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Free Edges in Epithelial Cell Sheets Stimulate Epidermal Growth Factor Receptor Signaling

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The ability of epithelia to migrate and cover wounds is essential to maintaining their functions as physical barriers. Wounding induces many cues that may affect the transition to motility, including the immediate mechanical perturbation, release of material from broken cells, new interactions with adjacent extracellular matrix, and breakdown of physical separation of ligands from their receptors. Depending on the exact nature of wounds, some cues may be present only transiently or insignificantly. In many epithelia, activation of the epidermal growth factor receptor (EGFR) is a central event in induction of motility, and we find that its continuous activation is required for progression of healing of wounds in sheets of corneal epithelial cells. Here, we examine the hypothesis that edges, which are universally and continuously present in wounds, are a cue. Using a novel culture model we find that their presence is sufficient to cause activation of the EGFR and increased motility of cells in the absence of other cues. Edges that are bordered by agarose do not induce activation of the EGFR, indicating that activation is not due to loss of any specific type of cell–cell interaction but rather due to loss of physical constraints.

INTRODUCTION

Wound healing in the skin and cornea is the result of complex and carefully orchestrated processes that include inflammation, cell migration and division, and extracellular matrix production and remodeling (Fini and Stramer, 2005; Gurtner *et al*., 2008). Wounding generally induces marked motility in epithelia, and they move to cover lesions and restore their function as physical barriers. The cells near edges in epithelia respond initially to wounding by extensive changes in transcription, and the phenotype is consequently modified allowing initiation of movement within a few hours. In many epithelia, including the gut, airway, epidermis, and cornea, activation of the epidermal growth factor receptor (EGFR) is a key event that controls the transition to motility (Puddicombe *et al*., 2000; Zieske *et al*., 2000; Myhre *et al*., 2004; Repertinger *et al*., 2004; Shirakata *et al*., 2005).

Wounding is a complex event that generates many potential cues that can promote motility in epithelia, but their relative roles are still incompletely understood (Jacinto *et al*., 2001; Martin and Parkhurst, 2004). The cues include the initial mechanical insults that may be registered by cells through mechanosensors (Reichelt, 2007), elevated concentrations of many growth factors (Klenkler and Sheardown, 2004; Gurtner *et al*., 2008), and intracellular components released from broken cells (Sponsel *et al*., 1995; Dignass *et al*., 1998; Boucher *et al*., 2007; Yin *et al*., 2007). Also, interaction of cell surface receptors with extracellular matrix that is laid bare after wounding can stimulate movement (DeMali and Burridge, 2003; Guo and Giancotti, 2004), and the breakdown of the epithelial barrier that occurs upon wounding may allow interactions of ligands and receptors that are separated in the intact epithelium (Vermeer *et al*., 2003). Finally, the cells at wound edges are in a unique environment because they have free edges that are not in contact with other cells (Desai *et al*., 2009).

The epithelium in any particular wound is therefore probably influenced by a variety of cues that may have redundant, and in some cases unique, effects. However, the presence of many of the cues is expected to be highly variable depending on the exact nature of the wounds. For example, growth factors and released ATP may be present in insufficient amounts or degraded quickly, and the extent and nature of adjacent extracellular matrix is expected to vary highly in different types of wounds. Cues that are present only at the time of wounding, for example, the trauma and high levels of extracellular ATP from damaged cells, may affect healing only initially.

There are striking similarities in the mechanics of movement of epithelial sheets during wound healing and development (Martin and Parkhurst, 2004). The similarities extend to the underlying signaling events: wounding of epithelia causes activation of the EGFR by stimulating proteolytic release of transmembrane precursors of ligands for the receptor (Block *et al*., 2004; Myhre *et al*., 2004; Xu *et al*., 2004), and epithelial sheet movement in eyelid closure in mice has also been shown to be driven by EGFR activation through this type of mechanism (Mine *et al*., 2005). One prominent similarity of wounded epithelia and epithelial

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Abbreviations used: AR, amphiregulin; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; HCLE, human corneal epithelial limbal; MMP, matrix metalloprotease; PBS, phosphate-buffered saline; polyHEMA, poly(2-hydroxyethyl methacrylate).

sheets migrating during development is the presence of edges, and indeed the general propensity of epithelia to move when edges are present has been long recognized (Rand, 1915). In this study, we examined the possibility that the presence of an edge is a cue that induces activation of the EGFR and stimulates motility in epithelial cell sheets.

MATERIALS AND METHODS

Antibodies and Reagents

Antibodies against the EGFR and extracellular signal-regulated kinase (ERK) were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against β-actin and poly(2-hydroxyethyl methacrylate) (polyHEMA) were from Sigma-Aldrich (St. Louis, MO). Ki67 antibody was from Thermo Fisher Scientific (Waltham, MA), E-cadherin antibodies were from BD Biosciences (San Jose, CA), and zona occludens (ZO)-1 antibodies were from Zymed Laboratories (South San Francisco, CA). Vybrant DiO, DiD, and Alexa Fluor conjugates were from Invitrogen (Carlsbad, CA). Grade VII potato apyrase was from Sigma-Aldrich. Cell culture reagents were from Mediatech (Herndon, VA), and other reagents and supplies were from Thermo Fisher Scientific, unless noted.

General Tissue Culture Procedures, Treatments, and Immunoblotting

Human corneal limbal epithelial (HCLE) cells were cultured as described previously (Block and Klarlund, 2008). Before analysis, the cultures were incubated overnight in keratinocyte serum-free medium (Invitrogen) without pituitary extract and epidermal growth factor (EGF). Where indicated, the cells were incubated for 3–4 h with 10 μg/ml neutralizing antibodies (Millipore, Billerica, MA) or for 30 min with 50 μM GM 6001, 50 μM GM 6001 negative control, or 1 μM tyrphostin AG 1478 (all from EMD Biosciences, San Diego, CA). For immunoblotting, the cells were washed once in ice-cold phosphate buffered saline (PBS), and 200 μl 1% SDS in water was added. After sonication and normalization for protein content, SDS-polyacrylamide gel electrophoresis and immunoblotting were performed according to standard procedures. Densitometry of autoradiographs was performed using Quantity One software (Bio-Rad Laboratories, Hercules, CA). Thymidine incorporation was measured by incubating the cells with 0.25 μCi/ml of [^{3}H]thymidine for 6 h, trypsinizing the cells, and precipitating with trichloroacetic acid, and then counting the radioactivity in a beta-counter. Wounding assays were performed as described previously (Block and Klarlund, 2008).

Biochemical Assays

Lactate dehydrogenase in supernatants was measured using a commercial assay kit (Promega, Madison, WI). ATP was measured in supernatants after clarification by centrifugation at 5000 rpm for 5 min by using a bioluminescence assay kit (Sigma Aldrich). Aphiregulin (AR) was measured in the supernatants using the DuoSet ELISA (R&D Systems, Minneapolis, MN) according to manufacturer's protocol. For measuring matrix metalloprotease (MMP)9 levels by zymography, tissue culture supernatants were collected, and proteins were separated in nonreducing polyacrylamide gels containing 0.08% gelatin. After incubating the gels to allow renaturation and digestion, and subsequent Coomassie staining, MMP9 appeared as translucent 92-kDa bands (for details of the procedure see, Snoek-van Beurden and Von den Hoff, 2005).

Time-Lapse Microscopy

Cells were grown in stratification medium without EGF (Gipson *et al*., 2003) for 2 d and then transferred to the same medium with 2% calf serum. Images were acquired at 10-min intervals with a Cascade 1K camera on an Ellipse Ti inverted microscope (Nikon, Tokyo, Japan) with a 10× phase contrast objective. The cells were incubated in an electric CO_2 MSI stage incubator (Okolab, Ottaviano, Italy) equilibrated to 37°C and 5% CO_2. Analysis was performed using MetaMorph 7.6.2 software (Molecular Devices, Sunnyvale, CA).

Cultures of Cells on Plastic Strips

Tissue culture dishes (3.5 cm) were coated with 1 ml of 10% polyHEMA in 95% ethanol and dried overnight at 37°C. The bottom parts of tissue culture dishes (Corning Life Sciences, Lowell, MA) were broken into pieces and a 5% (wt/vol) solution in chloroform was prepared in 50-ml conical tubes on an end-over-end rotator. Strips of the plastic solution were applied to the surface of the polyHEMA with narrow-bore 200-μl pipette tips. As controls, the entire polyHEMA-coated surfaces were covered with a thin layer of dissolved plastic. The plates were dried at 65°C for 2 d. HCLE cells were seeded as described previously (Block and Klarlund, 2008). Before assays, the cells were incubated with keratinocyte serum-free medium without pituitary extract and EGF for at least 24 h. To grow cells among agarose droplets, 0.25% agarose was sprayed on plates completely covered with plastic and dried at 65°C, and the agarose droplets were removed as described previously (Block *et al*., 2004). To examine protrusions of cells within cell layers, the pEGFP-actin vector (Clontech, Mountain View, CA) was transfected into HCLE cells by using Lipofectamine 2000. The following day, the cells were trypsinized and mixed with 3 times as many untransfected cells, seeded on dishes treated as described above, transferred to stratification medium (Gipson *et al*., 2003) the next day, and used 2 d later.

Confocal Microscopy

Rhodamine-dextran was serendipitously found to bind strongly to polyHEMA, and coated plates were stained by incubation with 1 mg/ml rhodamine-dextran (Invitrogen) in PBS for 1 h. The plates were then washed 5 times with PBS and dried at room temperature. Vybrant DiO (10 μl/ml) was added to the plastic solution before application as strips. Agarose was conjugated to fluorescein by dissolving 8 mg of agarose in 200 μl of dimethyl sulfoxide at 100°C and reacting overnight with 4 mg of fluorescein isothiocyanate (Sigma-Aldrich) at room temperature. It was diluted to 1.6 ml in water, chilled to 0°C, and the solidified agarose was washed extensively with water. The conjugate was dissolved by heating and diluted to 0.25% before application to plastic-covered plates. The cells were fixed with 3.7% formaldehyde in PBS, and cell membranes were stained with 100 μl/ml Vybrant DiD in PBS overnight. Images of the cultures were captured with an IX81 confocal microscope (Olympus, Tokyo, Japan) equipped with a 60× oil objective (numerical aperture [NA] 1.4), and xy and xz projections were generated using FluoView software (Olympus). For analysis of EGFP-actin–transfected cells, images were acquired on the same confocal system with a 40× (NA 1.3) oil objective.

Analysis of ERK1/2 Activation at Various Distances from Edges

One-half of polyHEMA-coated tissue culture plates were covered with dissolved plastic, creating a single edge in the middle of the plates. Cells were seeded and grown to confluence for 2–3 d, and they were starved for pituitary extract and EGF for 24 h before harvest. They were washed with ice-cold PBS and placed on an ice-water bath. Then, 0.5-cm-wide regions of the cell sheets were then sequentially removed by scraping, starting at the plastic/polyHEMA interface. Each region was scraped in 0.5 ml of PBS, pooled with corresponding regions from two other dishes, collected by centrifugation, and analyzed by immunoblotting.

RESULTS

Progression of Epithelial Sheet Movement Requires Continuous EGFR Signaling

Several studies have shown that introduction of gaps in sheets of corneal epithelial cells results in activation of the EGFR within minutes, and blocking the activity of the receptor with chemical inhibitors or antibodies completely abrogates induction of movement (Block *et al*., 2004; Xu *et al*., 2004; Boucher *et al*., 2007). First, we sought to examine whether EGFR activity is required throughout the healing phase. Some cues are expected to be present only initially; therefore, we also asked whether induction of movement requires activation of the EGFR at the same time that the wounds are inflicted.

To address these questions, we used a previously developed variant of the commonly used in vitro models of wound healing that reproduce many of the aspects of the cell sheet movements that occur in vivo (Martin and Parkhurst, 2004; Farooqui and Fenteany, 2005; Simpson *et al*., 2008; Friedl and Gilmour, 2009). We have previously developed a model in which cells are grown as five- to 10-cell-wide strips around agarose droplets, which can subsequently be removed allowing cells to initiate movement (hereafter described as "wounding"; Block *et al*., 2004). In this model, the majority of the cells are near a wound, which permits biochemical analysis of stimulated cells. We used HCLE cells, which have been immortalized by abrogation of p16INK4A/Rb and p53 functions and overexpression of the catalytic subunit of the telomerase holoenzyme (Gipson *et al*., 2003).

The EGFR was rapidly activated after wounding as detected by immunoblotting with an antibody against the receptor phosphorylated on tyrosine (tyr)-1173 (Figure 1A). Blotting with an antibody that recognizes the total amount of receptor revealed that it was subsequently down-regulated, but the ratio of the amounts of phosphorylated to total

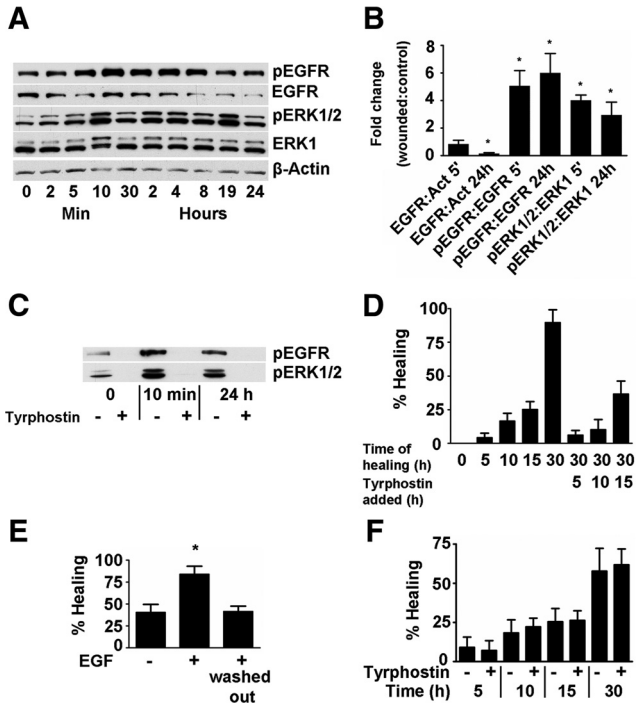

Figure 1. Continuous EGFR signaling is required for progression of wound healing. (A) Time course of activation of the EGFR and ERK1/2 after wounding. The blots were cut and relevant areas probed with antibodies to phospho-EGFR(1173), phospho-ERK1/2(204), or β-actin as a load control. The blots were then stripped and blotted with antibodies to total EGFR or ERK1. (B) Quantitation of down-regulation and activation of the EGFR after wounding. Asterisk (*) indicates significant differences from controls according to Student's *t* test ($n = 6$; $p < 0.001$). (C) Inhibition of EGFR signaling blocks ERK1/2 activation immediately and long after wounding. 1 μM tyrphostin AG 1478 was added 30 min before analysis. (D) Effects of blocking EGFR signaling at various times after wounding. Healing was measured after 0, 5, 10, 15, and 30 h. Where indicated, 1 μM tyrphostin AG 1478 was added at the indicated times, and the cells were allowed to heal for a total of 30 h ($n = 6$). (E) EGF is required continuously for acceleration of wound healing. EGF (4 nM) was added for the initial hour or continuously during wound healing. Asterisk (*) indicates significant difference from control according to Student's *t*-test ($n = 7$; $p < 0.001$). (F) EGFR signaling is dispensable during the initial hour after wounding. Tyrphostin AG 1478 (1 μM) was added for the first hour where indicated, and then the cells were washed and allowed to heal for the indicated times ($n = 6$). In this and the following figures, the values in the graphs are means and error bars are SDs. All experiments were performed at least three times with similar results.

receptor remained increased, suggesting that activation is prolonged after wounding (Figure 1B). ERK1/2 are important downstream targets for the EGFR that are central for induction of movement in a wide variety of cells (Dieckgraefe *et al*., 1997; Huang *et al*., 2004; Viala and Pouyssegur, 2004; Satoh *et al*., 2009). They are activated rapidly after wounding and remain active for prolonged periods. To determine whether the EGFR is actively signaling at both short and long time points after wounding, we examined the effects of the EGFR kinase inhibitor tyrphostin AG 1478 (Ellis *et al*., 2006). As is seen in Figure 1C, the inhibitor blocked activation of ERK1/2 both at 5 min and 24 h, indicating that the EGFR is actively signaling and controls the activities of ERK1/2 both early and late after wounding.

To examine the role of the prolonged phase of EGFR signaling on motility, we added tyrphostin AG 1478 at various times after wounding. As is seen in Figure 1D, blocking EGFR signaling stopped healing at each time point tested, indicating that continuous activity of the receptor is required for progression of healing. We also tested whether acceleration of healing induced by exogenously added EGF requires its continued presence. As is seen in Figure 1E, acceleration was clearly seen when it was present constantly but not when it was present only during the first hour. To examine whether the initial burst of activation of the receptor at the time of wounding is necessary for induction of motility, the EGFR kinase was blocked by adding tyrphosin AG 1478 during the initial hour after wounding, and the drug was then washed out. As illustrated in Figure 1F, the cells migrated to approximately the same extents as untreated cells, demonstrating that the initial burst of activation is dispensable for induction of motility. Together, these results point to the absolute control of cell migration by the EGFR during wound closure: the cells move only when the EGFR is active, but it is not necessary that the EGFR is turned on simultaneously with the infliction of wounds.

EGFR-ERK1/2 Are Persistently Activated by the Presence of Free Edges in Sheets of Epithelial Cells

The results described above lead us to speculate that the presence of edges is a cue for activation of the EGFR, because edges exist throughout the healing process. To test this, we developed a procedure that allows comparison of epithelial cultures containing many free edges with control cultures containing no edges. Strips (0.5 mm wide) of dissolved plastic were applied on top of a layer of polyHEMA in tissue culture dishes and allowed to solidify (Figure 2A). HCLE cells were grown to form sheets on the strips that were ~15 rows of cells wide. The cells did not adhere to the polyHEMA, which is known to resist cell attachment and spreading (Folkman and Moscona, 1978). As controls, cells were grown on polyHEMA-coated plates that were completely covered with plastic.

The cells exhibited similar epithelial traits when grown on strips and in control conditions: they had an epithelial appearance when examined by phase contrast microscopy (Figure 2A), substantial amounts of F-actin were present in the subcortical regions of the cells, and E-cadherin was present in cell–cell junctions (Figure 2B). The cells extended over the edges of the plastic strips, and they were not physically constrained by the polyHEMA (Figure 2C). Rates of thymidine uptake and expression of the Ki67 protein, which is present in actively dividing cells (Brown and Gatter, 2002), were reduced similarly in cells grown on strips and in control cultures when they reached confluence (Figure 2, D and E). Levels of lactate dehydrogenase in the supernatants of cells grown in the two conditions were low and similar, indicating that growth of cells on the strips does not promote cell lysis (Loo and Rillema, 1998; Figure 2F). These results indicate that differences in cells grown on strips or controls are unlikely to be due to differences in proliferation states or cell lysis.

Immunoblotting for the EGFR revealed that the ratio of pEGFR(1173) to total EGFR was increased in cells grown on strips and the receptor seemed down-regulated (Figure 3, A and B). The ERK1/2 kinases were strongly activated in cells grown on strips (Figure 3, C and D), which was blocked by the presence of tyrphostin AG 1478 indicating that they are activated as a result of EGFR signaling (Figure 3E). Identical results were obtained when agarose was used as the antiadhesive base (data not shown), demonstrating that the results were not dependent on any particular property of polyHEMA. The status of the EGFR in cells grown on strips

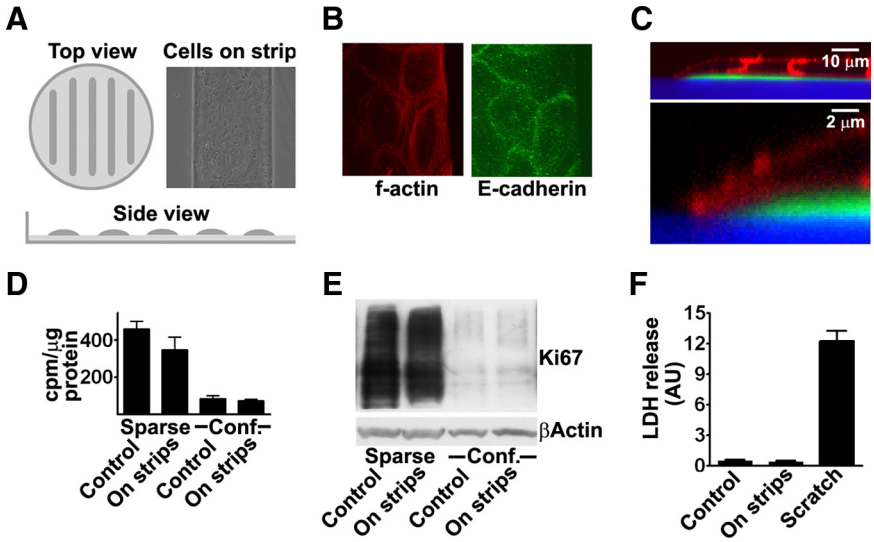

Figure 2. Tissue culture model for determination of the effects of free edges. (A) Schematic of plates covered with polyHEMA and plastic strips. Light gray, polyHEMA; dark gray, plastic; inset, phase contrast microscopy of HCLE cells grown on plastic strips. (B) The cells at edges of plastic strips were stained with Alexa Fluor 546-conjugated phalloidin or anti-E-cadherin antibodies. (C) The plastic strips and polyHEMA were labeled with flourophores (green and blue, respectively), and the cells were labeled with the membrane dye Vybrant DiD (depicted in red). (D) No significant differences were detected between the uptake of thymidine in cells grown on strips or as uninterrupted sheets (n = 6). (E) Expression of Ki67 protein was analyzed by immunoblots and appears as a high-molecular-weight smear. (F) Release of lactate dehydrogenase (LDH) was measured from HCLE cells grown on strips or corresponding controls (n = 4). For comparison, multiple scratches were introduced into confluent HCLE cell layers with a pipette tip. AU, arbitrary units.

seems similar to its status at late time points after wounding (Figure 1, A and B) in that the receptor is activated, downregulated, and controls ERK1/2 signaling.

Wounding induces activation of the EGFR by a process that is similar to the triple membrane-passing mode of activation by certain G protein-coupled receptors (Fischer *et al*., 2003). Precursors for some of its ligands in the cell membrane, predominantly heparin binding EGF-like growth factor and AR, are mobilized by proteolysis, and they subsequently bind to and activate the EGFR (Tokumaru *et al*., 2000; Block *et al*., 2004; Xu *et al*., 2004; Shirakata *et al*., 2005; Block and Klarlund, 2008). To examine whether the EGFR in cells grown on strips is activated similarly, the cells were preincubated with the LA-1 antibody, which blocks ligand binding to the EGFR (Johnson *et al*., 1993) or the broad-spectrum metalloprotease inhibitor GM 6001, and the activity levels of ERK1/2 were used as a readout of EGFR signaling. Both treatments blocked the ERK1/2 activation in cells grown on strips (Figure 4A). Furthermore, we analyzed release of AR, which is released by HCLE cells after wounding (Block and Klarlund, 2008). As expected, incubation with the protease inhibitor blocked release of AR from cells grown on strips (Figure 4B). As noted previously, we are unable to measure heparin binding EGF-like growth factor in the supernatants, presumably because it is adsorbed strongly to the glycocalyx of the cells (Block and Klarlund, 2008). Together, these results strongly support that the EGFR is indeed activated by a triple membrane-passing mechanism.

To determine how far from an edge activation occurs, cells were seeded on plates that were half covered with plastic and therefore contained a single edge. Immunoblotting extracts of cells located at various distances from the edge revealed that activation occurred within the most proximal 5 mm from the edge (Figure 4C).

EGFR-ERK1/2 Activation Is Not Induced by Extracellular ATP Signaling or by Breakdown of Segregation of Ligand and Receptor at Edges

Wounding acutely causes release of substantial amounts of extracellular ATP that may induce activation of the EGFR after binding to purinergic receptors on the cell surface (Boucher *et al*., 2007; Yin *et al*., 2007; Block and Klarlund, 2008). The level of ATP in the supernatants in cultures of cells grown on plastic strips was determined by a bioluminescence assay (see *Materials and Methods*) to be ~80 nM (Figure 5A). Titration of ATP revealed that ~1 μM is required for detectable activation of the EGFR (Figure 5B) in agreement with similar determinations (Boucher *et al*., 2007; Yin *et al*., 2007), and the amounts in the supernatants are therefore expected to be too low to activate the receptor. Transfer of supernatants from cells grown on strips did not induce activation of the EGFR (Figure 5C). Addition of apyrase, an ATP hydrolase, at concentrations that reduce ATP levels below the limit of detection in the medium and which blocks its signaling capacity in HCLE cells (Figure 5, A and B) did not block activation of the EGFR-ERK1/2 pathway in cells grown on strips (Figure 5D). Together, these data strongly suggest that activation of the EGFR in cells grown on strips is not mediated by extracellular ATP signaling.

A different mechanism of receptor activation in wounded epithelia is based on receptor/ligand segregation. In airway epithelium, the EGFR-related receptors ErbB2-3 are located in the basolateral membranes, whereas the ligand heregulin is present in the apical compartment and it can bind and activate the receptors only after the epithelium is disrupted by wounding (Vermeer *et al*., 2003). For such a mechanism to operate, the epithelial cells must separate the apical and basolateral compartments effectively, which mainly occurs at tight junctions. Specialized procedures are required for epithelial cells to form fully developed tight junctions in tissue culture (Cereijido *et al*., 1978; Utech *et al*., 2006); and, as expected, they were incomplete in HCLE cells grown in the culture conditions in this study (Figure 5E). Addition of EGF to the apical compartment gave rise to strong signals from activated EGFR, which seemed located mainly at the basolateral membranes, as expected (He *et al*., 2002; Figure 5F). This shows that a ligand present in the apical compartment can gain access to the EGFR in basolateral membranes under the culture conditions even in the absence of wounding, and activation of the EGFR can therefore not occur by the mechanism identified by Vermeer *et al*. (2003) in this system.

Activation of EGFR-ERK1/2 Signaling Requires the Presence of Unconstrained Edges

To establish whether the lack of physical constraints is a prerequisite for activation of the EGFR-ERK1/2 pathway,

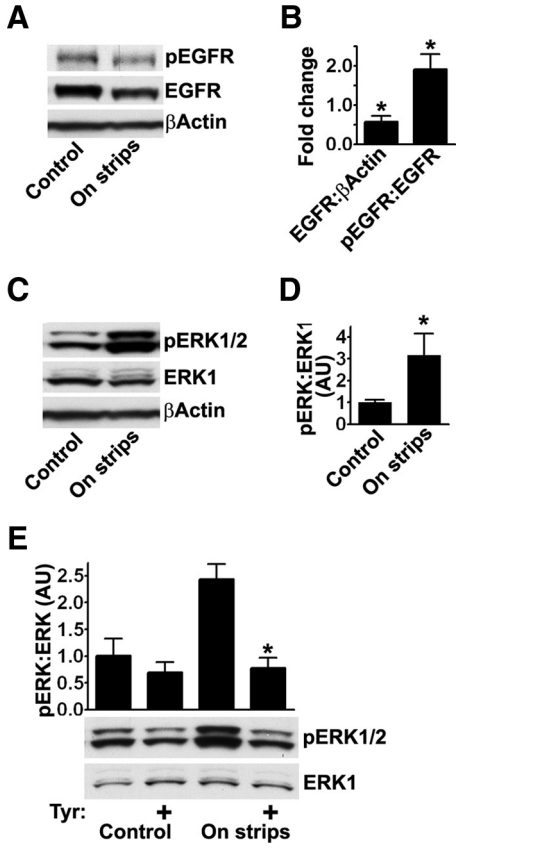

Figure 3. Activation of the EGFR and ERK1/2 by free edges. (A) Immunoblot of extracts with an antibody against EGFR phosphorylated on tyr-1173. The blots were stripped and reprobed with antibodies that recognize total amounts of the EGFR. The same blots were also probed with an antibody against β-actin as load control. (B) Quantitation of immunoblots by densitometry. Asterisk (*) indicates significant differences from controls by Student's *t*-test ($n = 6$; $p < 0.001$). (C) Extracts were stained with an antibody against ERK1/2 phosphorylated on tyr-204. The blots were stripped, and reprobed with antibodies that recognize total amounts of ERK1. (D) Quantitation as in B ($n = 6$; $p < 0.001$). (E) The EGFR controls ERK1/2 activation in cells grown on strips. Cells were treated with 1 μM tyrphostin AG 1478 30 min before harvest.

we seeded HCLE cells on plastic-covered polyHEMA-coated tissue culture plates that were sprayed with agarose droplets (Block *et al*., 2004). The cells grew to form strips two to 15 cells wide among the agarose droplets, comparable with, or less than the widths of the sheets of cells grown on plastic strips. Examination by confocal microscopy revealed that the cells were in direct contact with the agarose at their lower aspects and were thus physically constrained in contrast to the cells growing at edges on plastic strips (Figure 6A). The EGFR and ERK1/2 were not significantly activated in these conditions (Figure 6, B–E), even though addition of EGF to cells grown either as uninterrupted sheets or with agarose droplets resulted in similar levels of EGFR and ERK1/2 activation (Figure 6, F and G). We conclude that edges in epithelial cell sheets that are physically constrained by an inert material such as agarose do not induce activation of EGFR-ERK1/2 signaling.

Edges Induce Increased Motility of HCLE Cells

Time-lapse microscopy has shown that HCLE cells, like many other epithelial cells, exhibit appreciable spontaneous movement within sheets, and that they react to wounding by increasing their velocities by 60–70% near wound edges (Block and Klarlund, 2008). Cells in the center of the strips moved apparently randomly, although in coordination with neighboring cells. Near the edges, movement of cells was significantly more rapid (Figure 7, A and B, and Supplemental Video 1). The cells tended to retract from the edges upon prolonged exposure to tyrphostin AG 1478, precluding useful analysis of the effects of the drug in these conditions. The unconstrained facets of the cells facing the space between the strips were very active and extended and retracted ruffle- and filopodia-like structures rapidly, possibly exploring the surrounding environment (Figure 7C and Supplemental Video 2). To determine whether projections are unique to the cells at the edges of the strips, we transfected 5–10% of the cells with a vector coding for EGFP-actin, which allows visualization of the outline of individual cells because most expressing cells are surrounded by nontransfected cells (Farooqui and Fenteany, 2005). We found that the cells had irregular shapes with many protrusions and there were no discernible differences in the numbers and shapes of the protrusions of cells at the edges on plastic strips, near agarose blocks, or within confluent cell layers (Supplemental Figure 1). Also, no differences were evident in cells at the edges of scratch wounds and inside a confluent cell sheet (data not shown). This differs from results with Madin-Darby canine kidney (MDCK) cells that form a compact cuboidal epithelial sheet at confluence and flatten and send out numerous lamellipodia in cells near, or at wound edges (Matsubayashi *et al*., 2004; Farooqui and Fenteany, 2005).

High Levels of MMP9 Production Requires Access to Adjacent Extracellular Matrix

In addition to inducing motility in cells, wounding also induces other traits in epithelial cells, including enhanced secretion of certain cytokines and other proteins (Pastore *et al*., 2008). To examine whether the presence of free edges is sufficient for induction of other aspects of the motile phenotype, production of MMP9 was examined (Legrand *et al*., 1999; Bjorklund and Koivunen, 2005; Fini and Stramer, 2005). Cells that were grown with unconstrained edges on plastic strips exhibited modest, although significant, increases in MMP9 production compared with controls (Figure 8A). The wounding model used in this study provides at least two types of stimuli: edges in the cell sheets are unconstrained and the cells gain access to adjacent cell-free surface area (Block *et al*., 2004). After wounding, large increases in secretion of MMP9 were seen (Figure 8A), which is reminiscent of the substantial induction of MMP9 that occurs at wound edges in vivo (Matsubara *et al*., 1991; Sivak *et al*., 2004). Large increases were seen at both 0–24 and 24–48 h after removal of the agarose droplets, indicating that high levels of MMP9 production are maintained even long after wounding. To see whether EGFR signaling has a role in the increased production of MMP9, we inhibited its activation with tyrphostin AG 1478 or with the blocking antibody LA1. Either treatment reduced MMP9 production to near-background levels (Figure 8B). Together, these data indicate that full MMP9 production requires both EGFR signaling and interactions with adjacent extracellular matrix.

DISCUSSION

When edges occur in epithelial sheets, after wounding or during development, the epithelia are usually induced to move and cover adjacent free space. In this study, we have

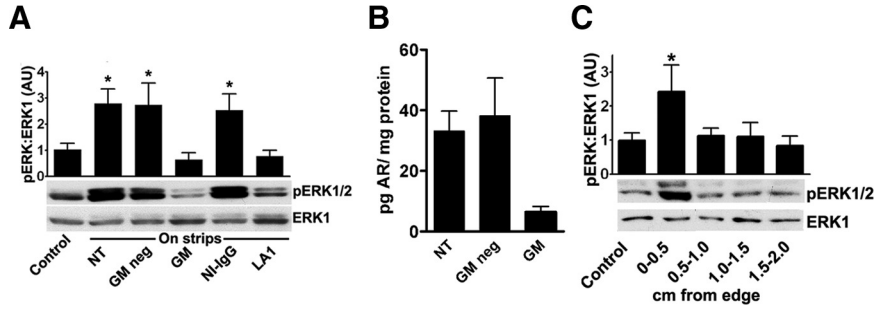

Figure 4. ERK1/2 are activated as a result of proteolytic activation of ligands for the EGFR, and activation occurs locally at free edges. (A) Cells were incubated with GM 6001 negative control (GM neg), GM 6001 (GM), nonimmune immunoglobulin (NI-IgG), or the LA1 antibody as indicated. The presence of GM 6001 did not affect EGFR phosphorylation by added EGF (Block *et al.*, 2004; data not shown). Asterisk (*) indicates significant differences from controls after one-way analysis of variance and the Bonferroni test for multiple comparisons ($n = 3$; $p < 0.001$). (B) Inhibition of AR release by GM 6001. See *Materials and Methods* for experimental details ($n = 4$). (C) Regions (0.5 cm) of HCLE cells were analyzed at various distances from an unconstrained edge. "Control" is a similar strip taken from a confluent culture. Asterisk (*) indicates significant differences according to the Bonferroni test for multiple comparisons ($n = 7$; $p < 0.001$).

determined that the presence of an edge is in itself a signal that causes activation of the EGFR, which is a potent inducer of cell motility in numerous epithelia (Puddicombe *et al.*, 2000; Zieske *et al.*, 2000; Myhre *et al.*, 2004; Repertinger *et al.*, 2004; Shirakata *et al.*, 2005).

Wounding is a complex event that generates many potential cues that can promote movement of epithelia, and the location, geometry, and mode of infliction of the wounds determines the range of cues that may be present. Interaction with extracellular matrix proteins can induce activation of the EGFR and other receptors (Streuli and Akhtar, 2009), and interactions with soluble factors, including numerous growth factors, can cause potent activation of the EGFR. However, the extent and composition of adjacent extracellular matrix and the amounts of growth factors released at wounding sites is expected to vary greatly in different types of wounds, and soluble factors may be washed out or degraded to different degrees. Some cues may only be present temporarily, for example, the initial mechanical forces and the large amounts of ATP that are released by broken cells (Yin *et al.*, 2007; Block and Klarlund, 2008). Because we found that introduction of wounds can be temporally dissociated from activation of the receptor without any discernible retardation of subsequent healing (Figure 1F), it is unlikely that cues present only at the time of wounding are necessary for healing. Specifically, we and others have reported that healing proceeds efficiently in the absence of the initial cell damage that is induced by mechanical wounding (Block *et al.*, 2004; Poujade *et al.*, 2007).

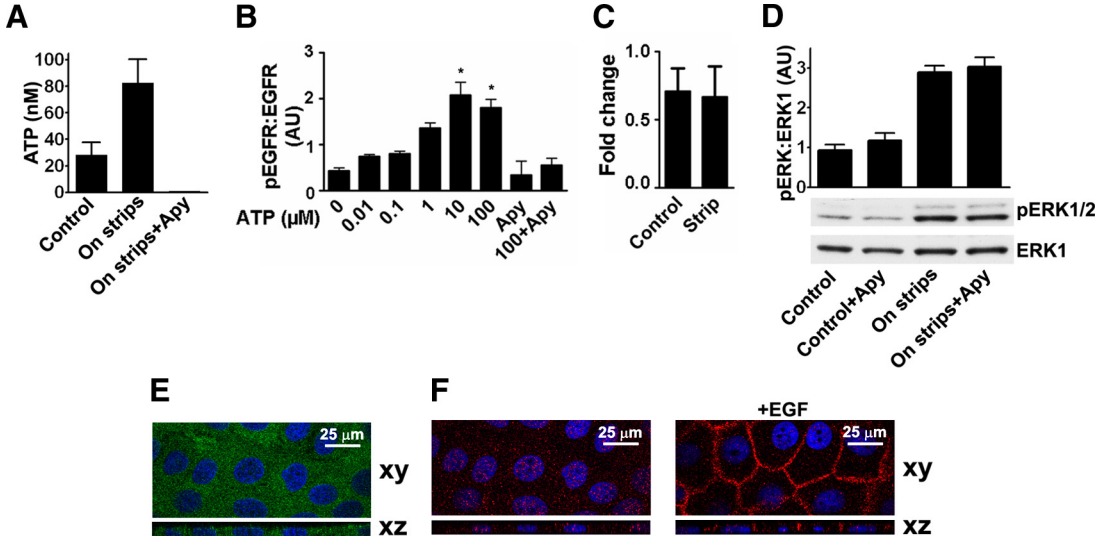

Figure 5. Edges do not stimulate EGFR activation through extracellular ATP signaling or through disruption of ligand/receptor segregation. (A) ATP levels in supernatants of cells grown under control conditions, on strips, or on strips and treated with 5 U/ml apyrase (Apy) for 3 h ($n = 6$). (B) Titration of activation of the EGFR by ATP. ATP was added for 10 min before harvest of the cells. Significant differences from controls are indicated by asterisks. Apy, 5 U/ml apyrase was added to the cells either alone or with 100 μM ATP ($n = 3$). (C) Transfer of supernatants from cells grown on strips does not induce activation of the EGFR. Cells grown as confluent controls or as strips were incubated for 24 h with starvation medium, and supernatants were collected and incubated on separately starved cells for 10 min. The data are normalized to transfer of starvation medium that was not incubated on cells. (D) Removal of ATP by treatment with 5 U/ml apyrase for 3 h does not result in any significant changes in the activation state of ERK1/2 in cells grown on plastic strips ($n = 6$). (E) HCLE cells were cultured on plastic-covered polyHEMA and were stained with an antibody to ZO-1. The activity of the antibody was verified by staining tight junctions in sections of mouse corneas (data not shown). Confocal microscopy was performed, and a representative xy and xz projection is shown. (F) Cells cultured on plastic-covered polyHEMA were untreated or treated with 100 ng/ml EGF for 2 min before fixation and analysis of phosphorylated EGFR by immunofluorescence confocal microscopy. Representative projections from xy and xz planes are shown.

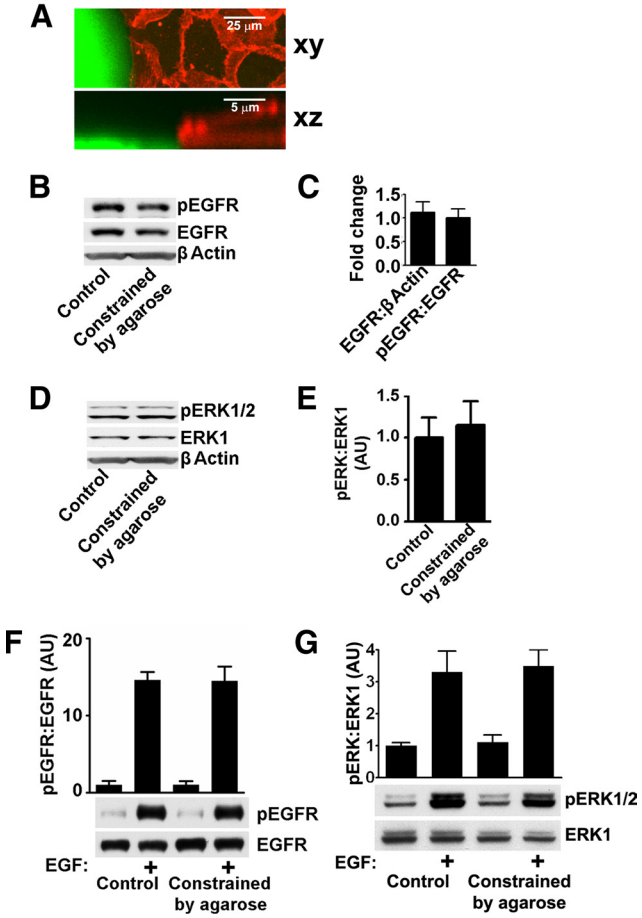

Figure 6. Edges that are physically constrained do not activate the EGFR. (A) Cells were labeled with the membrane dye Vybrant DiD (red), and agarose droplets were labeled with fluorescein (green). The plastic below the cells was left unlabeled for clarity. (B–E) Immunoblots of extracts of HCLE cells cultured without and with agarose droplets. The blots were probed as described in Figure 3, A–D. (F and G) Stimulation of HCLE cells grown either as uninterrupted sheets or around agarose droplets with 10 ng/ml EGF for 10 min results in similar levels of EGFR and ERK1/2 activation.

We speculated that the presence of edges, which are invariably and continuously present in wounds, is a signal that induces activation of the EGFR. We created a tissue

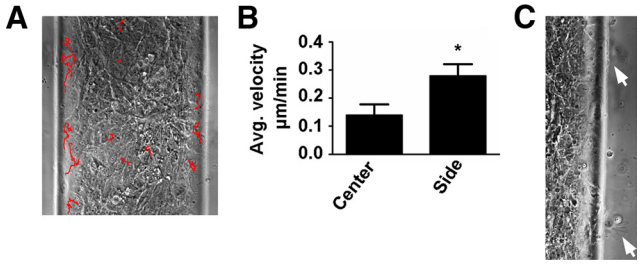

Figure 7. Motility of cells growing on plastic strips. (A) Representative trajectories of cells during a 4.5-h period are indicated (also see Supplemental Video 1). (B) Trajectories of 25 or 29 cells were followed, respectively. Asterisk (*) indicates significant difference from control ($p < 0.001$). (C) Close-up of movement of cells at edge of plastic strips. Note the extensions of lamellipodia- and ruffle-like structures (top and bottom arrow, respectively). Also see Supplemental Video 2.

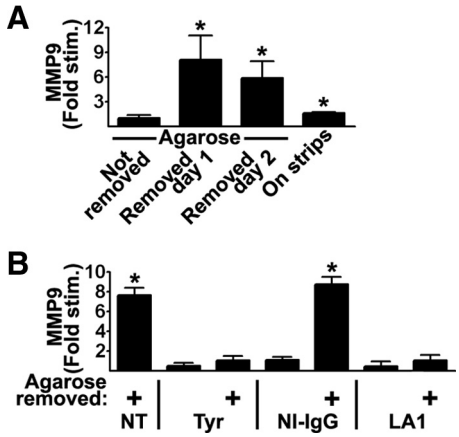

Figure 8. Full production of MMP9 requires cues in addition to the presence of edges. (A) Effect of removal of agarose droplets. MMP9 in medium incubated 24 h on cells grown among agarose droplets, and in supernatants collected 0–24 or 24–48 h after removal of agarose droplets (day 1 and 2, respectively). Mock-removal from cultures without agarose droplets did not result in enhanced secretion of MMP9 (data not shown). Also shown are levels of MMP9 in medium incubated 24 h on cells grown on plastic strips. Asterisk (*) indicates significant differences from controls by Student's *t* test ($n = 5$; $p < 0.001$). (B) Levels of MMP9 in cultures treated as indicated were compared with untreated controls, and the fold change was plotted. Cells were treated with tyrphostin AG 1478, nonimmune immunoglobulin (Ig)G, or the LA1 antibody. Asterisk (*) indicates significant differences from controls according to one-way analysis of variance and the Bonferroni test for multiple comparisons ($n = 3$; $p < 0.001$).

culture model in which epithelial cells are seeded on narrow (≈0.5-mm) strips of plastic, and which therefore contain numerous free edges, and found that the EGFR was potently activated and down-regulated compared with control cultures containing no edges. This model system is likely to be generally useful in investigating the effects of free edges in epithelial cell sheets. For example, understanding the defective healing in chronic wounds is clearly very important, and it is noteworthy that the EGFR was reported to be down-regulated in epidermis that borders chronic venous ulcers (Brem *et al*., 2007).

How do cells coordinate their movements to migrate as a sheet into wounds? Time-lapse microscopy shows that epithelial cells within a confluent monolayer move vigorously in an apparent random manner, and because cells generally move away from one another after collisions, they tend to move into unoccupied areas, i.e., into the free space created by wounds. Bindschadler and McGrath (2007) have performed calculations based on hypothetical cells that respond to contact with other cells by changing direction of movement and probability of entering cell division using empirically determined parameters. This, and similar models (Walker *et al*., 2004; Hunt *et al*., 2009), predict collective cell migration that approximates experimentally observed cells. However, the models do not account for the increased speeds of cells at edges of wounds of MDCK and HCLE cells (Farooqui and Fenteany, 2005; Block and Klarlund, 2008) and at the edges of the plastic strips. So, in addition to changing direction, the cells also react to wounds by changing speeds. This implies the existence of an additional sensing mechanism, and the data presented here suggest that the presence of an edge in cell sheets is a cue that triggers signals that increase the speed of the cells.

Activation of the EGFR is key to wound healing in HCLE cells, but how might the presence of a free edge translate into a biochemical signal that eventually results in EGFR activation? We have excluded several possible mechanisms: activation is not due to extracellular ATP signaling (Boucher *et al*., 2007; Yin *et al*., 2007) or to breakdown of spatial segregation of the EGFR and its ligands (Figure 5) (Vermeer *et al*., 2003), and the transient mechanical perturbation that is induced immediately after wounding does not exist in cells grown on plastic strips. Also, cells grown on plastic strips are not exposed acutely to adjacent extracellular matrix, as is the case in more conventional scratch wounding models and the wounding model used in this study (Figure 1; Cabodi *et al*., 2004). The observation that edges constrained by agarose do not cause activation is significant, because it strongly suggests that suppression of activation is not due to any specific interaction between cells. For example, contact inhibition of cell locomotion is usually thought to result from activation of receptors on cells that recognize ligands on adjacent cells (Abercrombie, 1979; Carmona-Fontaine *et al*., 2008). Cell surface receptors in the outward-facing membrane of cells at the edges are not able to interact with other receptors. This could have significant consequences, for example, connexins are not expected to be able to form gap junctions, but rather they form hemichannels that have very different properties and that may allow influx of calcium ions and other molecules (Evans *et al*., 2006; Spray *et al*., 2006). Also, E-cadherin cannot dimerize with molecules on abutting cells (Desai *et al*., 2009). However, membrane proteins are also unable to interact with binding partners on adjacent cells in cells that are constrained by agarose blocks. In that situation the EGFR is not activated (Figure 6), and this type of mechanism therefore seems unlikely.

We suggest instead that activation of the EGFR is due to the physical environment of the cells. The intracellular distribution of forces within cells is complex (Cai and Sheetz, 2009; Howard, 2009; Wang *et al*., 2009), and is very likely to be different in cells inside a cell sheet from the forces in cells at free edges. It is becoming increasingly clear that forces detected by cells can have profound effects on almost every aspect of cell behavior (Knies *et al*., 2006; Chen, 2008; Ingber, 2008; Jaalouk and Lammerding, 2009; Mammoto and Ingber, 2009; Vogel and Sheetz, 2009). Forces are presumably detected at many subcellular structures including focal adhesions, the cytoskeleton, and cell–cell junctions. One of the most plausible mechanisms that can convert mechanical forces into a biochemical event is by partial unfolding of proteins that uncovers cryptic phosphorylation or binding sites for other proteins. This has clearly been described in talin and in p130Cas, but many other proteins seem to change conformation or assembly upon application of mechanical stress (Sawada *et al*., 2006; Johnson *et al*., 2007; del Rio *et al*., 2009). Another structure of interest is the cell membrane facing outward to the free space at edges. Cell components may exert outward forces that would be unopposed by counterforces from adjacent cells or agarose blocks, causing the membrane to stretch, which could trigger stretch-activated channels (Martinac, 2004).

Our finding that the existence of a free edge is sufficient to induce EGFR activation and cell motility certainly does not exclude that other cues may affect the healing process. In living organisms, wounded epithelia are inundated with stimuli that can fine-tune the phenotype of the cells. In this work, we found that the presence of edges only results in modestly enhanced secretion of MMP9 but that the levels of MMP9 production are greatly increased when the cells are allowed to spread on adjacent tissue culture space (Figure 8). Production of MMP9 is known to be influenced by integrins binding to extracellular matrix proteins (Han *et al*., 2006; Cortes-Reynosa *et al*., 2008; Lamar *et al*., 2008), and signals derived from such interactions are likely to be cues that induce maximal MMP9 production in combination with signals from the EGFR.

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