. D.; Vermeul, K.; Poot, A. A.; Feijen, J.; Vermes, I. A microfluidic wound-healing assay for quantifying endothelial cell migration. *Am. J. Physiol.* **2009**, *298*(2), H719–725.

(8) Tamada, M.; Perez, T. D.; Nelson, W. J.; Sheetz, M. P. Two distinct modes of myosin assembly and dynamics during epithelial wound closure. *J. Cell Biol.* **2007**, *176*(1), 27–33.

(9) Block, E. R.; Matela, A. R.; SundarRaj, N.; Iszkula, E. R.; Klarlund, J. K. Wounding induces motility in sheets of corneal epithelial cells through loss of spatial constraints: role of heparin-binding epidermal growth factor-like growth factor signaling. *J. Biol. Chem.* **2004**, *279*(23), 24307–24312.

(10) van Horssen, R.; Galjart, N.; Rens, J. A.; Eggermont, A. M.; ten Hagen, T. L. Differential effects of matrix and growth factors on endothelial and fibroblast motility: application of a modified cell migration assay. *J. Cell. Biochem.* **2006**, *99*(6), 1536–1552.

(11) Poujade, M.; Grasland-Mongrain, E.; Hertzog, A.; Jouanneau, J.; Chavrier, P.; Ladoux, B.; Buguin, A.; Silberzan, P. Collective migration of an epithelial monolayer in response to a model wound. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*(41), 15988–15993.

(12) Kovalchin, J. T.; Wang, R.; Wagh, M. S.; Azoulay, J.; Sanders, M.; Chandawarkar, R. Y. In vivo delivery of heat shock protein 70 accelerates wound healing by up-regulating macrophage-mediated phagocytosis. *Wound Repair Regener.* **2006**, *14*(2), 129–137.

(13) Witte, M. B.; Barbul, A. General principles of wound healing. *Surg. Clin. North. Am.* **1997**, *77*(3), 509–528.

(14) Birge, R. B.; Ucker, D. S. Innate apoptotic immunity: the calming touch of death. *Cell Death Differ.* **2008**, *15*(7), 1096–1102.

(15) Parnaik, R.; Raff, M. C.; Scholes, J. Differences between the clearance of apoptotic cells by professional and non-professional phagocytes. *Curr. Biol.* **2000**, *10*(14), 857–60.

(16) Fullard, J. F.; Kale, A.; Baker, N. E. Clearance of apoptotic corpses. *Apoptosis* **2009**, *14*(8), 1029–1037.
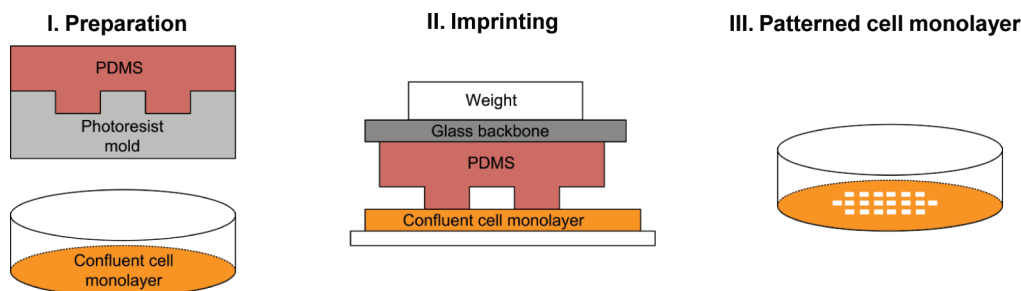
I. Preparation

PDMS

Photoresist mold

Confluent cell monolayer

II. Imprinting

Weight

Glass backbone

PDMS

Confluent cell monolayer

III. Patterned cell monolayer

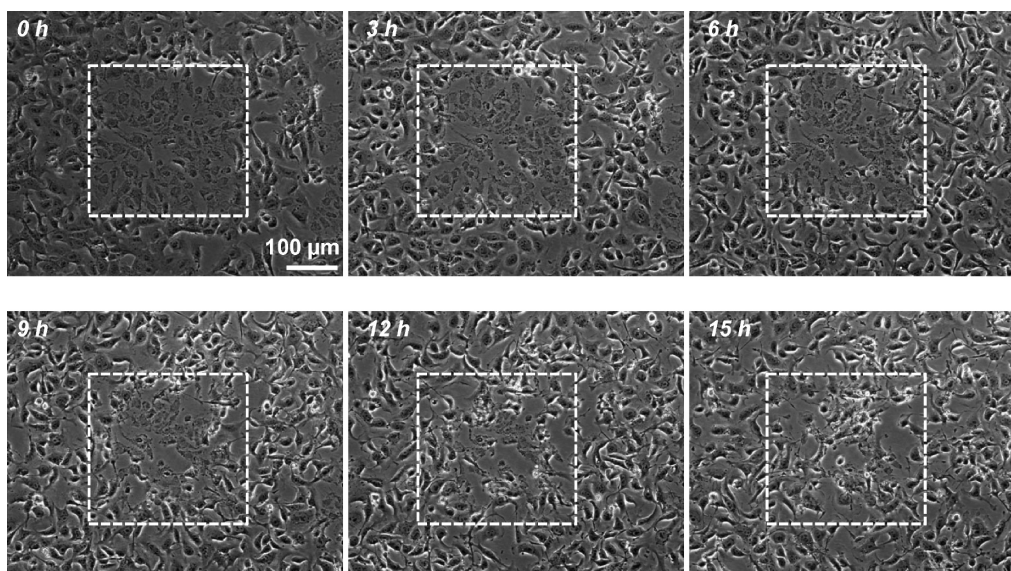**Figure 1.** Schematic diagram of stamp wound assay.

Figure 2. Time lapse images of stamped Het1A cells. Neighboring cells (outside of dashed line) started migrating into patterned cells (inside of dashed line) after removing the stamp. Stamping caused flattened cells (stamped wound).

for 20 min in a vacuum and then cured at 60 °C in an oven for 2 h. After peel off, the PDMS mold was sterilized with 70% ethanol and washed several times with sterilized DI water. Any remaining solvent and prepolymer was removed by baking the PDMS mold attached to a glass slide at 120 °C in an oven for 2 h.

Cell Culture and Soft Imprinting with the PDMS Mold. Human esophageal epithelial cells (Het1A) were cultured in LHC-9 medium (Invitrogen, Eugene, OR) supplemented with 5% donor bovine serum (DBS) (Gibco, Grand Island, NY). Cells were passed to glass bottom dishes (MatTek, Ashland, MA). When cells were confluent, the fabricated PDMS mold was placed on the cell monolayer with an 86.3 g weight on top. After 20 min, the weight and the PDMS mold were carefully removed from the cell culture dish.

Time-Lapse Microscopy. After imprinting the cell monolayer with a PDMS mold, cell culture dishes were washed with phosphate-buffered saline (PBS) once for removing nonadherent cells and new media was added to the dish. Phase contrast imaging was performed for 18 h on the Nikon TE 2000 microscope with a humidified incubator (In Vivo Scientific, St. Louis, MO). Images were collected every 5 or 10 min using 10×, 20×, and 60× objectives.

Cell Viability Assay. The live/dead viability/cytotoxicity kit for mammalian cells (Invitrogen, Eugene, OR) was used for observing live and dead cells. Stamped cells were washed with PBS once and incubated with 4 μM calcein AM and 4 μM ethidium homodimer-1 (EthD-1) for 30−45 min. After washing

with PBS, new media was added to the dish. Cells were imaged with time lapse microscopy as described above.

Stress Fiber and Nuclei Staining. Cells were stained for F-actin and the nucleus using previously published methods.[17] Briefly, cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. F-actin was stained with phalloidin conjugated with Alexa Fluor 594 (Invitrogen, Eugene, OR), and the nucleus was stained with 4′-6-diamidino-2-phenylindole (DAPI) (Sigma Aldrich, St. Louis, MO).

Blebbistatin Assay. Imprinted cells were washed with PBS once, and 5 μM blebbistatin (Calbiochem, San Diego, CA) in cell media was added to the dishes. Images were collected every 10 min using a 10× objective for 18 h on the Nikon TE 2000 microscope. The area of the wound was measured with Image J, and the time-dependent wound closure ratio was pooled and averaged.

Results

We utilized the soft imprint technology to stamp a wound on a confluent monolayer of cultured cells. Figure 1 shows the experimental scheme. Briefly, PDMS prepolymers were poured on the photoresist master mold and then cured. The raised features on the PDMS were square in shape with a size of 250 μm. Brief imprinting of the PDMS mold on the confluent human epithelial cell monolayer caused cells to have a flattened morphology in the stamped area (Figure 2). On tracking the wounded areas for several hours, cells were observed to heal the wound at 15 h (Figure 2).

We next stained cells to measure viability of the stamped cells. All the cells in the stamped area were found to be dead with

(17) Lee, J.; Chu, B. H.; Chen, K. H.; Ren, F.; Lele, T. P. Randomly oriented, upright SiO2 coated nanorods for reduced adhesion of mammalian cells. *Biomaterials* **2009**, *30*(27), 4488–4493.
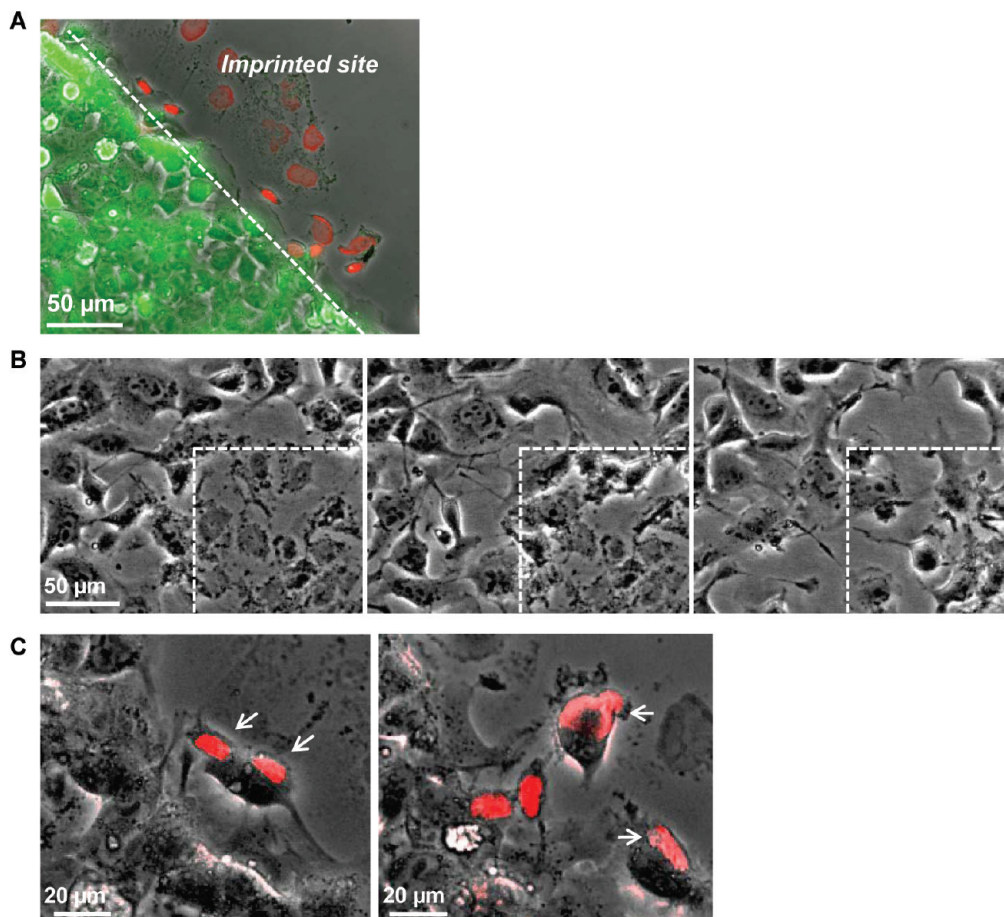
DOI: 10.1021/la103542y 16673

Figure 3. Epithelial cells migrate to the wound after first clearing dead cells. (a) Overlaid image of phase contrast and fluorescent microscopic images (red fluorescence indicates dead cells; green fluorescence indicates live cells). (b) Phase contrast images of healing of stamped Het1A cells. Live cells can be seen to clear neighboring dead cells. Dashed line indicates the boundary of stamped cells. (c) Overlaid images of phase contrast and fluorescent microscopic images during the healing process. White arrows mark live cells ingesting red fluorescent, dead cell debris.

predominant nuclei, suggesting that the brief stamping caused substantial damage to the cell (red fluorescent nuclei in Figure 3A). Cells in the stamped area did not display significant F-actin staining although they had prominent misshapen nuclei (Figure 1S in the Supporting Information). This suggests that the stamping process caused substantial damage to the cytoplasm and greatly deformed the nucleus. Time lapse images further revealed that the dead cell debris (inside of dashed line in Figure 3B) was "cleared" by inwardly migrating cells. Cells were observed to clear dead cell debris first and then migrate further into the wound (Figure 3B). The clearance process was remarkably efficient with no traces of the debris visible on the dish (see the movie in the Supporting Information). Overlaid images of phase contrast and fluorescent images in Figure 3C show that cleared dead cells were engulfed by neighboring live cells.

To create "empty" wounds, the PDMS mold was imprinted on the cell monolayers, shifted laterally while maintaining contact with the cells and then peeled off from the confluent cells. On lateral shifting, the stamped cells were found to be removed and clean wounds were created (Figure 4A). The rate of wound closure in wounds without dead cells was comparable to that measured in the "stamped wound" with dead cells (Figure 4B).

Given that wound closure in the stamp-wound assay is accompanied by the clearance of dead cell debris, we hypothesized that this process may require contractile forces generated by the

inwardly migrating cells. We therefore stamped cells and then added blebbistatin (5 μM) which inhibits non-muscle myosin II specifically.[18] Upon addition, blebbistatin was not washed out to prevent possible recovery of myosin II activity during the slow healing process. Interestingly, cells were not cleared after blebbistatin treatment and the wound remain unhealed even after 15 h (Figure 5). However, wound healing without dead cells was only slightly slowed in the presence of blebbistatin (Figure 6). These results suggest a fundamental requirement for actomyosin contractility in wound closure in the "stamped wound" assay in contrast to the conventional wound healing assay.

Discussion

Soft imprinting technology is widely used for fabricating patterns at the nano- and micrometer scale.[19] This method has been used to pattern extracellular matrix proteins (such as fibronectin) on the substrate and confine cell adhesion on individual protein islands.[20−22] Here, we used a similar soft imprinting method to create wounds

(18) Russell, R. J.; Xia, S. L.; Dickinson, R. B.; Lele, T. P. Sarcomere mechanics in capillary endothelial cells. *Biophys. J.* **2009**, *97*(6), 1578–1585.
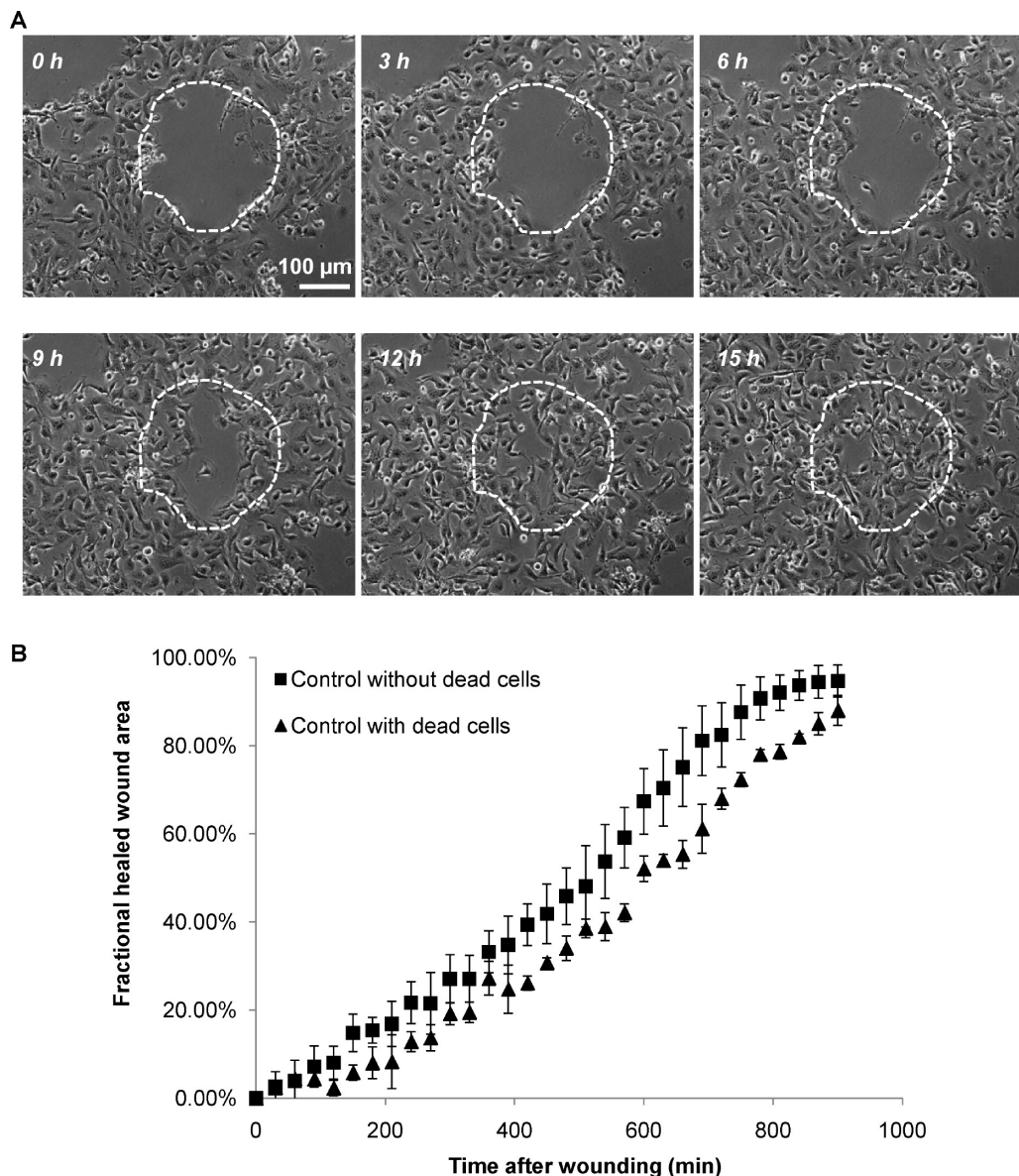
(19) Xia, Y. N.; Whitesides, G. M. Soft lithography. *Angew. Chem., Int. Ed.* **1998**, *37*(5), 551–575.

(20) Brock, A.; Chang, E.; Ho, C. C.; LeDuc, P.; Jiang, X. Y.; Whitesides, G. M.; Ingber, D. E. Geometric determinants of directional cell motility revealed using microcontact printing. *Langmuir* **2003**, *19*(5), 1611–1617.

(21) Kane, R. S.; Takayama, S.; Ostuni, E.; Ingber, D. E.; Whitesides, G. M. Patterning proteins and cells using soft lithography. *Biomaterials* **1999**, *20*(23−24), 2363–2376.

(22) Whitesides, G. M.; Ostuni, E.; Takayama, S.; Jiang, X.; Ingber, D. E. Soft lithography in biology and biochemistry. *Annu. Rev. Biomed. Eng.* **2001**, *3*, 335–373.

Figure 4. Wound healing rates of stamped wounds are comparable to wounds without dead cells. (a) Wounds without dead cells are healed on the order of 15 h, similar to rates in stamped wounds. (b) Quantification of wound healing rates. Both wounds were completely closed after 15 h. Bars indicate the standard error of the mean (SEM) (*n* = 3 for each condition).

with dead cell debris. This stamp-wound assay can be used to study both the migration of cells into the wound and simultaneous phagocytosis of dead cell debris in the wound site. Alternative methods to achieve dead cells surrounded by live cells include electric pulses to kill cells locally.[23]

An interesting finding in our study was that the stamped wound did not heal when non-muscle myosin II was inhibited. This is likely due to the fact that cells cannot phagocytose dead cell debris

efficiently in the absence of myosin activity.[24–27] While the surrounding live cells closed the wound partially by migrating into vacant spaces, they were not able to migrate over the cell debris. This supports the claim that the stamped wound assay is a more realistic approach to study wound healing in vitro.

That this assay allowed us to study wound healing in the presence of cell debris is significant because local necrosis of cells causes the release of chemotactic factors locally that influence the directionality of cell migration.[28–30] Therefore stamp-wound

(23) Keese, C. R.; Wegener, J.; Walker, S. R.; Giaever, I. Electrical wound-healing assay for cells in vitro. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*(6), 1554–1559.

(24) Swanson, J. A.; Johnson, M. T.; Beningo, K.; Post, P.; Mooseker, M.; Araki, N. A contractile activity that closes phagosomes in macrophages. *J. Cell Sci.* **1999**, *112*(Pt 3), 307–316.

(25) Tsai, R. K.; Discher, D. E. Inhibition of "self" engulfment through deactivation of myosin-II at the phagocytic synapse between human cells. *J. Cell Biol.* **2008**, *180*(5), 989–1003.

(26) Arora, P. D.; Conti, M. A.; Ravid, S.; Sacks, D. B.; Kapus, A.; Adelstein, R. S.; Bresnick, A. R.; McCulloch, C. A. Rap1 Activation in Collagen Phagocytosis Is Dependent on Nonmuscle Myosin II-A. *Mol. Biol. Cell* **2008**, *19*(12), 5032–5046.

(27) Jaffe, A. B.; Hall, A. Rho GTPases: biochemistry and biology. *Annu. Rev. Cell Dev. Biol.* **2005**, *21*, 247–269.

(28) Hebda, P. A. Stimulatory effects of transforming growth factor-beta and epidermal growth factor on epidermal cell outgrowth from porcine skin explant cultures. *J. Invest. Dermatol.* **1988**, *91*(5), 440–445.

(29) Schor, S. L.; Ellis, I. R.; Harada, K.; Motegi, K.; Anderson, A. R.; Chaplain, M. A.; Keatch, R. P.; Schor, A. M. A novel "sandwich" assay for quantifying chemo-regulated cell migration within 3-dimensional matrices: wound healing cytokines exhibit distinct motogenic activities compared to the transmembrane assay. *Cell Motil. Cytoskeleton* **2006**, *63*(5), 287–300.

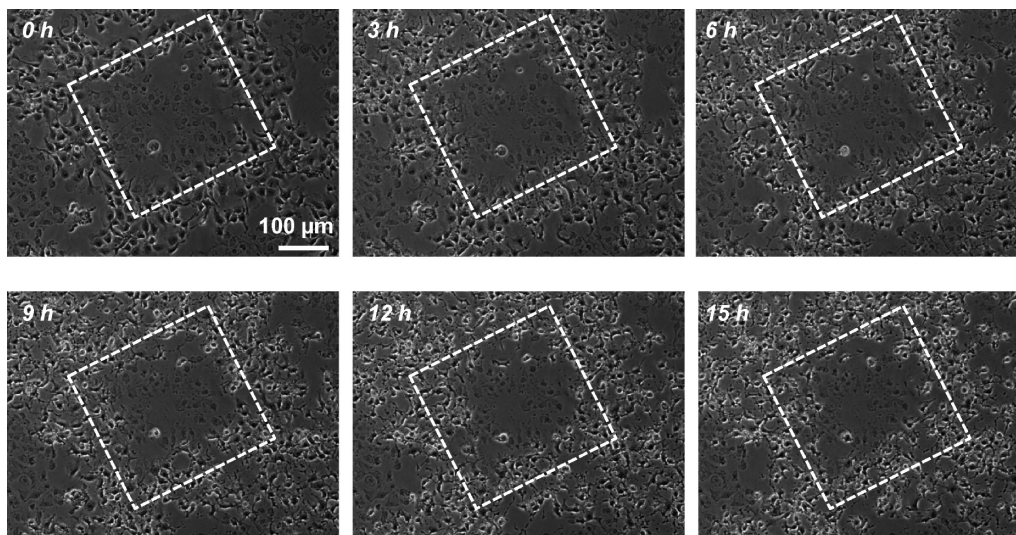(30) Coussens, L. M.; Werb, Z. Inflammation and cancer. *Nature* **2002**, *420*(6917), 860–867.

Figure 5. Time lapse images of stamped Het1A cells with blebbistatin treatment. Stamped wound closure was not completed even after 15 h.
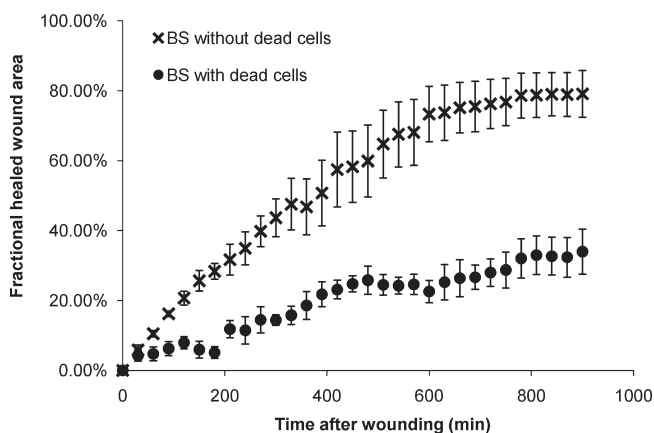

Figure 6. Inhibiting non-muscle myosin II inhibits wound healing in the stamped wound assay but not in wounds without dead cells. Cells were treated with blebbistatin (BS) during the healing process. After 15 h, BS treated cells in the stamped-wound assay did not close the wound (only 30%), while BS treated cells without dead cells completed 80% of wound healing. Bars indicate SEM (n = 4 for each condition). The differences in the rates are statistically significant ($p < 0.05$).

healing may occur not only because of the disruption of cell–cell junctions but also because of a local burst in chemotactic factors. We found that the rate of wound closure in the "stamped wound" with dead cells was comparable to that measured in the conventional wound without dead cells. This is remarkable given that, in the stamped wound, the cells have to clear dead cell debris which includes significantly large objects (on the size scale of the cell). We speculate that chemotactic factors released in the wounded area as a result of stamping may accelerate the migration of surrounding live cells into the wound, thereby resulting in only a small decrease in wound healing rates (despite the additional clearance step). The efficiency with which cells clear cell debris is surprising: no structures are evident on the dish surface once clearance occurs (movie in the Supporting Information). The clearance process itself appears similar to phagocytosis, and it is remarkable that cells continue to migrate and close the wounds with this extra payload at rates only slightly slower than wound assays without dead cells (Figure 4B). These results suggest that this assay could be very useful in studying phagocytosis by macrophages in a co-culture assay with wounded epithelial cells.

Conclusions

A new method for creating realistic wounds in adherent cell monolayers was proposed. The results suggest that cell migration in the stamped-wound assay may involve a complex interplay between chemotaxis, migration due to disruption of cell–cell junctions, and myosin-dependent cell clearance. This novel assay is expected to greatly improve our understanding of the process of wound healing.

Supporting Information Available: Fluorescent images of F-actin and nuclei stained cells in the stamped wound. Phase contrast time lapse movie (avi format) of wound healing in the stamped wound assay (total time of 15 h). This material is available free of charge via the Internet at http://pubs.acs.org.