# Characterization of the Downstream Effects of Apical Mistrafficking of Epiregulin and Potential for Transformation in Polarized Epithelial Cells

Saba Rehman, Galina Bogatcheva, Robert Coffey, and Bhuminder Singh

KEYWORDS. Epiregulin (EREG), Basolateral, Apical, Trafficking, Cancer

BRIEFS. Loss of epithelial polarity and mistrafficking of Epiregulin may be a driving force for cancer in polarized epithelial cells.

ABSTRACT. A loss of epithelial polarity during transformation has been considered a late event in cancer progression; however, recent reports show that this may occur during early stages and act as a driver of transformation. Epiregulin (EREG) is an epidermal growth factor receptor (EGFR) ligand that localizes to the basolateral surface of polarized Madin Darby Canine Kidney (MDCK) epithelial cells or to the apical surface of mutant EREG (Y156A) cells. EREG mistrafficking to the apical surface is an example of the loss of polarity associated with increased transformation potential. In this study, EREG expression was induced, and activation of downstream signaling by EREG expression (wild-type versus mutant) in MDCK cells was analyzed to test whether EREG mistrafficking drives epithelial transformation. Conditioned media from apical and basolateral compartments expressing different EREG forms were incubated with EGFR-expressing A431 cells, which were tested for activation of EGFR and downstream signaling molecules AKT and ERK1/2 by western blotting. These results showed greater activation from the apical media of mutant cells than from the basolateral media of wild-type cells. Such evidence suggests that EREG mistrafficking may be responsible for driving transformation of epithelial cells, something that can be applied to achieve a wider understanding of the origin and treatment options of related cancers.

## INTRODUCTION.

More than 90% of cancers arise from epithelial cells, which line the majority of organs, body cavities, and glands [1]. Most epithelial cells display a molecular asymmetry in which the cell membrane is divided into distinct apical and basolateral compartments [1]. Epiregulin (EREG) is one of seven epidermal growth factor ligands found in epithelial cells that can bind to epidermal growth factor receptor (EGFR) in order to signal cell growth [1]. EREG is produced in the cell and then trafficked to EGFR in the cell membrane, where it can begin a downstream phosphorylation cascade (Supplemental Figure 1) [1]. In normal cells, EREG is typically trafficked to the basolateral surface, where it can bind to and activate EGFR in order to activate downstream signaling molecules AKT and ERK1/2 [2]. Activation of AKT allows the cell to survive, whereas activation of ERK1/2 allows the cell to proliferate [2]. In mutant cells, however, it has been found that EREG is trafficked to the apical surface rather than to the basolateral surface [3].

After delivery to the cell surface, EREG is cleaved by metalloproteases (MMPs/ADAMs) to release soluble ligand that then binds to and activates EGFR and downstream signaling. In mutant cells, EREG is trafficked to the apical surface where it is hypothesized that this might be a "driver" event in cancer [1].

Until recently, such loss of epithelial polarity has been considered to be a late stage in the transformation of cancerous cells [4]. However, this study, as well as a past work, seem to suggest that loss of epithelial polarity is one of the hallmarks of cancer and that it drives the initial stages of transformation of epithelial cells into cancerous cells, an idea that has been not been given much attention until now. This information is crucial because it points to a root cause of cancer that can hopefully be targeted with drugs in order to treat cancer at an early stage.

One piece of evidence for the proposition that the mistrafficking of EREG drives transformation is the sustained phosphorylation of EGFR when EREG is trafficked to the apical surface rather than to the basolateral surface [3]. This sustained phosphorylation of EGFR found in a previous study is suggestive of increased downstream signaling, which would potentially lead to increased proliferation and eventual transformation of the cell [3]. Based on previous pulse-chase experiments, it is expected that similar amounts of EREG are delivered to the apical and basolateral surfaces, indicating that the cause of EREG mistrafficking-induced transformation might lie in the asymmetric activation of EGFR from the apical and basolateral compartments [1,5]. The objective of this study, then, was to observe the release of EREG into extracellular medium as well as subsequent EGFR activation and downstream signaling to test the hypothesis regarding the proposition that the mistrafficking of EREG drives cellular transformation. It was hypothesized that there would be increased signaling of EGFR and downstream molecules AKT and ERK1/2 in cells cultured with conditioned media from mutant EREG expressing cells. In order to investigate this proposition, media from mutant and wild-type cells expressing EREG were cultured with recipient cells expressing EGFR. It is hoped that the results from this study can be used as a further evidence to counter the status quo on how loss of epithelial polarity can drive cancer progression.

## MATERIALS AND METHODS.

### *Cell Lines.*

Madin-Darby Canine Kidney (MDCK) cells were used in all experiments. MDCK parental cells not expressing Epiregulin (EREG) were used as a control to compare the wild-type and mutant Y156A MDCK cells. The wild-type and mutant cells were induced with Doxycycline (DOX) in a tet-inducible system to express wild-type EREG (trafficked primarily to the basolateral surface) and mutant EREG (trafficked primarily to the apical surface), respectively [1]. The tet-inducible allowed the wild-type and mutant forms of EREG to be activated in the appropriate cells by inducing transcription in the presence of DOX. In some experiments, human A431 cells that only express the epidermal growth factor receptor (EGFR) were used as recipient cells to visualize and interpret the data.

The MDCK cells were grown either on Transwell filters or on plastic. In both cases, MDCK parental cells were cultured with DMEM and 10% Bovine Growth Serum (BGS), whereas the wild-type and mutant Y156A EREG-expressing cells were cultured in DMEM with G418 added to maintain selection. Both wild-type and mutant MDCK cells were treated with 500 ng/mL of DOX to induce EREG expression. For some experiments, the parental, wild-type, and mutant Y156A EREG expressing MDCK cells were grown on Transwell filters in order to expose distinct apical and basolateral compartments of the cells (Supplemental Figure 1). Apical media was harvested from the top of cells from the inner Transwell chamber, whereas basolateral media was isolated from the outer chamber. All A431 recipient cells were grown on plastic.

### *Effect of Conditioned Media from EGFR-Expressing MDCK Cells on A431 Recipient Cells.*

Parental, wild-type, and mutant Y156A MDCK cells were cultured on Transwell filters to expose the distinct apical and basolateral surfaces of the cells, and 500 ng/mL of DOX was used to induce EREG. Media from the apical and basolateral surfaces of these cells were added to A431 recipient cells expressing EGFR. EKI-785, an inhibitor that binds to the kinase domain of EGFR, and P1X, an antibody that binds to the EGFR ectodomain and blocks EREG from binding, were both added in different trials to assess whether the downstream activation of EREG and downstream molecules AKT and ERK1/2 could be appropriately

attributed to the binding of EREG to EGFR (Supplemental Figure 2). The results were developed on film (see *Western Blotting*).

*Doxycycline Titration to Normalize WT and Y156A EREG Expression in MDCK Cells.*

To investigate whether there is a difference between the responses to DOX for EREG expression in wild-type versus mutant Y156A MDCK cells, different concentrations of DOX were tested in order to compare and equalize expression in both cell types.

Parental, wild-type, and mutant (Y156A) MDCK cells were cultured and treated with DOX to induce EREG in a tet-inducible system. All cells were cultured with DMEM. Parental cells were cultured with 10% Bovine Growth Serum (BGS), whereas the wild-type and Y156A cells were cultured with G418 to maintain selection. The wild-type cells were treated with 500 ng/mL of DOX, and the mutant Y156A cells were treated with 500 ng/mL, 250 ng/mL, 100 ng/mL, 10 ng/mL, 1 ng/mL, and 0.1 ng/mL of DOX for comparison. The cells were incubated with DOX for 24 hours. In order to visualize the results of both experiments, a Western Blot was conducted (see *Western Blotting*).

*Western Blotting.*

All cells were lysed on ice using protease inhibitors NaF, Sodium Orthovanadate, Aprotinin, Leupeptins, Pepstatin A, and PMSF added to lysis buffer with a composition reported previously [3]. Phosphate-buffered saline (PBS) was used as a wash before the cells were allowed to lyse for 30 minutes at 4°C. The cell lysates were run on 7% SDS/PAGE gels which were then transferred onto nitrocellulose membranes overnight at 4°C.

In experiments concerning the effect of conditioned media from EREG-expressing MDCK cells on A431 recipient cells, the A431 cells were lysed and run on 7% SDS/PAGE gels, after which they were transferred onto nitrocellulose membranes. The membranes were blocked with TBS-T containing 5% non-fat dry milk for 2 hours at room temperature. Phospho-EGFR (pY1092) was the primary antibody used to identify activated EGFR by wild-type and mutant EREG; Phospho- AKT was used to identify the activation of AKT; Phospho-ERK1/2 was used to identify the activation of ERK1/2. EGFR (Millipore) was used to identify total EGFR to ensure that loading in the gels was used as a loading control. All primary and secondary antibodies were diluted in TBS-T containing 5% non-fat dry milk and incubated for 1 hour at room temperature, and washes for the membranes were performed in TBS-T.

In experiments concerning the titration of DOX to equalize levels of EREG expression in wild- type and mutant MDCK cells, primary antibody Phospho-EGFR (1092) was incubated with the membranes overnight in order to tag activated EGFR by the binding of EREG, and the membranes were washed with 5% milk before a HRP-conjugated secondary antibody was added. After incubation with the secondary antibody, the membranes were washed with TBS-T to wash away unbound antibodies. Membranes were then incubated with ECL reagent and exposed in the dark on photographic films to be developed.

RESULTS.

*Effect of Conditioned Medium from EREG-Expressing MDCK Cells on A431 Recipient Cells.*

The effect of conditioned medium from EREG-expressing MDCK cells on A431 recipient cells was analyzed in order to observe the results of what occurs after EREG is trafficked to the membrane. Downstream signaling is demonstrated by the relative amounts of activated EGFR and downstream molecules AKT and ERK1/2 after EREG has bound to EGFR. Inhibitor EKI-785 (binds to the C-terminus of EGFR) and antibody P1X (blocks the binding site of EGFR) were used to block signaling by EREG in order to test whether or not the EGFR and downstream AKT and ERK1/2 activation is a result of the EREG ligand binding to EGFR. Figure 1B displays the results for total EGFR. Similar signals for each well indicate that loading in the gels was equivalent. Figure 1A displays results for the activation of EGFR by wild-type and mutant EREG. It is shown that there is an increased signal for the media from the apical surface of the mutant cells, particularly when compared to the basolateral media from the wild-type cells. Figures 2 and 3, which show the results for the activation of AKT and ERK1/2, display a similar trend. In all cases, decreasing signal with the addition of inhibitor EKI-785 and antibody P1X indicate binding of EREG to EGFR in order to trigger subsequent activation of downstream molecules in the phosphorylation cascade.

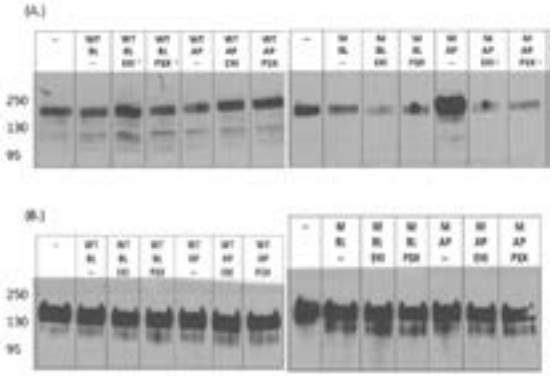

**Figure 1. (A.)** Activation of EGFR by Wild-Type and Mutant EREG. There is a greater activation of EGFR by the mutant (M) media from the apical (AP) surface, especially when compared to the activation of EGFR by the wild-type (WT) media from the basolateral (BL) surface. Decreasing signal with the addition of inhibitor EKI-785 and antibody P1X (marked by asterisk) indicate the binding of EREG to EGFR. **(B.)** Total EGFR. Similar signals for total EGFR in each well indicate that loading was equivalent.

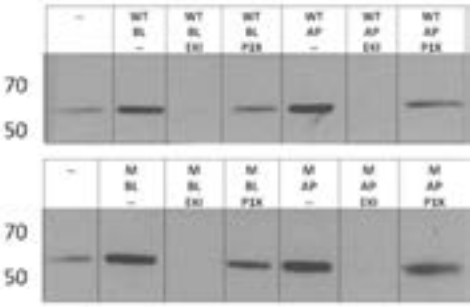

**Figure 2.** Activation of AKT by Wild-Type and Mutant EREG. This figure displays a slightly stronger signal for the activation of AKT by media from the apical (AP) surface of the mutant (M) cells. Decreasing signal with the addition of inhibitor EKI-785 and antibody P1X indicate the binding of EREG to EGFR.

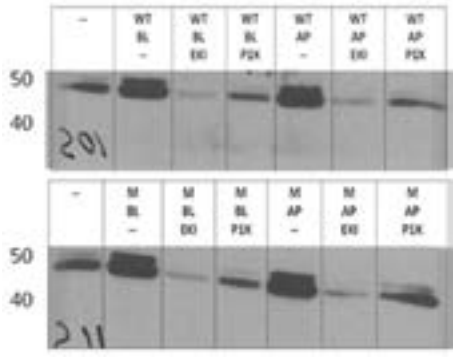

**Figure 3.** Activation of ERK1/2 by Wild-Type and Mutant EREG. This figure displays a slightly stronger signal for the activation of ERK1/2 by media from the apical (AP) surface of the mutant (M) cells. Decreasing signal with the addition of inhibitor EKI-785 and antibody P1X indicate the binding of EREG to EGFR.

*Doxycycline Titration to Equalize WT and Y156A EREG Expression in MDCK Cells.*

In order to assess whether the wild-type and mutant cells express the same level of EREG as each other in response to DOX, the concentration of DOX was titrated down for the Y156A mutant cells in comparison to the wild-type cells that were treated with a constant concentration of DOX. The results, displayed in Supplemental Figure 3, showed that the protein (EREG) concentrations for mutant cells treated with 500 ng/mL, 250 ng/mL, and 100 ng/mL of DOX were much higher than the concentration of protein for wild-type cells treated with 500 ng/mL of DOX; this suggested that the ideal concentration of DOX for the mutant cells would be below 100 ng/mL in comparison to a concentration of 500 ng/mL for the wild-type cells. The results also showed that the protein (EREG) concentrations for the mutant cells treated with 10 ng/mL, 1 ng/mL, and 0.1 ng/mL were much lower than the concentration of protein for wild-type cells treated with 500 ng/mL of DOX. These results seemed to suggest that the ideal concentration of DOX for mutant Y156A MDCK cells is between 10 ng/mL and 100 ng/mL when wild-type MDCK cells are treated with 500 ng/mL of DOX.

DISCUSSION.

When analyzed, the results showed EREG does induce activation of EGFR and downstream molecules AKT and ERK1/2 (Figures 1, 2, and 3). All results were analyzed in order to address the hypothesis regarding the proposition that mistrafficking of EREG is a driving force of cancer progression.

The DOX titration (Supplemental Figure 3), which was conducted in order to investigate whether there are different levels of EREG expression in wild-type versus mutant MDCK cells, suggested that there is a discrepancy in the responses of wild- type and mutant cells to treatment with DOX. In the future, it would be beneficial to utilize the equalized titrations of DOX for experiments concerning the effects of conditioned media from EREG-expressing cells on A431 recipient cells, which would be 500 ng/mL for wild-type cells and between 10 ng/mL and 100 ng/mL for mutant cells according to the gathered results. Alternatively, additional wild-type and mutant clones may be tested to compare for equivalent EREG expression at similar DOX concentrations.

The Western Blots, performed to determine protein (EREG and EGFR) concentration, for activated EGFR (pEGFR) displayed the activation of EGFR after EREG had bound to it. These results showed that there is increased activation of EGFR at the apical surface of mutant cells, particularly when compared to the basolateral medium from the same cells and the basolateral medium of the wild-type cells. This observation is supported by a previous study in which the mistrafficking of EREG to the apical surface rather than to the basolateral surface was first proposed to be a driving force in cancer progression [3]. In this past study, it was shown that trafficking of apical EREG resulted in sustained phophorylation (i.e., activation) of EGFR. It is important to note that the increase in pEGFR by apical EREG could be blocked by EGFR blocking antibody P1X and by EGFR kinase inhibitor EKI-785 (Supplemental Figure 2), suggesting that the sustained phosphorylation of EGFR by the binding of EREG could be attributed, at least in part, to the binding of EREG to the receptor. The results for activated AKT and ERK1/2, pAKT and pERK1/2, showed little differences between activation in wild-type versus mutant cells. This would typically suggest that mutant EREG is not responsible for eventual transformation. However, the use of EKI-785 and P1X showed that pAKT and pERK1/2 levels decreased when EREG did not bind to EGFR or when subsequent phosphorylation was blocked. This phenomenon suggests that EREG must play a part in the activation of these downstream molecules, although some other factor(s) must also be at play. This conclusion is in line with a previous study, which indicated that the activation of AKT and ERK1/2 is influenced greatly by the identity of the ligand, which is EREG in this case [6]. Although the past study focused on growth factor responses specifically for breast cancer cell lines, it can still be used to reflect on the current study in which MDCK and colon cancer cell lines (A431) were the primary ones used in experimentation. Based on the results for this study, it can be concluded that EREG is one of the ligands responsible for the activation of AKT and ERK1/2. Because the past study about growth factor responses in breast cancer cells showed that the activation of AKT and ERK1/2 is heavily influenced by ligand identity, it is highly possible that the other factor at play here may be another ligand similar to EREG [6]. This inference is also supported by the consideration that EREG is just one of seven epidermal growth factors, so it is likely that one of the other growth factors plays a role in the differential activation of downstream molecules in mutant versus wild-type MDCK cells. In the future, it would be beneficial to test the other six epidermal growth factor (EGF) ligands in order to understand what other components play a role in downstream activation and possibly transformation of epithelial cells. Another past study about the regulation of autocrine signaling through EGFR by mediating ligand release using metalloprotease inhibitors found that blocking the release of ligands amphiregulin (AREG) and transforming growth factor-α (TGFA) resulted in decreased migration and proliferation of the tested cell line [7]. Such work creates further support for the conclusion here that EREG and some other ligand(s) (such as AREG and TGFA) must be responsible for the differential downstream activation of AKT and ERK1/2 that results in eventual cellular transformation.

In light of past work, the current study gathers further evidence for the proposition that the mistrafficking of EREG is a driving force for the transformation of normal epithelial cells into cancerous ones. Although loss of epithelial polarity has long been considered a late stage in cellular transformation [4], emerging evidence such as that presented here is beginning to change the current frame of thought. It is hoped that by further understanding the role of the loss of epithelial polarity in cancer progression treatments can eventually be developed to target the disease in its earliest stages, perhaps to target the mutant form of the protein EREG.

ACKNOWLEDGMENTS.

I would like to extend great thanks to Dr. Bhuminder Singh and Galina Bogatcheva, who offered immense aid and guidance over the course of this project. I would also like to thank Dr. Robert Coffey for allowing me to work in his lab and utilize its facilities. A special thanks also goes out to the School for Science and Math at Vanderbilt for giving me the opportunity to be a part of the research community at Vanderbilt. I would also like to acknowledge Dr. Angela Eeds and Ms. Amanda Dixon for offering help and guidance along the way.

SUPPORTING INFORMATION.

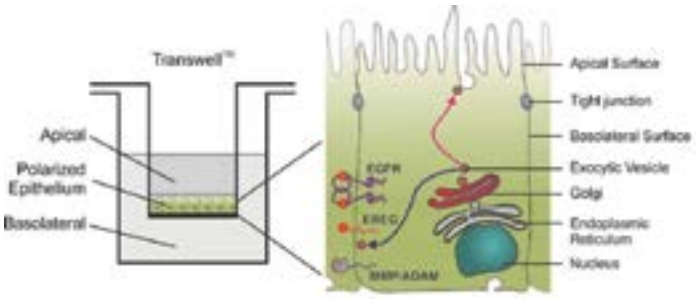


**Figure S1.** Trafficking of Epiregulin (EREG) in Polarized Epithelial Cells. On the left is a schematic for the Transwell culture system. Cells are seeded in the inner chamber that has permeable support. Cells grow to polarize so that the apical medium may be harvested from the inner chamber and the basolateral medium may be harvested from the outer compartment. On the right is a schematic for the trafficking of EREG in polarized epithelial cells. EREG is one of seven transmembrane EGFR ligands produced in the cell and trafficked to the cell membrane. Wild-type EREG is primarily trafficked to the basolateral surface of polarized epithelial cells.

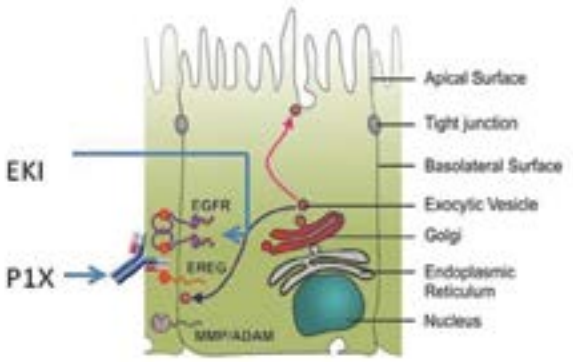


**Figure S2.** Binding of EKI-785 and P1X. EKI-785 is an inhibitor that binds to the kinase domain of EGFR to block subsequent phosphorylation, whereas P1X is an antibody that binds to the EGFR ectodomain and blocks EREG from binding. Both EKI-785 and P1X were added in different trials in order to assess whether downstream activation of EGFR, AKT, and EKT1/2 could be appropriately attributed to the binding of EREG to EGFR.

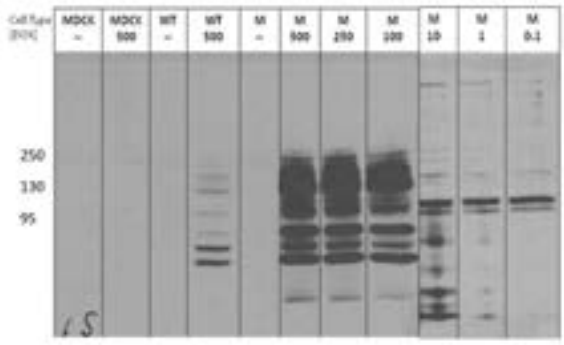


**Figure S3. Doxycycline Titration to Equalize WT and Y156A EREG Expression in MDCK Cells.** MDCK parental cells (MDCK) were used as a control. This figure shows that the signals were too strong for the mutant (M) cells treated with 500 ng/mL, 250 ng/mL, and 100 ng/mL of DOX in comparison to the wild-type cells (WT) treated with 500 ng/mL. The signals were too weak for mutant cells treated with 10 ng/mL, 1 ng/mL, 1 ng/mL, and 0.1 ng/mL of DOX. This suggests that the ideal concentration of DOX for the mutant cells would be between 10 ng/mL and 100 ng/mL of DOX when the wild-type cells are treated with 500 ng/mL of DOX.

REFERENCES.

1. B. Singh et al., "Trafficking of Epidermal Growth Factor Receptor Ligands in Polarized Epithelial Cells," *Annual Review of Physiology*, vol. 76, pp. 275-300, Nov. 2013.
2. D. Riese et al., "Epiregulin: Roles in Normal Physiology and Cancer," *Seminars in Cell and Developmental Biology*, vol. 28, pp.49-56, April 2014.
3. B. Singh et al., "Transformation of Polarized Epithelial Cells by Mistrafficking of Epiregulin," *PNAS*, vol. 110, pp. 8960-8965, May. 2013.
4. L. Alexandrov et al., "Signatures of Mutational Processes in Human Cancer," *Nature*, vol. 500, pp. 415-421, March 2013.
5. C. Wild-Bode et al., "A Basolateral Sorting Signal Directs ADAM10 to Adherens Junctions and is Required for its Function in Cell Migration," *J Biol Chem*, vol. 33, pp. 23824-9, Jun. 2006.
6. M. Niepel et al., "Analysis of Growth Factor Signaling in Genetically Diverse Breast Cancer Lines," *BMC Biology*, vol. 12, 2014.
7. J. Dong et al., "Metalloprotease-Mediated Ligand Release Regulates Autocrine Signaling Through the Epidermal Growth Factor Receptor," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, pp. 6235-6240, May 1999.
8. A. P. Mackenzie, Y. Maeno, *Rev. Mod.* Phys. 75, 657 (2003).

Saba Rehman is a student at Hume-Fogg Academic Magnet High School in Nashville, Tennessee; she participated in the School for Science and Math at Vanderbilt.