Dual modes of motility at the leading edge of migrating epithelial cell sheets

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Purse-string healing is driven by contraction of actin/myosin cables that span cells at wound edges, and it is the predominant mode of closing small round wounds in embryonic and some adult epithelia. Wounds can also heal by cell crawling, and my colleagues and I have shown previously that the presence of unconstrained, straight edges in sheets of epithelial cells is a sufficient signal to induce healing by crawling. Here, it is reported that the presence of highly concave edges, which are free or physically constrained by an inert material (agarose), is sufficient to induce formation of purse strings. It was determined that neither of the two types of healing required cell damage or other potential stimuli by using the particularly gentle procedure of introducing gaps by digesting agarose blocks imbedded in the cell sheets. Movement by crawling depends on signaling by the EGF receptor (EGFR); however, this was not required for purse-string contraction. A migrating epithelial cell sheet usually produces finger-like projections of crawling cells. The cells between fingers contain continuous actin cables, which were also determined to contain myosin IIA and exhibit additional characteristics of purse strings. When crawling was blocked by inhibition of EGFR signaling, the concave regions continued to move, suggesting that both mechanisms contribute to propel the sheets forward. Wounding epithelial cell sheets causes activation of the EGFR, which triggers movement by crawling. The EGFR was found to be activated only at straight and convex edges, which explains how both types of movement can coexist at leading epithelial edges.

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Agarose droplets provide inert physical barriers to the epithelial cells (23), and to test whether edges of epithelial cells also form purse strings at concave edges in the absence of such barriers, the cells were cultured on thin plastic strips (23) formed in zigzag patterns. As is seen in Fig. 1E, prominent actin cables spanning several cells formed at concave but not at straight or convex edges. They contained myosin IIA and were sensitive to blebbistatin and exoenzyme C3 transferase (Fig. S1). Hence, purse strings form whether the edges are constrained or free, but the edges must be concave.

Purse String Healing Proceeds Independently of EGFR Activation in Epithelial Cell Sheets. The agarose droplets were digested by treating with agarase. This was expected to be a very gentle way of removing the physical blocks provided by the droplets, and it did not result in detectable cell damage (Fig. 2A). After digestion, the holes in the cell layer closed within 10–12 h. Analysis by time-lapse microscopy revealed that contraction occurred initially with smooth edges, but after 2–4 h, ruffles appeared in some of the cells bordering the holes (Fig. 2B and Movie S1; see Fig. S6). To visualize the purse strings during healing, the cells were transfected with an expression vector coding for an enhanced green fluorescent protein/actin fusion protein. The actin cable was clearly visible in cells at the edges of transfected cells (Movie S2). The cells were very mobile, and individual cells moved away from the edges as the circumference of the wounds decreased during healing. The purse-string actin cables generally disappeared as the cells left the wound edges. This great mobility of cells at epithelial wound edges has been reported previously after mechanical wounding of corneas (30).

To test the requirement of EGFR signaling on purse-string healing, tyrphostin AG 1478, which potently inhibits the EGFR kinase (31, 32), was added. Importantly, the drug did not inhibit healing and had no noticeable morphological effect on the cells during purse-string contraction (Movie S3). Also, UO126, which inhibits activation of the important downstream targets of the EGFR, extracellular regulated kinase (ERK)1/2 (33), had no effect.

When HCLE cells were induced to migrate by digestion of agarose strips with straight edges, lamellipodia were apparent within 15–20 min in virtually all of the cells at the border, which is similar to observations with other epithelial cells at straight wound edges (24, 25) (Fig. S2). The early response at concave wound edges is, therefore, very different from that at straight edges. Importantly, and in sharp contrast to their effects after dissolution of agarose droplets, both UO126 and tyrphostin AG 1478 blocked healing in wounds with straight edges (Fig. 2B). This shows that the shapes of the edges determine whether EGFR-ERK1/2 signaling is required for motility. Inhibitors of Src family kinases [PP2 and Src kinase inhibitor I (SKI)] and phosphatidylinositol 3-kinase signaling (PI-103 and wortmannin) (34) blocked healing in wounds of either shape (Fig. 2C).

A limitation of the agarose-removal approach is that the effects of the inhibitors on formation of purse strings cannot be analyzed. Holes were, therefore, also induced by mechanical wounding, and although debris often obscured analysis, purse strings were clearly visible in about one-third of the holes, as detected by staining with phalloidin or antibodies to myosin IIA (Fig. 2D), and they formed even in the presence of tyrphostin AG 1478. Under these conditions, the small round wounds also closed independently of EGFR and ERK1/2 signaling (Fig. S3).

Role for Purse Strings in Forward Movement of Leading Edges of Epithelial Cell Sheets. Moving edges of HCLE cells segregate into regions that contain protruding cell clusters and concave regions that have few lamellipodia (Fig. 3A). The latter regions contained actin cables that were continuous from cell to cell; they also contained myosin IIA and were sensitive to exoenzyme C3 transferase and blebbistatin, and were characterized as purse strings (Fig. 3A and Fig. S4). To examine the role of purse-string-type movement, tyrphostin AG 1478, which reversibly
inhibits overall forward movement of HCLE cells (32), was added to moving cell sheets. The concave regions were seen to fill out whereas the advance of the protruding lamellipodia-rich regions was blocked (Fig. 3B and Movie S4). To support this conclusion, activation of the EGFR was also blocked with the LA1 antibody, which prevents EGFR activation by blocking its transactivation by ligands (32). The LA1 antibody blocked overall forward movement of the cell sheets (32) but did not prevent concave regions from filling out (Fig. S5). These observations suggest that both crawling and purse-string–type movement occurs at the leading edge and that both contribute to propel the cell sheet forward.

EGFR Activation Overrides Purse String Healing. Addition of EGF caused dissolution of the purse strings around agarose droplets, which was apparent after ∼8 h (Fig. 4A). When the agarose droplets were digested in the presence of EGF, the purse strings disassembled quickly and the cells extended numerous filbrillar actin (f-actin)-rich lamellipodia containing prominent focal adhesions (Fig. 4A, Fig. S6, and Movie S5). This was associated with increased rates of healing (Fig. 4B). Blocking cell division did not affect acceleration of healing, demonstrating that enhanced proliferation induced by EGF does not contribute significantly to acceleration of healing (Fig. S7).

In the leading edge of moving sheets of HCLE cells, purse strings are dynamic structures that form at seemingly random regions (Movie S6). After addition of EGF, the purse strings dissolved and large lamellipodia were uniformly present in the cells at the edge (Fig. 4C and Movie S7). As previously described, this is associated with an increased rate of healing of wounds with straight edges (23). Thus, in both of the examined systems, addition of EGF results in overriding the purse-string mechanism and in increased rates of wound closure.

EGFR Signaling Is Selectively Activated at Convex and Straight Wound Edges. The EGFR is activated upon wounding epithelial cell sheets (17–23). The finding that EGFR signaling disrupts purse strings suggested that activation occurs only at straight and convex edges. Direct localization of activated EGFR after wounding is complicated because of limitation of antibodies and down-regulation of the receptor (32). Transcription of c-fos is triggered by EGFR activation (35, 36), and c-fos accumulation was used as a marker of EGFR activity. c-fos accumulation occurred at protruding regions of the wound edges where lamellipodia were abundant but was not increased at concave regions (Fig. 5A). Also, no activation was seen in cells at edges after digestion of agarose droplets (Fig. S8). As controls, c-fos accumulation was inhibited when wounding was performed in the presence of...
tyrphostin AG 1478 (Fig. 5B) or the LA1 antibody (Fig. S9) (37). When EGF was added, c-fos accumulated in cells at edges of any shape, demonstrating that the EGFR signaling pathway was not blocked at concave edges (Fig. 5C).

**Discussion**

Impaired resolution of wounds is a very significant source of morbidity (38, 39), and it is, therefore, important to understand the healing process in depth. Epithelialization is an essential part of the proliferative phase of healing, and it is initially driven by cells moving to cover wounds. Here, it was found that the two known modes of epithelial movement, cell crawling and purse-string contraction, have different signaling requirements because the latter does not require signaling by the EGFR. Indeed, stimulation of the EGFR promotes transition of purse-string healing to cell crawling. It was found that the presence of a concave edge in an epithelial cell sheet is a sufficient signal for induction of purse strings, but that other stimuli, such as cell damage, are dispensable. The shape of a wound edge is, therefore, a critical signal that determines which type of healing is induced. In accordance with the differing signaling requirements of the two types of healing, closure of small round wounds does not require EGFR signaling, in contrast to healing of larger wounds. An implication of this is that developing procedures to enhance healing of large wounds may involve stimulating different signaling pathways from promoting epithelial resealing of small wounds. Small wounds can result, for instance, from extrusion of apoptotic cells from epithelia (14).

The cues that initiate the two types of epithelial healing are quite different. The presence of free straight edges in epithelial cell sheets is sufficient to induce EGFR activation and to promote cell crawling (23). The present data show that formation of purse strings does not depend on EGFR signaling but, rather, requires the presence of concave edges, in accordance with previous observations that purse strings form predominantly in small round wounds (6, 7, 15, 16). The edges can be free or constrained by an inert material such as agarose. This raises the issue of how curvatures of edges are detected: it is increasingly apparent that mechanical forces can have profound effects on almost every aspect of cell behavior and that cells communicate extensively by mechanical means (40–44). The intracellular distribution of forces within cells is complex and is likely to depend on the shapes of edges. Forces can be detected by a variety of different sensors, including components of the cytoskeleton, focal adhesions, and cell–cell junctions (45–48). Tension in the plasma membrane, which is detected by stress-activated ion channels (49), could also be different in cells at concave and convex edges. Our results suggest that apoptotic cells in epithelia

**Fig. 4.** Switch to the crawling mode of healing by EGF signaling. (A) Cells were grown around agarose droplets and were either untreated (Upper Left) or treated with 10 nM EGF for 10 h without removing the agarose droplets (Upper Right) and stained with Alexa Fluor 546-conjugated phalloidin. Cells were incubated with 10 nM EGF and photographed in phase contrast (Lower Left) or stained with phalloidin 3 h after removal of agarose (Lower Right). (Scale bar: 100 μm.) (B) Healing of holes after removal of agarose droplets. EGF (10 nM) was added where indicated. Means of triplicates ± SD. (C) Progression of moving epithelial sheets at the time of addition of 10 nM EGF and 7 h later (Movie S7).

**Fig. 5.** EGFR signaling is selectively activated at convex and straight edges. Moving edges of sheets of HCLE cells after no treatment (A), 4 h with 0.25 μM tyrphostin AG 1478 (B), or 1 h with 10 nM EGF (C). Upper images show staining with an anti-c-fos antibody, and the exposures are directly comparable. Lower images show staining with labeled phalloidin. (Scale bar: 100 μm.)
could be detected as inert bodies similar to agarose droplets and their recognition and extrusion may not require any specific signals released from the cells (14, 50).

The forces that produce movement are also different in the two types of healing. Movement by crawling depends on formation of lamellipodia, whereas purse-string healing is thought to, at least in part, depend on contraction of an actin/myosin cable that encircles the wounds (81, 51, 52). During cell crawling, forces that propel the sheet forward are generated in cells at the leading edge and in many rows behind them (3, 53–55). It is possible that cells behind the edge also contribute some forces to close purse-string wounds, although it is notable that blocking EGFR signaling, which drives lamellipodial crawling, has no detectable effects on purse-string healing.

EGF was found to induce a switch to movement by crawling, so although EGFR signaling is not required for purse-string closure of wounds, it profoundly affects healing. Purse strings can nonetheless form at the leading edges of moving epithelial cell sheets because EGFR activation occurs only at straight and convex edges. Wounding sheets of cornalian epithelial cells causes release of heparin-binding EGF-like growth factor and amphiregulin (19, 20, 22, 32). Both of these ligands bind strongly to negatively charged glycans in the extracellular matrix and the cell surface (36–38). This limits their diffusion and provides an explanation why the receptor is activated only very locally.

Edges of moving sheets of epithelial cells are typically segregated into protruding regions interspersed with concave regions that contain few lamellipodia (24, 25). The traditional view is that the leader cells at the tips of the protrusions pull neighboring cells forward and that the forces are propagated through actin cables that connect the cells in the concave regions (24, 25, 55). Here, it was found that the actin cables have the characteristics of purse strings and that the concave regions move forward when overall forward movement is blocked by inhibiting EGFR signaling. This suggests an active role of concave regions in the movement of the cell sheets and that both crawling and purse-string modes contribute to propel the sheets forward. Cells engaged in crawling protrude at the cell edge in accord with more rapid movement by this mechanism.

The presence of two different modes of generating force at the leading edge has important implications for understanding epithelial movement. Creation of mathematical models is complicated by the existence of the two modes of motility and the influence of the shapes of the edges. Models based on a single class of cells that obey a single set of rules presumably do not describe movement accurately (54, 55, 59–62). Also, our finding that stimulation of the EGFR causes a switch to cell crawling underscores that the many factors in the wound environment that can stimulate this receptor must be taken into account for a complete understanding of epithelial wound healing.

**Methods**

For a more detailed description, see SI Methods.

To create small circular holes in the cell layer, 3.5-cm Petri dishes were sprayed with a 0.125% low-melting point agarose (Promega) with 0.2% (wt/vol) glycerol and dried overnight at room temperature. Cells were then seeded using standard techniques. The plates were incubated in the same medium with 2% (vol/vol) newborn calf serum and 0.5 units of agarase for 10–15 min at 37 °C, and the cells were washed and incubated as required in the same medium with 10% (vol/vol) newborn calf serum. After appropriate treatments and incubations, the cells were fixed with 3.7% (wt/vol) formaldehyde and stained with 0.05% gentian violet. For quantitation, four areas of the dishes were photographed using a Nikon TS100 inverted microscope with a 2× objective and a Spot RT camera (Diagnostic Instruments). The areas of the holes were determined using Metamorph software (Molecular Devices) using the thresholding function. To create straight edges, strips of agarose were made with plastic molds as described (20) and digested as described. To examine the effects of unconstrained edges, cells were grown on plastic strips cast on top of agarose layers as described (23). Moving edges of epithelial cell sheets were produced by culturing and differentiating cells around agarose strips (20), transferring to MEM with 10% (vol/vol) newborn calf serum, and removing the strips 16 h before use.

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**Supporting Information**

**SI Methods**

**Antibodies and Other Reagents.** Antibodies to c-fos were from Santa Cruz Biotechnology, antibodies to nonmuscle myosin II heavy chain A were from Covance, and LA1 antibody was from R&D Systems. Alexa Fluor–conjugated secondary antibodies and phalloidin were from Invitrogen. Cell-permeable exoenzyme C3 transferase from *Clostridium botulinum* was from Cytoskeleton. UO126, PP2, and SKI were from EMD Biosciences, tyrphostin AG 1478 was from Enzo Life Sciences, and PI-103 was from Cayman Chemical. The green fluorescent protein–actin vector was from Clontech. Tissue culture plastics were from BD Falcon or Greiner Bio-One. Agarase was from Fermentas. EGF was from Invitrogen, cell culture reagents were from Mediatech, and other reagents and supplies were from Thermo-Fisher Scientific.

**Tissue Culture and Immunoﬂuorescence.** HCLE cells (1) were propagated in human keratinocyte serum-free medium (Invitrogen) supplemented with 0.3 mM CaCl$_2$, 25 μg/mL bovine pituitary extract, and 0.2 ng/mL EGF. When confluent, they were differentiated by incubation for 2 d in F12: Dulbecco’s modified Eagle’s medium (1:1) with 10% (vol/vol) newborn calf serum. The agarose strips or droplets were removed and cells transferred to MEM with 10% (vol/vol) newborn calf serum. Incubation in the latter medium was found to enhance the appearance of purse strings. To block division, cells were treated with mitomycin C (0.5 mM) for 2 h in differentiation medium, washed, and used for experiments. For immunofluorescence analysis, cells were fixed with 3.7% (wt/wt) formaldehyde at the end of experiments and processed as described previously (2). Images were acquired on a Nikon TE2000-E microscope with a CoolSNAP camera (Photometrics).

**Treatment with LA1 Antibody and Mechanical Wounding.** For antibody treatment, cells were cultured in 1.5-cm tissue culture wells with 1 × 11 mm agarose strips in 300 μL medium, treated as described above in the presence of 10 μg/mL LA1 antibody or nonimmune IgG. Small holes were induced by stabbing confluent cell layers with a 200-μL pipette tip sharpened by cutting with a razor blade. Because the holes were of very irregular shapes, suitable regularly shaped small round holes were first identified at the beginning of the assay (20–30 holes per dish), their locations were recorded with a laboratory marker, and the number of holes closed after 10 h was counted. To test for cell death, propidium iodide (1 μg/mL) was added to the tissue culture medium immediately after wounding, and the cells were washed after 3 min.

**Live-Cell Imaging.** Cells were seeded in six-well dishes containing agarose droplets or strips. Just before imaging, they were transferred to an incubation chamber (Oko Industries) to maintain constant temperature, CO$_2$ concentration, and humidity. Images were captured on a Nikon TI microscope and acquired with a Cascade camera (Photometrics).

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**Fig. S1.** Characterization of actin cables formed at concave edges in cells grown on thin plastic strips. Cells were stained with labeled phalloidin and antibodies to myosin IIA (*Upper Left* and *Right*, respectively). Cells were treated with 20 μM blebbistatin (2.5 h) or 5 μg/mL cell-permeable exoenzyme C3 transferase (6 h) and stained with labeled phalloidin (*Lower Left* and *Right*, respectively). Purse strings are indicated with arrows. (Scale bar: 100 μm.)
Fig. S2. Formation of lamellipodia after digestion of strips of agarose with straight edges. The times after initiation of digestion are indicated (min). Phase-contrast micrographs. (Scale bar: 100 µm.)

Fig. S3. Insensitivity of healing of small mechanically induced holes to EGFR inhibition. (A) The cells were incubated with the inhibitors used in Fig. 2B at the same concentrations. Values are means of the percentages of holes closed (triplicates ± SD). (B) To verify the efficacy of the tyrphostin AG 1478 and UO128 treatments, straight wounds were induced by scraping with a pipette tip, and the percentage of healing was calculated after photographing the wounds as described (1) (quadruplicates ± SD).


Fig. S4. Characterization of actin cables formed at concave edges in moving cell sheets. The cells were stained with labeled phalloidin after no treatment (Left), after treatment with 20 µM blebbistatin (2.5 h) (Center), or 5 µg/mL cell-permeable exoenzyme C3 transferase (6 h) (Right). Purse strings are indicated with arrows. (Scale bar: 100 µm.)
Fig. S5. Effects of LA1 antibody. (A) A strip of agarose was removed from a culture of HCLE cells, and healing was allowed to proceed for 12 h (Upper). LA1 antibody was added (10 μg/mL), and the cells incubated for a further 16 h (Lower). (Scale bar: 100 μm.) (B) Healing of small round wounds (Upper) or wounds with linear edges (Lower) with LA1 added, as indicated (four to six determinations ± SD).

Fig. S6. Lamellipodia induced by EGF. Agarose droplets were digested with agarase without (left images, showing ruffles) or with 10 nM EGF (right images). Cells were stained with labeled phalloidin (upper images) and with antibodies to paxillin (lower images). Focal adhesions are indicated with arrows. (Scale bar: 100 μm.)

Fig. S7. Purse-string healing in mitomycin C–treated cells. (A) Cells were treated with mitomycin C (MC) and 10 nM EGF, where indicated, and allowed to heal for 4 h. (B) To verify the efficacy of the MC treatment, HCLE cells were counted the day after seeding and treated with the drug where indicated, and the fold increases in cell number were calculated after 2 d of further incubation. Quadruplicates ± SD.
**Fig. S8.** c-fos transcription during purse-string healing. The cells were fixed at the indicated times after removal of the agarose droplets. (Scale bar: 100 μm.)

**Fig. S9.** Inhibition of c-fos transcription by the blocking antibody LA1. Twelve hours after removing the agarose to initiate movement, the cells were incubated with 10 μg/mL nonimmune IgG (A) or LA1 antibody (B) for 6 h before staining with antibodies to c-fos (upper images) and labeled phalloidin (lower images). After 6 h of incubation with LA1, the purse strings have largely disappeared. (Scale bar: 100 μm.)
Movie S1. Healing after digestion of agarose droplets. The movies were acquired with a 10× phase-contrast objective at one frame per 10 min in this and the subsequent movies, except where indicated. The numbers indicate times after initiation of the movies (h:min).

Movie S1

Movie S2. Localization of actin during healing. Cells were transfected with a vector coding for an enhanced green fluorescent protein/actin fusion protein. Micrographs in phase contrast (Left) or fluorescence (Right) (20×).

Movie S2
Movie S3. Healing after digestion of agarose droplets in the presence of 0.25 μM tyrphostin AG 1478. The drug was added at the start of digestion.

Movie S4. Effect of inhibition of the EGFR on the leading edge of moving HCLE cells. Cells were cultured around an agarose strip and acquisition of images started 16 h after removal of the strip. Tyrphostin AG 1478 was added 2.5 h after the start of the movie to a final concentration of 0.25 μM, and acquisition of images continued. Note that overall movement of the sheet is blocked but that the concave regions fill out.
Movie S5. Effect of added EGF on healing induced by digestion of agarose droplets. EGF (10 nM) was added at the start of the digestion. Note the formation of numerous lamellipodia and general increased motility of the cells.

Movie S6. Dynamics of cells at a moving leading edge of HCLE cells. The movie was started 16 h after removal of the agarose strip.
Movie S7. Effect of EGF on the leading edge of moving HCLE cells. Acquisition of images started 16 h after removal of the strip, and 10 nM EGF was added 2.5 h later. Compare with Movie S6.