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Specific Signaling Cascades Involved in Cell Spreading During Healing of Micro-Wounded Gastric Epithelial Monolayers

Marie-Pier Tétreault, Pierre Chailler, Jean-François Beaulieu, Nathalie Rivard,* and Daniel Ménard*

Department of Anatomy and Cellular Biology, CIHR Team on Digestive Epithelium, Faculty of Medicine and Health Sciences, Université de Sherbrooke, Sherbrooke, Québec, Canada

ABSTRACT

Mechanisms that specifically modulate cell spreading and/or cell migration following epithelial wounding are poorly understood. Using micro-wounded human gastric epithelial monolayers, we show herein that EGF and TGFα maximally increase spreading of epithelial sheets under a cell proliferation-independent mechanism. Treatment of confluent HGE-17 cells with the phosphatidylinositol 3-kinase inhibitor, LY294002, and the epidermal growth factor receptor inhibitor, PD153035, strongly reduced basal and TGFα-stimulated cell spreading. While pharmacological inhibition of pp60src-kinase activity also attenuated basal epithelial spreading, addition of the mTOR/p70S6K inhibitor rapamycin or a specific siRNA targeting ILK sequence did not affect the kinetic rates of wound closure. Epithelial wound healing was initiated by actin purse-string contraction followed by lamellae formation. Conversely, disruption of actin and tubulin stability with cytochalasin D and nocodazole, respectively, inhibited epithelial sheet spreading. Finally, antibodies directed against the α3 integrin subunit, but not against the α6 or α2 subunits, attenuated epithelial sheet spreading as well as lamellae formation. In conclusion, the current investigation establishes that EGF/TGFα and the α3β1 integrin, pp60c-src, EGFR and PI3K pathways are mainly associated with the cell spreading of the restitution process during healing of human gastric epithelial wounds. J. Cell. Biochem. 105: 1240–1249, 2008.

KEY WORDS: CELL SPREADING; WOUND HEALING; GASTRIC EPITHELIAL CELLS; EGF/TGFα; SIGNALING PATHWAYS

The epithelium of human gastric mucosa which is involved in secretory and digestive functions [reviewed in Ménard and Basque, 2001] represents a crucial barrier between a broad spectrum of luminal noxious and immunogenic substances. Consequently, loss of cells from the epithelium as a result of damage and disruption of this barrier could be disastrous for the gastric mucosa and the organism unless it is quickly repaired. Hence mucosal wounds can be efficiently resealed by a process involving cohesive flattening and migration of epithelial cell sheets into the damaged area. This process termed restitution does not require proliferation and appears to be sufficient to reseal superficial injuries in vivo [Lacy, 1995; Tarnawski et al., 1995].

A recent study performed on intestinal epithelial T84 cells has allowed to distinguish three morphological zones in migrating epithelial monolayers [Hopkins et al., 2004]. Indeed migrating cells undergo a remarkable transition in cell shape, changing from columnar cells (zone III) to flattened elongated cells (zone II) that are induced to migrate (zone I). Moreover, epithelial cell movement relies on the ability of cells to reorganize their actin cytoskeleton. At the leading edge of cells, actin is thought to provide the protrusive force via three characteristic structures: filopodia spikes, ruffling lamellipodia and flat cytoplasmic lamellae [Sheetz et al., 1998; Etienne-Manneville, 2004]. Studies of wound repair using cultured epithelial cells have led to the consensus that large wounds induced in cell monolayers are resealed by extension of lamellipodia from cells at the wound margin followed by migration of cells into the denuded area [Rutten and Ito, 1983; Nusrat et al., 1992]. However, it now appears that re-construction of small epithelial wounds is a very common phenomena that operates through purse-string contraction [Florian et al., 2002; Russo et al., 2005], sometimes accompanied by formation of lamellae [Lotz et al., 2000].

Several studies have demonstrated that in vitro restitution of gastric epithelial cell monolayers is stimulated by a variety of growth factors (GFs) such as epidermal growth factor (EGF), transforming growth factor-α (TGFα), hepatocyte growth factor (HGF) and insulin-like growth factors I and II (IGF-I, IGF-II)

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*Correspondence to: Nathalie Rivard, PhD, and Daniel Ménard, PhD, Department of Anatomy and Cellular Biology, Faculty of Medicine and Health Sciences, Université de Sherbrooke, 3001 N. 12th Avenue, Sherbrooke, Québec, Canada J1H 5N4. E-mail: nathalie.rivard@usherbrooke.ca; daniel.menard@usherbrooke.ca

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[Watanabe et al., 1994; Maehiro et al., 1997; Jones et al., 1999; Kato et al., 1999; Hollande et al., 2001]. We have recently confirmed the involvement of GFs in the modulation of epithelial restitution of a new human gastric epithelial cell line (HGE-17) able to form tight monolayers [Chailler and Ménard, 2005] and revealed that these GFs differentially regulate the regeneration of gastric epithelial cells through specific modulation of cell shape adaptation, migration and proliferation [Tétreault et al., 2005]. These results argued against a functional redundancy of GF actions supporting the concept that a coordination of their activities is necessary for a normal progression of post-wounding epithelial repair.

Our understanding of the specific intracellular signaling pathways that transmit extracellular cues and regulate gastric epithelial restitution upon wounding is still incompletely understood, especially in humans [Tarnawski, 2005]. We have recently succeeded at the identification of major signaling pathways mediating the gastric epithelial restitution using confluent HGE-17 cell monolayers wounded with a razor blade [Tétreault et al., 2008]. Indeed, using well-characterized pharmacological inhibitors of signaling proteins, blocking antibodies and/or specific siRNA, we have established that EGF receptor-dependent PI3K activation promotes the restitution of wounded human gastric epithelial monolayers. An important question remains however unanswered that is which signaling pathways specifically trigger the first phase of the epithelial restitution, i.e. cell spreading? The objective of the present investigation was therefore to determine the specific GFs and intracellular signaling pathways involved in triggering HGE-17 cell spreading upon micro-wounding of gastric epithelial monolayers.

MATERIALS AND METHODS

ANTIBODIES AND CULTURE REAGENTS

Dulbecco's Modified Eagle Medium (DMEM), Ham-F12 (F12), penicillin/streptomycin and 0.25% trypsin–0.53 mM EDTA solution were purchased from Invitrogen (Burlington, ON, Canada). Fetal bovine serum (Cellect-Gold FBS) was obtained from ICN Pharmaceuticals (Montréal, QC, Canada). EGF, TGFα and IGF-I were obtained from Becton Dickinson Biosciences (Bedford, MA). HGF and LY294002 were obtained from Calbiochem (La Jolla, CA). Rapamycin was purchased from Biomol Research Laboratories Inc (Plymouth Meeting, PA). Hydroxyurea, nocodazole and cytochalasin D were from Sigma–Aldrich (Oakville, ON, Canada). PD98059 was from New England Biolabs (Mississauga, ON, Canada). Transforming growth factor-β1 (TGFβ1) was from R&D Systems (Minneapolis, MN). Rat antibody G0H3 (integrin α6 subunit), mouse antibody PIB5 (integrin α3 subunit) and mouse antibody P1E6 (integrin α2 subunit) were purchased from Chemicon International (Temecula, CA).

CELL CULTURE

The HGE-17 cell line was cloned in our laboratory from the NCI-N87 parental strain (CRL-5822; differentiated-type human gastric carcinoma from ATCC, American Type Culture Collection, Manassas, VA, USA) and previously characterized [Chailler and Ménard, 2005].

MICRO-WOUNDING ASSAY

Assays were performed according to a method standardized for T84 cells as previously reported [Lotz et al., 2000]. HGE-17 cells were seeded in 24-well plates and allowed to reach confluence. Small circular wounds (75–100 μm) were made by aspiration with a needle. Cultures were rinsed and further incubated in serum-reduced (0.1% FBS) DMEM:F12 medium (1:1, v:v) with or without exogenous regulators. Growth-independent effects on epithelial spreading were assessed by simultaneous addition of the cell-cycle blocker hydroxyurea at a concentration of 20 mM [Tétreault et al., 2008]. The modulatory effects of growth factors on cell spreading were assessed at the following concentrations known to optimize restitution [Tétreault et al., 2005]: TGFα (5 ng/ml), EGF (5 ng/ml), HGF (20 ng/ml) and IGF-I (100 ng/ml). In addition, putative intracellular effectors of cell spreading were identified by the use of specific inhibitors: LY294002 (5 μM), rapamycin (25 nM), PP2 (5 μM), PD153035 (10 μM), nocodazole (3 μM), cytochalasin D (1 μM). The involvement of the α2β1, α3β1 and α6β1/β4 integrins on cell spreading was also investigated by using the specific neutralizing [Basora et al., 1999] anti-α2 (PIE6), anti-α3 (PIB5) and anti-α6 subunit (GOH3) antibody at a final concentration of 10 μg/ml. Images were captured at different time intervals to assess the progression of wound closure using a Leica DM ERBE inverted microscope with phase-contrast optics. Wounding diameters were measured using Metamorph software (Universal Imaging Corporation, Downingtown, PA). The initial and final wound diameters were used to determine effects of growth factors and pharmacological inhibitors on wound closure kinetics.

LIGHT AND ELECTRON MICROSCOPY

Cell morphology was routinely visualized on a DMIL phase-contrast microscope. Epithelial ultrastructure was studied by scanning electron microscopy (SEM) on a Cambridge Stereoscan S-120 microscope. For this purpose, specimens seeded on sterile glass coverslips were fixed with 2.8% glutaraldehyde diluted in 0.2 M cacodylate buffer pH 7.4 containing 7.5% sucrose, post-fixed with 2% osmium tetroxide and dehydrated through a series of graded ethanol (40–100%). Following the removal of ethanol by critical point drying using liquid carbon dioxide, the specimens were coated with gold in a sputter coater S150B (Edwards) prior to ultrastructural examination.

FLUORESCENCE MICROSCOPY

Cells were seeded on 24-well plates and allowed to reach confluence. A wound was made at the center of the specimen before further incubation and processing. Cultures were fixed with 3.7% formaldehyde for 12 min and permeabilized with 0.1% Triton X-100 diluted in phosphate buffered saline (PBS, pH 7.4) for 3 min. After rinsing in PBS, cells were incubated inside humidified chambers with diluted F-actin directly labeled with phalloidin-FITC (1:800) for 60 min. Fluorescent staining was observed on a Reichert Polyvar microscope (Leica) equipped for epifluorescence. Negative controls were included in all experiments where the primary antibody was omitted or replaced by a non-immune serum.

STATISTICAL ANALYSIS

Results were expressed as mean ± SEM, with statistical significance of differences between experimental conditions established at 95%. One-way ANOVA and Student's *t*-test were used to indicate the statistical difference between the groups.

RESULTS

CELL SPREADING DYNAMICS FOLLOWING WOUNDING OF GASTRIC EPITHELIAL MONOLAYERS

The spreading capacity of a human gastric epithelial sheet was evaluated by wounding coherent monolayers by aspiration with a needle, followed by incubation with 20 mM hydroxyurea. This agent, which inhibits the synthesis of deoxynucleoside triphosphates and arrests cells in G1/S phase, allowed us to specifically focus on the cell spreading process since all possible proliferative activities were blocked. Images were acquired every 2 h until complete closure of the wound. Diameter of the wound was measured, after which the values were compared with the initial diameter measurement and the percentage of wound closure determined for each time point. Following wounding, the initial wound diameter was approximately 75–100 μm. Figure 1 (panels 1–6) illustrates the response of newly confluent HGE-17 cultures in serum-reduced conditions. In basal conditions (absence of added GFs), the wound was completely closed after 14 h (Fig. 1A, panel 6). The sequence of events involved in the closure of HGE-17 wounds was also analyzed. After 4 h, a refractile purse string surrounding the wound edge was observed. At 6 h, lamellae protrusion began to be observed in cells bordering the wound and gradually, the wound was entirely covered by lamellae. Approximately at the same time, cells surrounding the wound edge started to elongate and significantly increased their surface area by twofold. Based on these observations, it appeared that a pulling force on bordering cells was produced by purse-string (Fig. 1A, panel 2, see arrows) and lamellae formation (Fig. 1A, panel 3, see arrows), which induced these cells to flatten. Scanning electron microscopy (SEM) was used to detail the ultrastructure of micro-wounded HGE-17 monolayers (Fig. 1B). Confirming microscopic observations, SEM revealed the persistence of cell–cell contacts and flattening of bordering cells, thus validating that closure of gastric epithelial micro-wounds occurs only by cell spreading. Moreover, purse-string ring (Fig. 1B, asterisks) and lamellae formation (Fig. 1B, arrowheads) were observed at the wound margin. Conversely, lamellipodia protrusions (migratory structures) were never observed during the entire closure process, whether it is in the absence or presence of TGFα. Therefore, the micro-wounding of the HGE-17 cell monolayers is an ideal model to specifically study cell spreading.

TGFα AND EGF ARE POTENT INDUCERS OF GASTRIC EPITHELIAL CELL SPREADING

The effects of different exogenous growth factors on the spreading capacity of human gastric epithelial sheets were subsequently evaluated. As shown in Figure 2, added TGFα and EGF were able to drastically accelerate the spreading response of wounded HGE-17 monolayers, reducing by 50% the time of wound closure comparatively to controls. HGF, at 20 ng/ml, constantly accelerated

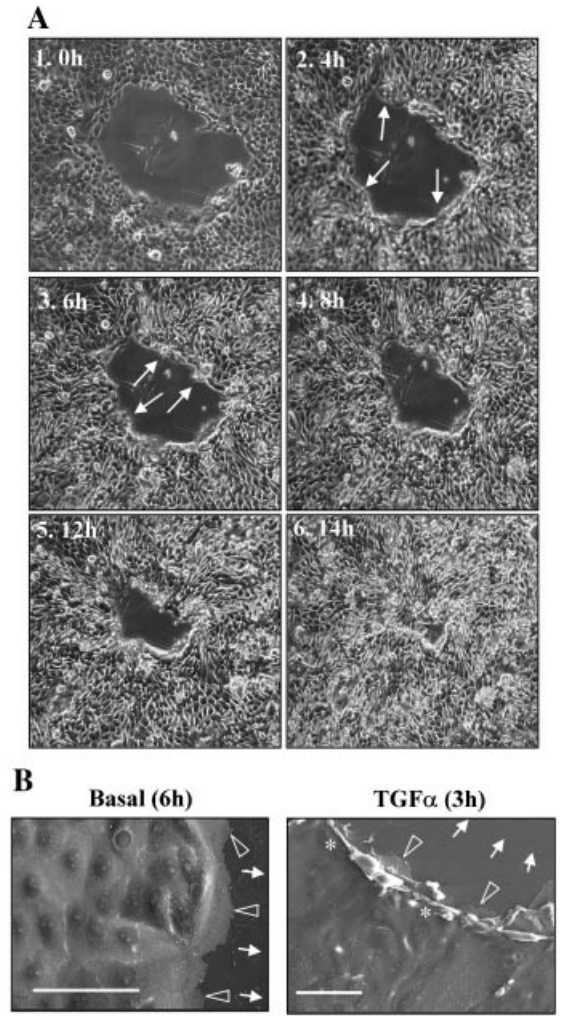

Fig. 1. Sequence of events occurring during wound closure of HGE-17 monolayers following micro-wounding. A: Newly confluent HGE-17 cells were micro-wounded by needle aspiration and treated with 20 mM hydroxyurea. The wound closure process was examined by time-lapse microscopy. Times in upper left corners refer to the number of hours elapsed after wounding. Panel 1: The first image of the wound was captured immediately after micro-wounding. Panel 2: At 4 h after wounding, a refractile ring surrounding the wound is evident. Panels 3–6: Following the appearance of the refractile ring, cells bordering the wound begin to elongate and their surface is also increased by twofold. As the refractile ring becomes less evident, formation of protrusions such as lamellae are observed at the surface of marginal cells. Panel 6: By 14 h, the wound is completely closed. B: Ultrastructural observations by scanning electron microscopy of spreading HGE-17 monolayers 6 h (left panel) and 3 h (right panel) after wounding. In 6 h controls, lamellae are observed at the surface of most bordering cells (open arrow head). The presence of 5 ng/ml TGFα elicits intense cytoplasmic spreading. At 3 h, lamellae protrusions are observed at the surface of edge cells. Lamellipodia were never observed during the entire closure process. White arrows indicate the direction of wound closure by cell spreading in each micrograph. Asterisks indicate the purse-string contraction of a ring of actin filaments that completely encircles the wound edge. Bars, 50 μm.

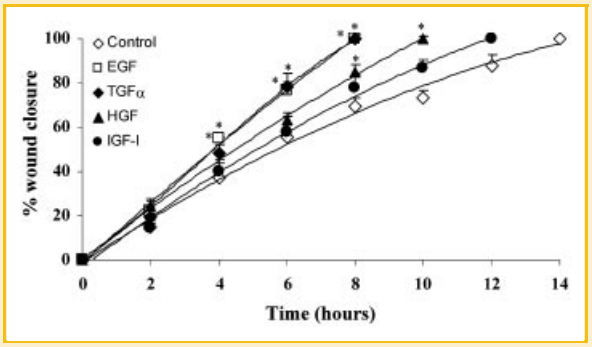

Fig. 2. Progression of wound closure of HGE-17 monolayers in the presence of 0.1% serum and various growth factors. Wound closure is determined by the area covered by spreading cells (in %) as a function of time (in h) by HGE-17 cells in the absence or presence of growth factors. Control; 5 ng/ml EGF; 5 ng/ml TGFα; 20 ng/ml HGF; 100 ng/ml IGF-I. Results are the mean ± SEM of five different experiments. $^{*}P < 0.05$ compared with control.

the progression of wound closure and was half as effective in surface covering activity compared to TGFα/EGF. By contrast, exogenous addition of IGF-I exerted no significant effect. Microscopic video time lapse analysis revealed that closure of TGFα-stimulated wounds involved the same morphologically defined events as recorded in basal conditions (purse-string ring formation, cell elongation and lamellae protrusion). At 2 h post-wounding, TGFα induced a purse-string ring. Thereafter, cells at the wound margin gradually produced lamellae protrusions. Finally, the wound was entirely covered by lamellae after 4 h (data not shown).

ROLE OF Src, EGFR AND PI3K-DEPENDENT PATHWAYS IN GASTRIC EPITHELIAL CELL SPREADING

The use of HGE-17 cell monolayers wounded with a razor blade and treated with specific pharmacological inhibitors of PI3K (LY294002), EGFR (PD15305) and pp60c-src (PP2) signaling proteins permitted the validation of their efficacy and optimal concentrations for inhibiting these signaling pathways [Tétreault et al., 2008]. These data were paramount as the small number of wounded cells in micro-wounding assays does not allow quantitative measurements. We were then in a unique position to evaluate the impact of these inhibitors solely on HGE-17 cell spreading. As shown in Figure 3A and B, the inhibition of PI3K activity by LY294002 resulted in a marked decrease in wound closure, in both the presence and absence of TGFα. To examine whether activation of the EGFR is necessary for cell spreading in micro-wound healing, an inhibitor of the EGFR kinase, PD153035, was added. This inhibitor was found to strongly decrease spreading of cells both in basal and TGFα stimulated wounds Figure 3A and B. Our previous observation that pp60c-src is rapidly activated upon epithelial wounding [Tétreault et al., 2008], led us to verify whether this effector was required for gastric cell spreading. As shown in Figure 3A, pretreatment with PP2, an inhibitor of members of the Src tyrosine kinase family [Hanke et al., 1996], markedly attenuated the course of wound closure. Therefore, the use of pharmacological inhibitors confirmed the paramount importance of Src/EGFR/PI3K

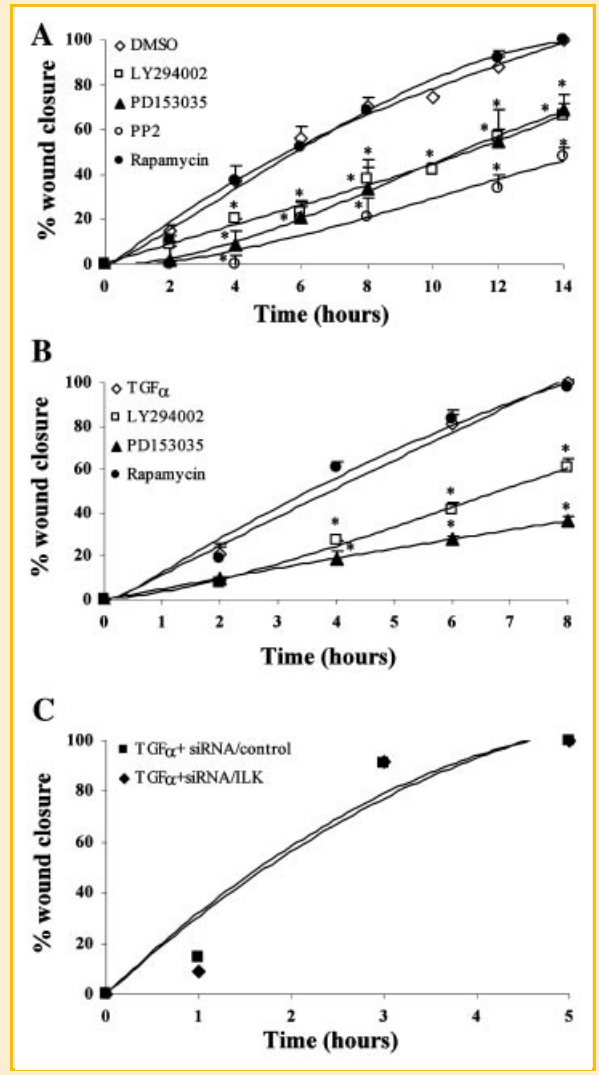

Fig. 3. Signaling through the EGFR/PI3K pathway is necessary for basal and TGFα-stimulated gastric cell spreading. A: Newly confluent HGE-17 cells were wounded and treated with 20 mM hydroxyurea, in absence (DMSO) or presence of 10 μM PD153035, 25 nM rapamycin, 5 μM LY294002 and 5 μM PP2. Images of the denuded wound area were acquired every 2 h until complete closure of the wound and the percentage of wound closure determined by comparison of the denuded area immediately following micro-wounding. B: Newly confluent HGE-17 cells were wounded and treated with 20 mM hydroxyurea, in the presence of TGFα (5 ng/ml) and in the absence (DMSO) or presence of 10 μM PD153035, 25 nM rapamycin and 5 μM LY294002. Images of the denuded wound area were acquired every 2 h until the complete closure of the wound and the percentage of wound closure determined by comparison of the denuded area immediately following micro-wounding. C: HGE-17 cells were nucleofected with a control siRNA or with siRNA against ILK which has been previously shown to down-regulate ILK protein expression in HGE-17 cells (Tétreault et al., 2008). Five days after nucleofection, confluent cells were wounded and treated with 20 mM hydroxyurea. Images of the denuded wound area were acquired every 2 h until the complete closure of the wound and the percentage of wound closure determined by comparison of the denuded area immediately following micro-wounding. Results are the mean ± SEM of at least three separate experiments. $^{*}P < 0.05$ compared with control.

pathways on human gastric epithelial cell spreading in basal conditions and of EGFR/PI3K pathways in TGFα-stimulated cell spreading following wounding.

PIP_3 is the major lipid product of PI3K that acts as a docking protein for several pleckstrin homology (PH) domain-containing proteins [Cantrell, 2001] and controls the activity of various signaling effectors. Among these, phosphoinositide dependent-kinase 1 (PDK1) mediates the activation of Akt, integrin-linked kinase (ILK) and p70S6K. To determine whether activation of mTOR/p70S6Kinase recorded following wounding of HGE-17 epithelial sheets [Tétreault et al., 2008] was specifically involved in regulating human gastric cell spreading, confluent cells were incubated in the presence of rapamycin (mTOR inhibitor), and their effects analyzed on wound closure kinetics following micro-wounding in basal and TGFα-stimulated conditions. As shown in Figure 3A and B, pretreatment of HGE-17 cells with rapamycin did not abolish cell spreading. The possible role of ILK which is also involved in the restitution process of HGE17 epithelial sheets upon wounding was also investigated. A validated synthetic double-stranded siRNA directed against human ILK gene sequence was transfected into HGE-17 cells to inhibit ILK protein synthesis. A commercial synthetic double-stranded control non-silencing siRNA was used as a control. Cells transfected with siRNA that targets ILK sequence exhibited strongly reduced ILK protein synthesis, in contrast to cells transfected with control siRNA [Tétreault et al., 2008]. This siRNA was subsequently used to determine whether reduced ILK expression affects HGE-17 wound closure following micro-wounding. Confluent HGE-17 cell monolayers transfected with siRNA/ILK were micro-wounded after which the percentage of wound closure was determined. As shown in Figure 3C, reduction of ILK expression did not affect wound closure kinetics induced by wounding when compared to cells transfected with a control siRNA. It is important to note that none of the inhibitors used affected HGE-17 cell viability [Tétreault et al., 2008], thus excluding the possible interference of cell death in the recorded signaling pathways following wounding.

MICRO-WOUNDS IN HGE-17 MONOLAYERS RESEALS BY ACTIN PURSE STRING FORMATION AND LAMELLAE PROTRUSION

Microscopic images of HGE-17 after wounding suggested that the refractile ring observed during the closure of a micro-wound, in the presence or absence of exogenous TGF-α, is an actin purse string phenomenon similar to what has been shown in other models [Martin and Lewis, 1992; Bement et al., 1993; Lotz et al., 2000] and that cytoskeleton rearrangements were important in the wound closure process accomplished only by cell spreading. Hence, further analyses of purse-string and lamellae using phalloidin-FITC for F-actin were performed on HGE-17 monolayers at different time intervals after wounding (Fig. 4, panels 1–6). Immediately after wounding, filamentous actin was mainly concentrated at the periphery of cells (panels 1 and 2, see arrows) and staining was more intense in the first three rows of bordering cells. At 4 h, cells bordering the wound displayed a continuous F-actin belt whereas F-actin was still localized at the periphery of cells away from the injury (panels 3 and 4). The wound was completely covered by lamellae at 8 h after wounding. Phalloidin-FITC staining demonstrated the presence of F-actin within the lamellae protruding into the wound (panels 5 and 6, see arrows). In parallel to actin staining, the distribution of microtubules, was also investigated during the wound closure process. For this purpose, α-tubulin staining was performed on HGE-17 monolayers at different time points after wounding. Figure 4A (panels 7–9) reveals the distribution of microtubules in the subset of bordering cells. By contrast, immunofluorescent staining for α-tubulin protein was localized in the cytoplasm of cells surrounding the wound with cells appearing to have a decreased amount of microtubules (Fig. 4, panels 8 and 9, see arrowheads). A similar distribution of F-actin and α-tubulin was observed in wounded monolayers stimulated by TGFα, albeit under a faster stimulation of cell flattening. Indeed, the actin purse string was observed within 2 h after wounding and lamellae formation was activated after 4 h (Fig. 4B) whereas microtubule polymerization occurred only into lamellae of bordering cells (data not shown). In order to ascertain that cytoskeletal reorganizations are necessary for micro-wound healing, inhibitors of actin organization and of microtubule polymerization, cytochalasin D and nocodazole, respectively, were added. These agents were found to significantly inhibit wound closure both in basal (data not shown) and TGFα stimulated conditions (Fig. 4C). These data strongly suggest that actin and to a lesser degree microtubule rearrangement are central in regulating the processes that induce gastric cell sheet spreading following wounding.

α3β1 INTEGRINS CONTROL CELL SHEET SPREADING OF HUMAN GASTRIC EPITHELIAL CELLS

Our recent observation that α3β1 and α2β1 integrins are involved in the restitution of gastric epithelial cells [Tétreault et al., 2006] prompted us to verify their role in cell spreading. Thus, α2-subunit and α3-subunit neutralization assays were performed during the healing process of wounded HGE-17 monolayers. As shown in Figure 5A and B, addition of 10 μg/ml anti-α3 resulted in a significant decrease (40%) in the speed of wound closure, either in absence (A) or presence of TGFα (B). Surprisingly, neutralization of α2-subunit had no significant effect on basal and TGFα-stimulated HGE-17 cell spreading (Fig. 5A and B). The potential involvement of α6-integrin subunit, which has been demonstrated to be implicated in both adhesion [Basora et al., 1999] and migration [Lotz et al., 2000] in intestinal epithelial cells, was also tested. As seen in Figure 5A and B, neutralization of α6-subunit did not significantly alter the rate of HGE-17 wound closure, in either basal or stimulated conditions.

To decipher how α3-subunit contributes to cell spreading in micro-wounded HGE-17 monolayers, immunolocalization of this subunit protein was assessed at various times during the healing process. Immunofluorescent staining for α3-subunit was enhanced in the cytoplasm of cells surrounding the wound (Fig. 6A, panel 1) and redistributed to lamellae of edge cells (Fig. 6A, panels 2–5, see arrows). Neutralization assays were then performed at 4 h after wounding and the respective distributions of F-actin and α-tubulin examined. As shown in Figure 6B (panels 1–2, see arrows), neutralization of α3-subunit but not α-2 subunit (not shown) strongly reduced lamellae protusion in basal and TGFα-stimulated

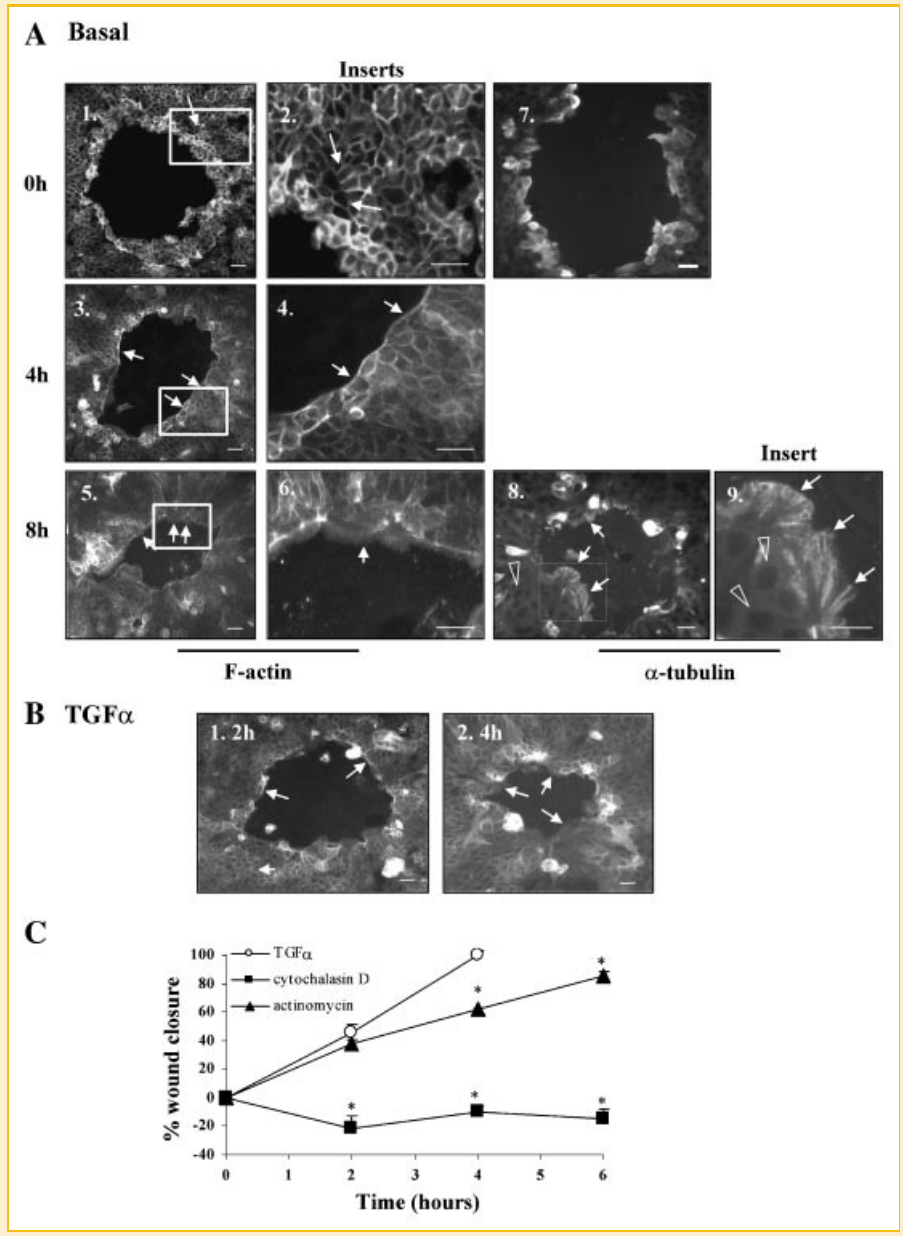

Fig. 4. Cytoskeletal rearrangement during spreading of HGE-17 cells following micro-wounding. A: Newly confluent HGE-17 cells were wounded by needle aspiration and treated with 20 mM hydroxyurea. Cells were fixed at different time intervals for immunofluorescence and stained for F-actin with FITC-phalloidin or stained for α-tubulin. Panels 1 and 2: Immediately after wounding, F-actin is seen localized at the periphery of cells. Panels 3 and 4: In controls, at 4 h after wounding, the micro-wound is surrounded by a continuous actin belt or purse-string (arrows). Panels 5 and 6: In controls, at 8 h after wounding, bordering cells elongate towards the denuded area and exhibit lamellae along the periphery of the wound (arrows). Panel 7: Immediately following epithelial wounding, α-tubulin is mainly localized in the cytoplasm. Panels 8 and 9: 8 h after wounding, α-tubulin is mainly redistributed to the surface lamellae of edge cells (arrows) while it remains localized in the cytoplasm as in Panel 7 (arrowheads). Panels 2, 4, 6 and 9 represent higher magnifications of the images contained within the boxed areas in panels 1, 3, 5 and 8, respectively. B: Newly confluent HGE-17 cells were wounded and treated with 20 mM hydroxyurea, in the presence of 5 ng/ml of TGFα. Panel 1 (arrows): Actin purse-string formation can be seen around the circumference of the wound after 2 h. Panel 2: The wound is surrounded by lamellae (arrows). C: Confluent HGE-17 monolayers were wounded and treated with 20 mM hydroxyurea, in the presence of 5 ng/ml of TGFα for 6 h. Cells were also treated for 6 h with cytochalasin D (1 μM) or nocodazole (3 μM). Images of the denuded wound area were acquired every 2 h until complete closure of the wound and the percentage of wound closure determined by comparison of the denuded area immediately following micro-wounding. Results are the mean ± SEM of at least three separate experiments. $^{*}P < 0.05$ compared with control. Bars, 50 μm.

conditions. Neutralization of α3-subunit did not prevent actin ring assembly around the wound edge, but cells surrounding the wound edge were less elongated and less flattened. Moreover, treatment with anti-α3 markedly attenuated the polymerization of micro-tubules in cells bordering the wound (Fig. 6B, panels 3 and 4, see arrows). These observations suggest that α3β1 integrin contributes to generate the pulling force that induce bordering cells to flatten and to lamellae formation.

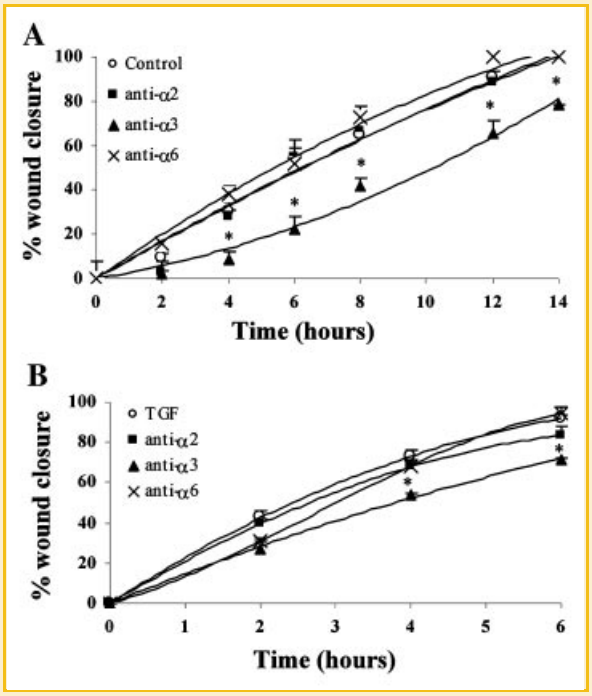

Fig. 5. α3β1 integrin is necessary for basal and TGFα stimulated gastric epithelial cell sheet spreading following micro-wounding. Wounds were made in the absence (A) or presence (B) of TGFα and in the presence or absence of neutralizing antibody against the α3, α2 or α6 integrin subunit. Images of the denuded wound area were acquired every 2 h until the complete closure of the wound and the percentage of wound closure determined by comparison of the denuded area immediately following micro-wounding. Results are the mean ± SEM of at least three separate experiments. $^{*}P < 0.05$ compared with control.

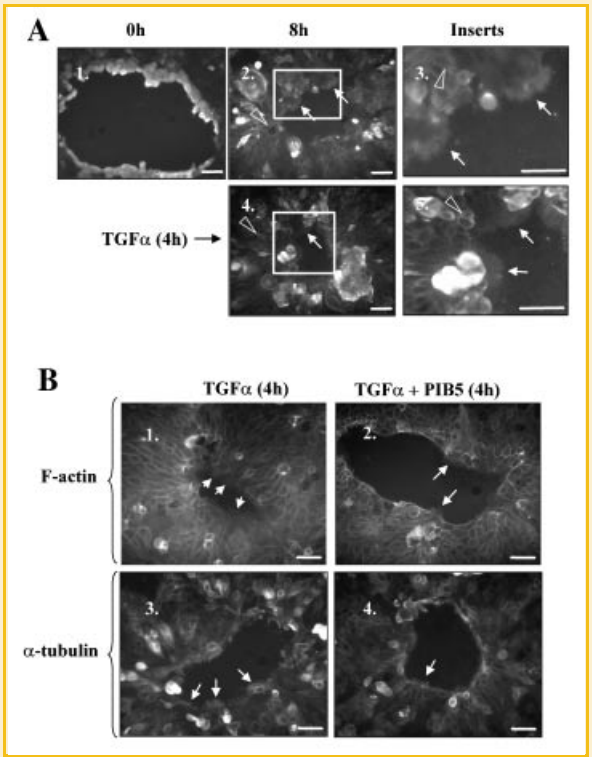

Fig. 6. α3β1 Integrin regulates lamellae formation after micro-wounding of gastric epithelial monolayers. A: Wounded HGE-17 monolayers, stimulated or not by TGFα 5 ng/ml, were fixed for immunofluorescence and stained for expression of the α3 integrin subunit. Panel 1: Immediately after wounding, α3 integrin subunit staining is more intense in the damage cells surrounding the wound. Within 8 h of wounding in basal conditions (Panels 2 and 3) and/or 4 h of wounding in TGFα-stimulated conditions (Panels 4 and 5), staining for α3 integrin subunit revealed its presence in the cytoplasm of cells adjacent to the wound (arrowheads) and to the outermost edge of the lamella (white arrows). B: Wounded HGE-17 monolayers stimulated with TGFα 5 ng/ml, in the presence or absence of a neutralizing antibody against the α3 integrin subunit (PIB5, 10 μg/ml), were fixed for immunofluorescence and then stained for F-actin with phalloidin-FITC (panels 1 and 2) or stained for the detection of α-tubulin (panels 3 and 4). In the wounds treated with TGFα for 4 h, F-actin (panel 1) and α-tubulin (panel 3) staining demonstrated lamellae formation in cells surrounding the wound (white arrows). Panels 2 and 4: Neutralization of the α3 integrin subunit did not prevent the formation of a ring of actin that completely encircles the wound, but unlike the control wounds, only few lamellae are seen (white arrows). In response to wounding, cells that were treated with a neutralizing antibody against the α3 integrin subunit are less flatten and less elongated compared to wounded HGE-17 monolayers stimulated with TGFα. Bars, 50 μm.

DISCUSSION

Rapid re-establishment of mucosal epithelial continuity is an ordered process of cell spreading and motility into the denuded area that is characterized by persistence of cell–cell contacts and monolayer integrity. This process, which is termed restitution, is crucial for rapidly repairing any form of injury to the gastric mucosa. While our understanding of the intracellular signaling pathways that control gastric epithelial restitution is now starting to be elucidated [Tétreault et al., 2008], little is known regarding the molecular events that specifically control its initial phase, i.e., cell spreading. Hence, the present study was conducted to discern which growth factors and intracellular signaling pathways are involved in the regulation of epithelial cell spreading during gastric epithelial sheet restitution following micro-wounding in vitro. For this purpose, the human gastric epithelial cell model HGE-17 was used. HGE-17 represents the first human gastric cell line which displays a true epithelial phenotype forming a dense coherent monolayer which steadily expresses epithelial-type junction components as well as generating transepithelial electrical resistance upon reaching confluence [Chailler and Ménard, 2005]. In an experimental wounding assay, a confluent monolayer of HGE-17 cells is able to regenerate as a fully coherent epithelial sheet via a coordination of cell spreading, migration and proliferation [Tétreault et al., 2005]. As shown herein, small wounds (75–100 μm) made in a newly confluent monolayer of HGE-17 only repair by cell sheet spreading, a process characterized by the persistence of cell–cell contacts and the absence of lamellipodia formation during the entire process. By contrast to the razor blade method, this micro-wounding model allowed us to eliminate the cell migration component and was therefore auspicious in focusing uniquely on the cellular and molecular events involved in cell spreading of gastric monolayers following epithelial wounding. This micro-wound model was also

successfully used to study colonic epithelial cell spreading following wounding of T-84 cell sheets [Lotz et al., 2000].

Wound closure involves a coordinated process of actin purse-string formation, cell flattening and lamella protrusion at the wound margin, corresponding precisely to cellular events already described in other intestinal epithelial cells lines (T84, Caco-2) [Bement et al., 1993; Lotz et al., 2000]. Indeed, gastric epithelial wound closure begins with an actin purse string which generates a pulling force that is communicated to neighboring cells, inducing them to flatten and stretch toward the injury. Thereafter, cells at the wound margin extend lamellae to cover additional surface of the denuded area and to generate a supplementary pulling force on the adjacent monolayer. A novel finding from the present investigation is the visualization of microtubule polymerization into emerging lamellae following epithelial micro-wounding. Extension of microtubules towards the leading edge has also been demonstrated to contribute to membrane delivery, thereby promoting membrane extension [Bretscher, 1996]. Moreover, in microtubule-rich regions of the protrusion of slow migrating astrocytes, these organelles have been postulated to select, stabilize and potentiate the formation of focal adhesions [Etienne-Manneville and Hall, 2001]. Hence, our findings suggest that microtubule polymerization stabilizes the flattened cytoplasm of spread gastric epithelial cells.

Interestingly, closure of the micro-wounded gastric epithelial sheet by cell spreading was relatively slow in basal conditions and greatly accelerated by exogenous growth factors. Two growth factors belonging to the same family, TGFα and EGF, were potent inducers of HGE-17 cell spreading after micro-wounding, as determined by wound area measurement and time lapse microscopy of wound edge movement. In the presence of HGF, epithelial sheet spreading was moderately accelerated compared to EGF and TGFα. These results are in accordance with our previous microscopic observations where HGF was found to stimulate cell spreading during gastric restitution, but not as intensively as EGF or TGFα [Tétreault et al., 2008]. The data further highlight the major role of EGF and TGFα in the regulation of gastric epithelial regeneration following wounding and support previous observations reporting the induction a novel EGF-secreting cell lineage during healing of gastric mucosal ulceration in vivo [Wright et al., 1990]. Hence, we suggest that EGF/TGFα action during gastric epithelial regeneration likely favors the phenotypic adaptation of differentiated cells in order to better respond to cell migration and proliferation stimuli.

In order to gain better insight into mechanisms underlying the gastric regulation of cell spreading upon micro-wounding, we also examined the intracellular pathways known to be involved in HGE-17 epithelial restitution [Tétreault et al., 2008]. Indeed, we have previously established that wounding gastric epithelial monolayers transactivates gastric cell EGFR through a pp60c-src-dependent manner and that this pp60c-src activation is upstream of EGFR. We demonstrate herein that inhibition of PI3K and EGFR activities strongly reduced micro-wound closure kinetics by cell spreading in both the presence and absence of TGFα. Furthermore, our results indicate that c-src is required for gastric cell spreading in basal conditions. The present data are therefore in agreement with the previously described signaling pathway sequences providing evidence of the early implication of EGFR/PI3K pathway in the process of gastric cell spreading, the latter being activated by stimulation with TGFα or by activation of pp60c-src [Tétreault et al. 2008]. Another noteworthy finding from this investigation is the demonstration that p70S6K and ILK effectors are not required for cell spreading of gastric epithelial monolayers. In the light of previous results observed on larger gastric epithelial wounds in which healing requires epithelial restitution [Tétreault et al., 2008], it is clear that activation of mTOR/p70S6K and ILK only promotes cell migration following wounding. The role of mTOR/p70S6K [Chandrasekher et al., 2001; Qian et al., 2004] and ILK [Zhang et al., 2002; Qian et al., 2005] in cell migration has been well-established in different cellular models. The present investigation, for the first time, identifies specific signaling effectors associated with the early (cell spreading) and late phase (cell migration) of human gastric epithelial restitution.

Cell spreading is also known to be initiated by integrin-mediated cell-matrix interaction [Cuvelier et al., 2007]. Gastric epithelial restitution likely involves α2β1 and α3β1 integrins [Tétreault et al., 2006]. Since α3β1 and α6β4 integrins have been reported to contribute to wound closure in intestinal T84 cells [Lotz et al., 2000], the potential implication of α2, α3 and α6 integrin subunits was therefore examined. Herein, neutralization of the α3-integrin subunit strongly reduced the progression of wound closure following epithelial micro-wounding. Because neutralization of α3 integrin subunit did not prevent the formation of an actin ring at the wound edge, our data suggest that α3β1 integrin is not necessary for the initial phase of wound closure following epithelial micro-wounding. Our observation that stretching and flattening of cells surrounding the wound is altered following neutralization of α3-subunit suggests that α3β1 integrin is involved in the transmission of the force of contraction across cells in response to epithelial micro-wounding. Moreover the observation that α3-subunit is specifically redistributed toward the emerging lamellae of bordering cells further suggests that α3β1 integrin participates in lamellae formation. Moreover, neutralization assays with anti-α3 during wound closure strongly abolished lamellae extension, corroborating this central role of α3β1 integrin in lamellae formation. It would be quite interesting to explore the effects of α3β1 inhibition on Src, EGFR and P13K activation. Unfortunately, the small number of wounded cells in the micro-wounded epithelial sheet does not yet allow such quantitative studies. Such role of α3β1 integrin lamellae protrusion has also been reported in intestinal cells [Lotz et al., 2000]. Antibody inhibition assays also demonstrated that the α2-subunit is likely not involved in the modulation of cell sheet spreading following epithelial wounding. Since α2β1 integrin has been shown to be an important modulator of gastric epithelial restitution [Tétreault et al., 2006], this result strongly suggests the specific involvement of this integrin in the control of the migratory phase during gastric epithelial restitution. Finally the observation that neutralization of α6-subunit did not affect the rate of wound closure discards the potential involvement of this integrin-subunit in the control of cell sheet spreading following micro-wounding. This latter result is in contrast to that of a previous study performed on intestinal T84 cells which demonstrated the role of α6β4 integrins in cell retraction and lamellae formation during wound healing indicating that the

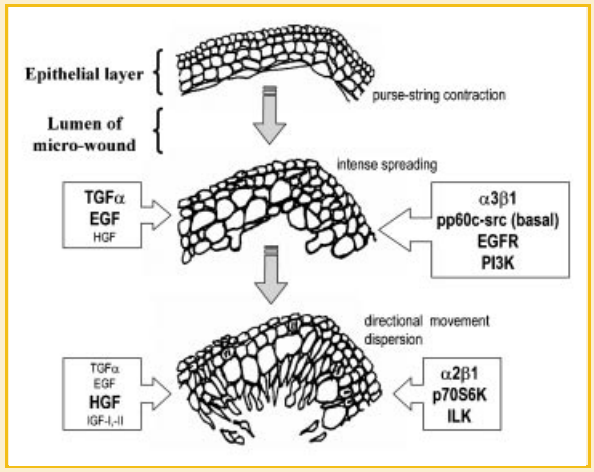

Fig. 7. Schematic illustrations of growth factor bioactivities and of proposed specific pathways involved in the regulation of cell spreading (present investigation) and cell migration (Tétreault et al., 2008) during human gastric epithelial restitution.

mechanisms controlling cell spreading during epithelial restitution may vary according to the tissue or the functionality of α6β4 receptors known to depend on specific isoform expression [Teller and Beaulieu, 2001; Ni et al., 2005].

In conclusion, Figure 7 summarizes our current understanding of the regulatory processes involved in the restitution sequence: purse-string formation, spreading and motility. Data shown herein clearly demonstrate that growth factors EGF/TGFα are crucial for intensifying cytoplasmic spreading of epithelial sheets following epithelial micro-wounding of human gastric epithelial cells. The initial stimulatory signal is still unknown but may relate to interactions of α3β1 integrins with appropriate ligands and/or to mechanical stress in cells, induced by wounding and activating pp60c-src. These events trigger phosphorylation and activation of EGFR. In addition, EGFR can be activated by soluble ligands present in the wound milieu such as TGFα and EGF. Finally, subsequent activation of EGFR results in the activation of PI3K which engages the directional movement and dispersion of gastric epithelial cells resulting in subsequent healing of the gastric epithelial wound.

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REFERENCES

Basora N, Herring-Gillam FE, Boudreau F, Perreault N, Pageot LP, Simoneau M, Bouatrouss Y, Beaulieu JF. 1999. Expression of functionally distinct variants of the beta(4)A integrin subunit in relation to the differentiation state in human intestinal cells. J Biol Chem 274:29819–29825.

Bement WM, Forscher P, Mooseker MS. 1993. A novel cytoskeletal structure involved in purse string wound closure and cell polarity maintenance. J Cell Biol 121:565–578.

Bretscher MS. 1996. Getting membrane flow and the cytoskeleton to cooperate in moving cells. Cell 87:601–606.

Cantrell DA. 2001. Phosphoinositide 3-kinase signalling pathways. J Cell Sci 114:1439–1445.

Chailler P, Ménard D. 2005. Establishment of human gastric epithelial (HGE) cell lines exhibiting barrier function, progenitor, and prezymogenic characteristics. J Cell Physiol 202:263–274.

Chandrasekher G, Kakazu AH, Bazan HE. 2001. HGF- and KGF-induced activation of PI-3K/p70 s6 kinase pathway in corneal epithelial cells: Its relevance in wound healing. Exp Eye Res 73:191–202.

Cuvelier D, Théry M, Chu YS, Dufour S, Thiéry JP, Bornens M, Nassoy P, Mahadevan L. 2007. The universal dynamics of cell spreading. Curr Biol 17:694–699.

Etienne-Manneville S. 2004. Actin and microtubules in cell motility: Which one is in control? Traffic 5:470–477.

Etienne-Manneville S, Hall A. 2001. Integrin-mediated activation of Cdc42 controls cell polarity in migrating astrocytes through PKCzeta. Cell 106:489–498.

Florian P, Schoneberg T, Schulzke JD, Fromm M, Gitter AH. 2002. Single-cell epithelial defects close rapidly by an actinomyosin purse string mechanism with functional tight junctions. J Physiol 545:485–499.

Hanke JH, Gardner JP, Dow RL, Changelian PS, Brissette WH, Weringer EJ, Pollok BA, Connelly PA. 1996. Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor. Study of Lck- and FynT-dependent T cell activation. J Biol Chem 271:695–701.

Hollande F, Blanc EM, Bali JP, Whitehead RH, Pelegrin A, Baldwin GS, Choquet A. 2001. HGF regulates tight junctions in new nontumorigenic gastric epithelial cell line. Am J Physiol Gastrointest Liver Physiol 280:G910–G921.

Hopkins AM, Bruewer M, Brown GT, Pineda AA, Ha JJ, Winfree LM, Walsh SV, Babbin BA, Nusrat A. 2004. Epithelial cell spreading induced by hepatocyte growth factor influences paxillin protein synthesis and post-translational modification. Am J Physiol Gastrointest Liver Physiol 287:G886–G898.

Jones MK, Tomikawa M, Mohajer B, Tarnawski AS. 1999. Gastrointestinal mucosal regeneration: Role of growth factors. Front Biosci 4:D303–D309.

Kato K, Chen MC, Nguyen M, Lehmann FS, Podolsky DK, Soll AH. 1999. Effects of growth factors and trefoil peptides on migration and replication in primary oxyntic cultures. Am J Physiol 276:G1105–G1116.

Lacy ER. 1995. Rapid epithelial restitution in the stomach: An updated perspective. Scand J Gastroenterol (Suppl 210):6–8.

Lotz MM, Rabinovitz I, Mercurio AM. 2000. Intestinal restitution: Progression of actin cytoskeleton rearrangements and integrin function in a model of epithelial wound healing. Am J Pathol 156:985–996.

Maehiro K, Watanabe S, Hirose M, Iwazaki R, Miwa H, Sato N. 1997. Effects of epidermal growth factor and insulin on migration and proliferation of primary cultured rabbit gastric epithelial cells. J Gastroenterol 32:573–578.

Martin P, Lewis J. 1992. Actin cables and epidermal movement in embryonic wound healing. Nature 360:179–183.

Ménard D, Basque JR. 2001. Gastric digestive functions. In: Delvin EE, Lentze MJ, editors. Gastrointestinal functions, Nestlé Workshop Series, vol. 46. Philadelphia: Vevey/Lippincott Williams & Wilkins. pp 147–163.

Ni H, Dydensborg AB, Herring FE, Basora N, Gagné D, Vachon PH, Beaulieu JF. 2005. Upregulation of a functional form of the beta4 integrin subunit in colorectal cancers correlates with c-Myc expression. Oncogene 24:6820–6829.

Nusrat A, Delp C, Madara JL. 1992. Intestinal epithelial restitution. Characterization of a cell culture model and mapping of cytoskeletal elements in migrating cells. J Clin Invest 89:1501–1511.

Qian Y, Corum L, Meng Q, Blenis J, Zheng JZ, Shi X, Flynn DC, Jiang BH. 2004. PI3K induced actin filament remodeling through Akt and p70S6K1: Implication of essential role in cell migration. Am J Physiol Cell Physiol 286:C153–C163.

Qian Y, Zhong X, Flynn DC, Zheng JZ, Qiao M, Wu C, Dedhar S, Shi X, Jiang BH. 2005. ILK mediates actin filament rearrangements and cell migration and invasion through PI3K/Akt/Rac1 signaling. Oncogene 24:3154–3165.

Russo JM, Florian P, Shen L, Graham WV, Tretiakova MS, Gitter AH, Mrsny RJ, Turner JR. 2005. Distinct temporal–spatial roles for rho kinase and myosin light chain kinase in epithelial purse-string wound closure. Gastroenterology 128:987–1001.

Rutten MJ, Ito S. 1983. Morphology and electrophysiology of guinea pig gastric mucosal repair *in vitro*. Am J Physiol 244:G171–G182.

Sheetz MP, Felsenfeld DP, Galbraith CG. 1998. Cell migration: Regulation of force on extracellular–matrix–integrin complexes. Trends Cell Biol 8:51–54.

Tarnawski AS. 2005. Cellular and molecular mechanisms of gastrointestinal ulcer healing. Dig Dis Sci 50 (Suppl 1): S24–S33.

Tarnawski A, Tanoue K, Santos AM, Sarfeh IJ. 1995. Cellular and molecular mechanisms of gastric ulcer healing. is the quality of mucosal scar affected by treatment? Scand J Gastroenterol (Suppl 210): 9–14.

Teller IC, Beaulieu JF. 2001. Interactions between laminin and epithelial cells in intestinal health and disease. Expert Rev Mol Med 2001:1–18.

Tétreault MP, Chailler P, Rivard N, Ménard D. 2005. Differential growth factor induction and modulation of human gastric epithelial regeneration. Exp Cell Res 306:285–297.

Tétreault MP, Rivard N, Ménard D. 2006. Phosphatidylinositol 3-kinase and specific integrins modulate growth factor stimulated human gastric epithelial cell spreading. Can J Gastroenterol 20:64A.

Tétreault MP, Chailler P, Beaulieu JF, Rivard N, Ménard D. 2008. Epidermal growth factor-dependent PI3K-activation promotes restitution of wounded human gastric epithelial monolayers. J Cell Physiol 214:545–557.

Watanabe S, Hirose M, Wang XE, Maehiro K, Murai T, Kobayashi O, Nagahara A, Sato N. 1994. Hepatocyte growth factor accelerates the wound repair of cultured gastric mucosal cells. Biochem Biophys Res Commun 199:1453–1460.

Wright NA, Pike C, Elia G. 1990. Induction of a novel epidermal growth factor-secreting cell lineage by mucosal ulceration in human gastrointestinal stem cells. Nature 343:82–85.

Zhang Y, Guo L, Chen K, Wu C. 2002. A critical role of the PINCH–integrin-linked kinase interaction in the regulation of cell shape change and migration. J Biol Chem 277:318–326.