Juxtacrine activation of EGFR regulates claudin expression and increases transepithelial resistance

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Singh AB, Sugimoto K, Dhawan P, Harris RC. Juxtacrine activation of EGFR regulates claudin expression and increases transepithelial resistance. Am J Physiol Cell Physiol 293: C1660–C1668, 2007.—Heparin-binding (HB)-EGF, a ligand for EGF receptors, is synthesized as a membrane-anchored precursor that is potentially capable of juxtacrine activation of EGF receptors. However, the physiological importance of such juxtacrine signaling remains poorly described, due to frequent inability to distinguish effects mediated by membrane-anchored HB-EGF vs. mature “secreted HB-EGF.” In our studies, using stable expression of a noncleavable, membrane-anchored rat HB-EGF isoform (MDCKrat5aa cells) in Madin-Darby canine kidney (MDCK) II cells, we observed a significant increase in transepithelial resistance (TER). Similar significant increases in TER were observed on stable expression of an analogous, noncleavable, membrane-anchored human HB-EGF construct (MDCKhuman5aa cells). The presence of noncleavable, membrane-anchored HB-EGF led to alterations in the expression of selected claudin family members, including a marked decrease in claudin-2 in MDCKrat5aa cells compared with the control MDCK II cells, and prevented the increases in TER. Ion substitution studies indicated increased paracellular ionic permeability of Na+ in MDCKrat5aa cells, further indicating that the altered claudin-2 expression mediated the increased TER seen in these cells. In a Ca2+-switch model, increased phosphorylation of EGF receptor and Akt was observed in MDCKrat5aa cells compared with the control MDCK cells, and inhibition of these pathways inhibited TER changes specifically in MDCKrat5aa cells. Therefore, we hypothesize that juxtacrine activation of EGF by membrane-anchored HB-EGF may play an important role in the regulation of tight junction proteins and TER.

heparin-binding-epidermal growth factor; membrane-anchored; epidermal growth factor receptor

Epithelial cells serve as selective permeability barriers, separating fluid compartments with very different chemical compositions. Specific substrates may cross the epithelial cell layers by either transcellular or paracellular routes. Transcellular transport is principally catalyzed by carrier-mediated proteins on the apical and basolateral membrane surfaces and represents a very important focus of research in epithelial cell physiology. Less is known about the mechanism(s) regulating paracellular transport. The tight junctions are the most apical cell-cell junctions and provide a barrier that impedes the paracellular leakage of small molecules (1, 47). In addition, functional tight junctions are essential for the maintenance of the polarized architecture of the epithelial cells (40). In renal tubules, the paracellular pathway is now recognized to play an important role in vectorial transport, with nephron segment-specific selectivity for transported ions, such as sodium, chloride, calcium, and magnesium (42, 51, 52). Transepithelial resistance (TER) reflects paracellular ionic conductance, and TER varies dramatically among different nephron segments (37). It may also vary within a specific epithelial type in response to changes in physiological, pharmacological, or pathological conditions (7, 8, 34). Recently, claudins, the proteins integral to the tight junction, have been shown to display selective permeability for specific ions, suggesting they may be involved in the regulation and maintenance of paracellular ionic movement (4, 42, 48, 52).

EGF receptor (EGFR) activation plays fundamental roles in development, proliferation, and differentiation (44). By contrast, constitutive activation of the EGFR is implicated in the development and progression of numerous types of human cancers (3, 44, 49). Loss of cell polarization is a key event on transformation of the epithelial cells, and loss of functional tight junctions has been correlated with tumorigenesis (27, 30). Therefore, a possible correlation between EGFR activation and regulation of tight junctions can be postulated.

Among the EGF family of growth factors, heparin-binding EGF-like growth factor (HB-EGF) is of special interest, as it is synthesized as a type 1 transmembrane protein that can be shed enzymatically to release a soluble 14- to 20-kDa growth factor (19, 20). Both the transmembrane (proHB-EGF) and the mature soluble form may serve as ligands for EGFRs (32, 35, 45, 46). The soluble form of HB-EGF can interact with its receptors in an autocrine and/or paracrine manner, is a potent mitogen, and induces proliferation and migration in a variety of cells (24, 41). Under physiological conditions, the majority of HB-EGF is found as the uncleaved transmembrane precursor (proHB-EGF) that is suggested to activate EGFRs in a juxtacrine fashion (17). In addition, proHB-EGF interacts with other integral membrane proteins, especially integrins, via the tetraspanin, CD9/DRAP27 (26, 31). It is noteworthy that the HB-EGF-CD9-integrin complex and EGFR are both targeted to cell-cell junctions in polarized epithelial cells (13, 31). In the mammalian kidney, HB-EGF expression is primarily localized to the collecting duct, the nephron segment with the highest TER (21).

The Madin-Darby canine kidney (MDCK) cell line has been widely used as an in vitro model of polarized epithelium, including the study of mechanisms underlying formation and regulation of tight junctions (15, 29, 43, 50). Four strains of MDCK cells have been established, MDCK strains I, II, 7, and 11 (16, 38). These established strains of MDCK cells exhibit functional and biochemical differences, including a marked
difference in TER (4, 15, 28). MDCK strains I and 7 exhibit 10- to 100-fold higher TER than MDCK strains II or 11. The former strains model a “tight” epithelium, whereas the latter strains model a “leaky” epithelium. All four MDCK strains express claudin-1, claudin-3, claudin-4, claudin-7, and occludin, as well as zona occludens-1 (ZO-1), the tight junction-related protein (4, 15). The differences between TER in MDCK strains I or 7 vs. II or 11 are due, at least in part, to differences in expression of claudin-2, with expression only in the strains with lower TER (4, 15). Furthermore, MDCK strain I or 7 cells can be converted to a leaky epithelium, if exogenous claudin-2 is expressed in these cells (4, 15). In addition, exogenous claudin-2 expression in MDCK-7 cells also induces cation-selective paracellular current in the tight junctions (4). In the mammalian kidney, claudin-2 expression is restricted to selective paracellular current in the tight junctions (4). In the mammalian kidney, claudin-2 expression is restricted to

**MATERIALS AND METHODS**

**Cell culture and transfections.** MDCK II control and cells expressing either noncleavable, membrane-anchored rat HB-EGF or noncleavable, membrane-anchored human HB-EGF were grown in Dulbecco modified Eagle’s medium containing Earle’s balanced salt solution supplemented with 10% fetal bovine serum, 100 μg/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B in 5% CO2/95% air at 37°C. The details of rat HB-EGF constructs and generation of stable populations of HB-EGF mutants and characterization have been described previously (45). Two separate clones of MDCK rat5aa cells with similar expression levels were used for the studies, unless otherwise stated. For stable reexpression of claudin-2 in the MDCKrat5aa cells, full-length canine claudin-2 cDNA subcloned in pcDNA-4-Zeocin mammalian expression vector was used, as the original selection was done using neomycin (G418 1 mg/ml). MDCKhuman5aa cells, and two separate clones were studied.

**Immunoblotting.** Equal amounts of proteins were subjected to SDS-PAGE and immunoblotted with the antigen-specific antibodies. Signals were detected using an enhanced chemiluminescence detection kit (Amersham Biosciences). Equal protein loading was assessed by immunoblotting with anti-β-actin antibody (Sigma-Aldrich, St. Louis, MO) after stripping the respective membrane.

**Immunofluorescence microscopy.** Immunofluorescence staining and confocal analysis were performed as described previously (43). In brief, filters were rinsed with ice cold ×1 PBS and fixed with ice-cold methanol. Cells were further permeabilized with 0.2% Triton X-100 in PBS for 10 min. Thereafter, cells were washed three times with ice-cold PBS. After being blocked in PBS containing 5% normal goat serum and 1% bovine serum albumin, the samples were incubated with primary antibodies for 1 h in a moist chamber at 37°C or overnight at 4°C. The samples were then washed three times with PBS, followed by incubation for 30 min with the respective conjugated secondary antibody. The samples were again washed five times with PBS, embedded in glycerol/PBS-based mounting medium, and examined using a fluorescent microscope. Confocal images were obtained with Zeiss 510 confocal microscope available at the Vanderbilt University Medical Center Cell Imaging Core Resource. Image analysis was performed using the Metamorph cell-imaging program (Universal Imaging, Downingtown, PA).

**Statistics.** Graphic data are presented as means ± SE. Statistical analysis was performed, where appropriate, using the Student t-test. Differences with P < 0.05 were considered statistically significant.
RESULTS

Epithelial cells polarize upon culture on permeable filter supports, and TER across the confluent epithelial cell layer is a measure of tight junction function (9). Our laboratory has previously shown that a confluent monolayer of MDCK cells stably expressing a noncleavable form of the transmembrane form of rat proHB-EGF (MDCKrat5aa cells) developed significantly higher TER compared with control MDCK cells (45). In agreement with our laboratory’s published findings, in the present study, TER values in MDCKrat5aa cells were approximately threefold higher compared with the control MDCK cells (Fig. 1A). To determine whether the observed effects were unique to rat HB-EGF, we developed analogous noncleavable human HB-EGF construct (PVENP, the cleavage site, was deleted) and stably expressed it in MDCK II cells (MDCKhuman5aa cells). We confirmed that the human HB-EGF mutant lacking the putative cleavage site (PVENP) was not cleaved under normal conditions or in response to stimulation with phorbol myristate acetate ester (1 μM) (data not shown). Similar to the MDCKrat5aa cells, TER in MDCKhuman5aa cells was also 2.5- to 3-fold higher than that in the control cells (Fig. 1B).

We further examined possible changes in the paracellular flux between MDCKrat5aa and control MDCK cells using [3H]inulin (5 kDa), as described in the MATERIALS AND METHODS. Samples were collected from the basal compartment of the transwells 4 h after application of [3H]inulin in the upper compartment. As predicted, the amount of [3H]inulin collected from the basal chamber was significantly lower (P < 0.05) in MDCKrat5aa cells, suggesting formation of a tighter epithelium (Fig. 1C). Microscopic examination also confirmed tight cuboidal epithelial morphology of MDCKrat5aa similar to the control MDCK cells (Fig. 2A). MDCKhuman5aa cells showed similar morphology (data not shown).

Expression of tight junction proteins varied between MDCKrat5aa and control MDCK cells. As described above, claudins are integral to the structure and function of tight junctions (14). Therefore, we examined differences in claudin expression in response to expression of membrane-associated HB-EGF. MDCK II cells express claudin-1, -2, -3, and -4 and occludin (15, 28, 43). In addition, they express the tight junction-associated protein ZO-1 (43). We also examined expression of the adherens junction proteins E-cadherin and β-catenin. Immunoblotting indicated higher expression of claudins-1, -3, and -4, as well as occludin and ZO-1 in MDCKrat5aa cells compared with the control MDCK cells (Fig. 2B, I and II). In contrast, expression of claudin-2 in these cells was markedly decreased compared with the control cells. No major alterations were observed in the expression of either E-cadherin (Fig. 2B, I and II) or β-catenin (data not shown) between MDCKrat5aa and control MDCK cells. Similar decreases in claudin-2 expression were noted in MDCKhuman5aa cells, expressing the noncleavable human HB-EGF construct (data not shown).

In addition to the changes in total cellular expression, appropriate cellular localization of cell-cell adhesion proteins is essential for their normal cellular functioning. Therefore, we examined the cellular distribution of the proteins under investigation in MDCKrat5aa and control MDCK cells. As shown in Fig. 3, distinct membrane localization of tight junction proteins claudin-1, -3, and -4 and ZO-1 and adherens junction protein E-cadherin was observed in MDCKrat5aa cells, with relatively stronger expression compared with the control MDCK cells. Also, Z-sectioning showed interesting lateral membrane distribution for claudins-1 and -3 in the MDCKrat5aa cells compared with the exclusively apical expression in the control MDCK cells. While claudin-2 was expressed in distinct apical membrane localization in the control MDCK cells, expression levels as well as the membrane localization were severely compromised in MDCKrat5aa cells (Fig. 3).

Reexpression of claudin-2 decreased TER in MDCKrat5aa cells. We have previously shown that acute administration of EGF to polarizing MDCK II cells increased TER in a time-dependent manner and was associated with a simultaneous decrease in claudin-2 expression (43); however, a cause-and-effect relationship between the decreased claudin-2 expression and increased TER was not determined. Also, in our present study, we observed a marked decrease specifically in the expression of claudin-2 in MDCKrat5aa cells compared with the control MDCK cells. EGF activation decreases claudin-2 expression predominantly through the regulation of mRNA expression (43). Therefore, we stably transfected MDCKrat5aa cells with full-length canine claudin-2 cDNA. These cells are indicated as MDCKrat5aaCl2 cells. Expression of claudin-2 in MDCKrat5aaCl2 cells was confirmed by Western blot analysis, and membrane localization was confirmed using immunofluorescence analysis (Fig. 4, A1 and B). At least 70–80% of cells in MDCKrat5aaCl2 cells were claudin-2 positive, and, therefore, no further clonal selection was performed, which ensured similar expression levels of membrane-anchored HB-EGF in MDCKrat5aa and MDCKrat5aaCl2 cells (Fig. 4A1, top panel).

In MDCKrat5aa cells, reexpression of claudin-2 resulted in partial reversal of increases in the total cellular levels of claudin-1 and ZO-1, whereas expression of claudin-3, occludin, and E-cadherin remained largely unchanged (Fig. 4A, I and II). Reexpression of claudin-2 in MDCKrat5aaCl2 cells also resulted in decreased TER (Fig. 5A). In addition, [3H]inulin flux reverted to the levels seen in MDCK control cells (Fig. 5B).

Increased TER in MDCKrat5aa cells depended on changes in paracellular ionic permeability. It has been suggested that claudin-2 forms cation-selective paracellular channel (4, 48). Since reexpression of claudin-2 largely rescued the increases in TER in the MDCKrat5aa cells, we examined contribution of possible changes in paracellular ionic permeability in the increased TER in MDCKrat5aa cells. Effect of ion substitution was examined on TER using control, MDCKrat5aa, and MDCKrat5aaCl2 cells, where TER was measured in solutions of defined ionic composition. With 140 mM NaCl (P1 buffer), TER was higher (250% increase) in MDCKrat5aa cells than that of control MDCK cells (Fig. 5C). On substitution of sodium+ with arginine+ (buffer A) or lysine+ (buffer B), TER in the control MDCK cells increased dramatically, but TER in MDCKrat5aa cells was largely unaffected compared with the TER in buffer P1. Replacing Na+ with arginine+ or lysine+ increased the TER in the control MDCK cells by 6.5- and 6.9-fold, respectively, while only increasing TER by 0.48- and 0.54-fold, respectively, in MDCKrat5aa cells compared with the TER in buffer P1 (Fig. 5C). However, in MDCKrat5aaCl2 cells, TER increased 2.5- and 2.58-fold, respectively, compared with the TER in buffer P1 upon substitution of Na+ ion with...
arginine$^+$ or lysine$^+$. By contrast, when chloride was replaced by aspartate (buffer C), TER in control MDCK cells, MDCK$^{rat5aa}$, and MDCK$^{rat5aaCl2}$ cells showed modest and equivalent increases (0.83, 0.76, and 0.73) compared with the TER in buffer P1 (Fig. 5C). These findings indicated that differences in paracellular permeability to sodium underlay the variations in observed TER and further supported a role for changes in claudin-2 expression with the increased TER in the MDCK$^{rat5aa}$ cells.

Increased activation of EGFR and PI3 kinase were observed in MDCK$^{rat5aa}$ cells upon Ca$^{2+}$ switch. The membrane-anchored HB-EGF is a functional ligand of EGFR receptors and is potentially capable of juxtaacrine activation (17, 32). Therefore, we examined signaling mechanisms underlying development of increased TER in MDCK$^{rat5aa}$ cells. We used the Ca$^{2+}$-switch model to distinguish signaling changes upon initiation of cell-cell contact. As shown in Fig. 6, an early and

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**Fig. 1.** Changes in transepithelial resistance (TER) and paracellular permeability. A: TER in Madin-Darby canine kidney (MDCK) II cells stably expressing noncleavable, membrane-anchored rat heparin-binding EGF (HB-EGF) mutant construct. Cells (2 × 10$^5$) were plated in transwell filters (0.4-μm pore size), and TER was measured as described in MATERIALS AND METHODS. Values represent means ± SE of peak TER values from each cell line. **P < 0.01. B: TER in MDCK cells stably expressing noncleavable, membrane-anchored human HB-EGF mutant construct. Cells were plated and TER was measured as described above. D1–D7, days 1–7. Values represent means ± SE. *P < 0.05, **P < 0.01, ***P < 0.001. C: change in the paracellular permeability. Cells (2 × 10$^5$) were plated in transwell filters (0.4 μm pore size), and [3H]inulin (4 kDa) was added to the top chamber when cells achieved peak TER values (day 4). Determination of [3H]inulin leakage was performed as described in the MATERIALS AND METHODS. Values represent mean of total counts per minute (CPM) collected from the lower chamber ± SE. *P < 0.05.

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**Fig. 2.** A: representative phase-contrast images of confluent cells expressing noncleavable, membrane-anchored rat HB-EGF mutant construct and control MDCK II cells cultured on transwell filters. Cells expressing membrane-anchored HB-EGF (MDCK$^{rat5aa}$) retained epithelial morphology similar to the control MDCK II cells. Cells expressing noncleavable membrane-anchored human HB-EGF mutant construct (MDCK$^{human5aa}$) demonstrated similar cell morphology (data not shown). Original magnification ×100. B: I: comparison of the expression profile of tight and adherens junction proteins between MDCK$^{rat5aa}$ and control MDCK cells. Total cell lysates from confluent cells were electrophoresed on appropriate percentage of SDS-PAGE and immunoblotted with antigen-specific antibodies. Equal protein loading was determined by probing for β-actin following stripping of the respective membranes. II: densitometric analysis of the expression of the proteins under investigation. Densitometric values for each protein were normalized with respective actin values, and data are presented as relative increase or decrease compared with the control MDCK cells. ZO-1, zonula occludens-1.
time-dependent increase in the activation of EGFR (Fig. 6A, I and II) and Akt phosphorylation (Fig. 6B, I and II) was observed in MDCK\textsuperscript{ratSaa} cells compared with control MDCK cells. However, no major changes were observed in the activation of ERK1/2 in MDCK\textsuperscript{ratSaa} cells compared with control MDCK cells (Fig. 6C, I and II). There was decreased expression of claudin-2 but increased expression of claudin-1 in MDCK\textsuperscript{ratSaa} cells compared with control MDCK cells at all time points after the switch (Fig. 6D, I and II).

**Inhibition of EGFR, PI3 kinase activation, \(\beta_1\)-integrin, or CD-9 prevented the increased TER in MDCK\textsuperscript{ratSaa} cells.** To explore the causal relationship of the observed changes in EGFR and PI3 kinase activation with the increased TER in MDCK\textsuperscript{ratSaa} cells, we inhibited relevant signaling pathways. Antigen-specific pharmacological inhibitors PD-153035 (1 \(\mu\)M; an EGFR inhibitor), U-0126 (10 \(\mu\)M; an ERK1/2 MAP kinase inhibitor), and LY-294002 (20 \(\mu\)M; a PI3 kinase inhibitor) were used. Since both \(\beta_1\)-integrin and CD9 have been reported to associate with, and enhance juxtacrine activity of, membrane-anchored HB-EGF (31, 32), we also tested functional blocking antibodies against \(\beta_1\)-integrin and CD9. As shown in Fig. 7, inhibition of EGFR, PI3 kinase, or ERK1/2 MAP kinase activation resulted in small decreases in TER in the control MDCK cells [PD-153035 (10.3%), LY-294002 (11.5%), and U-0126 (9.1%)], while inhibition of \(\beta_1\)-integrin or CD9 had no effect on TER. By contrast, in MDCK\textsuperscript{ratSaa} cells, inhibition of EGFR or PI3 kinase resulted in marked decreases in TER [PD-153035 (36%), LY-294002 (48%)], while inhibition of ERK1/2 had no significant effects on TER. Blocking antibodies against either CD9 or \(\beta_1\)-integrin resulted in a similar extent of decrease in TER (38.8–40%) in MDCK\textsuperscript{ratSaa} cells (Fig. 7).

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**Fig. 3.** Immunofluorescent localization of tight and adherens junction proteins. Control MDCK and MDCK\textsuperscript{ratSaa} cells were plated on transwell filters, and TER was measured. Cells were fixed when they achieved peak TER values and processed for immunofluorescence staining, as described in MATERIALS AND METHODS. All images were treated identically. The bottom panels show the XZ sectioning of the corresponding panels. Z-sectioning of the confocal image stack showed distinct apical membrane staining for all tight junction proteins and lateral membrane staining for E-cadherin in the control MDCK cells. In the MDCK\textsuperscript{ratSaa} cells, a more lateral distribution for claudin-1 and claudin-3 was observed. Original magnification \(\times 400\).
DISCUSSION

Epithelial paracellular permeability varies among different tissues as well as within the same organ. In the kidney, TER (a functional measure of paracellular permeability) varies significantly among different nephron segments. Claudin composi-

Fig. 4. Reexpression of claudin-2 in MDCK<sup>rat5aa</sup> cells. A. I: Western blot analysis of the expression of claudin-2 in control MDCK, MDCK<sup>rat5aa</sup>, and MDCK<sup>rat5aaCl2</sup> cells. Total cell lysates from confluent cells were subjected to appropriate percentage of SDS-PAGE and immunblotted with antigen-specific antibodies. II: densitometric analysis of the expression of the proteins under investigation. Densitometric values for each protein were normalized with respective actin values, and data are presented as relative increase or decrease compared with the control cells. B: immunofluorescent localization of claudin-2 in MDCK<sup>rat5aa</sup> and MDCK<sup>rat5aaCl2</sup> cells. Original magnification ×400.

Fig. 5. Effect of reexpression of claudin-2 in MDCK<sup>rat5aa</sup> cells upon TER and paracellular permeability for [3H]inulin and charged ions. A: TER in control MDCK, MDCK<sup>rat5aa</sup>, and MDCK<sup>rat5aaCl2</sup> cells. Values represent means ± SD. ***P < 0.001, **P < 0.01. B: change in the paracellular permeability upon reexpression of claudin-2 in MDCK<sup>rat5aa</sup> cells. Cells were plated on transwell filters, and determination of [3H]inulin flux was performed as described in the MATERIALS AND METHODS. Data are presented as relative change compared with the control MDCK cells ± SE. *P < 0.05. C: TER in response to alterations in extracellular ionic composition. TER measurements were performed in cells grown on transwell filters. Buffer P contained 140 mM NaCl, and TER in buffer P was no different compared with the TER in normal culture medium. In buffer A, 140 mM NaCl was replaced by 140 mM arginine-HCl. In buffer B, 140 mM NaCl was substituted by 140 mM lysine-HCl. In buffer C, 140 mM NaCl was replaced with 140 mM sodium aspartate. Data are presented as means ± SE. *P < 0.01; **P < 0.001.
tion also varies among different nephron segments. In addition, various physiological and pathological changes result in altered epithelial paracellular permeability, which appear to be due in large part to changes in the expression and cellular distribution of different claudins (5, 6). However, the physiological regulation of tight junction structure and function remain largely undefined.

Studies that have genetically manipulated expression of specific claudin family members in cultured cells have confirmed that changes in the expression and cellular localization of claudins are central to the observed alterations in tight junction structure and function. Overexpression of various claudins, including claudins-1 and -4, leads to increased TER (2, 10, 23). By contrast, overexpression of claudin-2 results in remarkable decreases in TER in high-TER clonal variants of MDCK cells (4, 15). Conversely, RNA interference knockdown of claudin-2 in low-TER MDCK II cells results in significant increases in TER. Interestingly, similar knockdown of claudin-1 in the same cell line did not result in substantial changes in TER, although knockdown of claudins-4 and -7 did decrease TER to low levels (22). In vivo, claudin-2 expression is restricted to leaky epithelia, including the kidney proximal tubule and thin descending limb, and is absent from tight epithelia (25). We have previously reported a role for EGFR activation in the regulation of TER, whereby acute EGF administration resulted in a time-dependent and significant

Fig. 6. Phosphorylation (P) of EGF receptor (EGFR; A), Akt (B), and ERK (C) and expression of claudin-1 and -2 (D) following Ca\(^{2+}\) switch. Cells (0.8 \(\times\) 10\(^6\)) were plated in 6-well transwell filters, and Ca\(^{2+}\) switch was induced as described in MATERIALS AND METHODS. Samples were collected after indicated time points, and total cell lysates were prepared. Immunoblotting was performed using antigen-specific phospho-antibodies. A, I–C, I: immunoblot analysis for P-EGFR, P-Akt, and P-ERK1/2. Blots were stripped and reprobed with nonphospho-antibodies to confirm equal protein loading. A, II–C, II: densitometric analysis of the respective blots. Values for phospho-proteins were normalized to the values from nonphospho-protein, and data are presented as relative change compared with control MDCK cells. D, I: expression of claudin-1 and -2 was examined in the same cell lysates using antigen-specific antibodies. Actin was used for normalization. D, II: densitometric analysis of the respective blots. Values were normalized to actin values from the same blot and presented as relative change compared with the control MDCK cells.

Fig. 7. Effects of inhibition of signaling pathways and molecules on TER. Cells were incubated with PD-153035 (inhibitor of EGFR phosphorylation, 1 \(\mu\)M), U-0126 (inhibitor of mitogen-activated protein kinase, ERK1/2, 10 \(\mu\)M), LY-294002 (inhibitor of phosphatidylinositol 3-kinase, 20 \(\mu\)M), A2B2 (functional blocking antibody for \(\beta_1\)-integrin, 4 \(\mu\)g/ml), or MEM-61 (functional blocking antibody for CD9, 2 \(\mu\)g/ml) before switching cells to normal Ca\(^{2+}\) medium for 30 min. An IgG of the same class (2 \(\mu\)g/ml) was used as control. All inhibitors were also present in the normal Ca\(^{2+}\) medium used for the switch and remained present throughout the study. Values represent the means \(\pm\) SE of the peak TER from each cell line. \(* P < 0.01\)
increase in TER in MDCKII cells (43). This increase in TER was accompanied by specific decreases in claudin-2 expression (43). Recent studies have identified EGF as the factor in human urine that increases TER in MDCK II cells (12). In the present studies, expression of noncleavable, membrane-associated HB-EGF not only significantly decreased expression of claudin-2, but also increased expression of other claudins (1, 3, and 4) known to increase TER. In addition, Z-sectioning demonstrated interesting lateral membrane distribution of claudins-1 and -3 compared with the apical membrane localization in the MDCK cells. Similar lateral membrane localization for claudins-1 and -3 have been described in other studies (18, 36, 39), but the physiological importance remains unknown.

As with certain other EGFR ligands, the transmembrane HB-EGF precursor (proHB-EGF), as well as its secreted mature product (soluble HB-EGF), are both potentially capable of activating EGFR, although the mode of receptor activation is different. While transmembrane HB-EGF precursor activates EGFR in a juxtacrine fashion, the secreted soluble HB-EGF activates EGFR in an autocrine/paracrine fashion. In fact, simple substitution of the transmembrane domain of the human proEGF with the corresponding domain of human HB-EGF renders human proEGF capable of activating EGFR in a juxtacrine fashion (11). The current studies confirm a role for EGFR activation in the regulation of tight junction composition and function and provide evidence regarding a possible physiological function of the membrane-anchored HB-EGF and hence juxtacrine activation of EGFR, in vivo. Of note, during normal physiological conditions, HB-EGF is predominantly expressed as the membrane-anchored precursor (17), and, in this regard, in the kidney, HB-EGF is expressed predominantly in the collecting tubules (21), the nephron segment that demonstrates the highest TER among nephron segments and does not express claudin-2 (25).

The possibility that the observed effects on tight junctions in MDCKKrat5aa cells may represent more than simple juxtacrine activation of EGFR cannot be ruled out, as the ability of membrane-anchored HB-EGF to form a complex with accessory proteins, such as CD9, seems to be crucial for full activity (26). Accessory proteins may be required to remove a steric constraint imposed by another structural feature of the ligand, such as the heparin-binding domain. Alternatively, the accessory protein could be required to “present” the ligand to the receptor by forming part of a multiprotein complex. In this regard, functional blocking antibodies against either CD9 or β1-integrin decreased TER in MDCKKrat5aa cells. Both of these proteins are known to associate with membrane-anchored HB-EGF and have been colocalized with EGFR on lateral cell borders (31).

Taken together, we postulate that the juxtacrine activation and protein complexing by the membrane-anchored HB-EGF provide local and tightly regulated control of cell-matrix and cell-cell adhesion and maintenance of the normal epithelial architecture. In contrast, pathological disruption of cell-cell junctions will induce cleavage of HB-EGF, resulting in the release of mature secreted soluble HB-EGF, which may be involved in proliferation, migration, and dedifferentiation. Dedifferentiation of the epithelial cells has been recognized as a necessary and important step in regeneration following epithelial injury (33). Our laboratory has previously shown that, in contrast to acute EGFR activation, which, as mentioned above, resulted in increased TER, stable expression of secreted HB-EGF resulted in transformed cells with decreased cell-matrix, cell-cell adhesion, increased cell proliferation, migration, and complete loss of TER (45). These results suggested different responses with acute vs. chronic EGFR activation. Indeed, chronic administration of exogenous EGF to MDCK II cells also resulted in dedifferentiation and loss of functional tight junctions (unpublished data). Normal functioning of EGFR is essential for epithelial growth and differentiation, whereas dysregulated expression/activation of EGFR induces dedifferentiation of epithelial cells and is correlated with neoplastic transformation. However, it is important to note that constitutive expression of the noncleavable, membrane-anchored HB-EGF and hence juxtacrine signaling resulted in a contrasting phenotype characterized by increased cell-matrix and cell-cell adhesion, decreased migration, and significantly increased TER, suggesting qualitative/quantitative differences between the juxtacrine vs. autocrine/paracrine signaling.

In summary, we demonstrate that the presence of transmembrane HB-EGF in MDCK II cells results in tighter epithelium and enhanced TER. MDCK II cells display certain properties of proximal tubular epithelium, including low TER and claudin-2 expression. The presence of membrane-anchored HB-EGF changed the characteristics of MDCK II cells from a more proximal tubular epithelial cell (low TER) toward a phenotype more consistent with distal nephron epithelia (higher TER). These findings, combined with the fact that, in the kidney, HB-EGF expression is largely restricted to the distal nephron segments and is expressed predominantly as membrane-anchored precursor under normal physiological conditions, suggest a possible physiological function of the membrane-anchored HB-EGF in vivo. However, further studies are required to confirm this hypothesis.

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