Muc4/Sialomucin Complex, the Intramembrane ErbB2 Ligand, Translocates ErbB2 to the Apical Surface in Polarized Epithelial Cells*

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Victoria P. Ramsauer‡, Coralie A. Carothers Carraway§, Pedro J. I. Salas‡, and Kermit L. Carraway¶
From the Departments of ‡Cell Biology and Anatomy and §Biochemistry and Molecular Biology, University of Miami School of Medicine, Miami, Florida 33101

Intramembrane ligand for the receptor tyrosine kinase ErbB2, inducing a limited phosphorylation of the receptor. The mucin subunit ASGP-1 (600 kDa) endows the molecule with anti-adhesive properties and contributes to the ability to evade immune recognition (13, 14). Subunit ASGP-2 (~600 kDa) endows the molecule with anti-adhesive properties and contributes to the ability to evade immune recognition (13, 14). Subunit ASGP-2 (~120kDa) tethers the complex to the cell surface and serves as an intramembrane ligand for the receptor tyrosine kinase ErbB2 via an epidermal growth factor-like domain (11). This interaction induces phosphorylation of ErbB2 in the absence of a soluble ligand and potentiates the phosphorylation of the ErbB2-ErbB3 heterodimer in the presence of the ErbB3-soluble ligand neurogulin. The Muc4/SMC-ErbB2 complex was first observed in highly metastatic rat ascites 13762 mammary adenocarcinoma cells, wherein the receptor and several of its associated intracellular signaling proteins appeared constitutively tyrosine-phosphorylated (15). The Muc4/SMC-ErbB2 in-

ErbB2 is a 185-kDa class I receptor tyrosine kinase that is structurally related to the epidermal growth factor receptor EGFR. The EGFR (ErbB) family includes four distinct members, i.e. epidermal growth factor receptor (EGFR, HER1, or c-ErbB1), c-ErbB2 (HER2, p185^new), c-ErbB3 (HER3), and c-ErbB4 (HER4) (1). Binding of a specific ligand to one of the ErbB receptors triggers the formation of specific homo and heterodimers (2). This dimerization is followed by phosphorylation at one or more of several possible tyrosine residues in the cytoplasmic domains, which provide specific binding sites for cytoplasmic signaling proteins that, in turn, initiate downstream signaling (3). The pathways activated may lead the cells to proliferation, differentiation, or apoptosis (1, 3). ErbB2 is the preferred signaling partner and a crucial component of active receptor heterodimers (1). These interactions have been implicated in numerous developmental processes in normal tissues such as the heart, brain, and mammary gland (3) as well as in cancers of the breast, ovary, colon, kidney, bladder, stomach, and salivary gland, wherein ErbB2 is aberrantly expressed and regulates the ErbB receptor network by acting as a co-receptor (6, 7).

A potential physiological role for the apical localization of ErbB2 is indicated by the fact that ErbB2 phosphorylated at tyrosine 1248 is found predominantly in Muc4/SMC-transfected cells, but not in untransfected cells in the same culture. The change in localization of ErbB2 was confirmed by cell surface biotinylation of apical and basolateral proteins, followed by streptavidin precipitation and the subsequent detection of ErbB2 by immunoblotting in contrast, Na⁺/K⁺-ATPase maintains its basolateral localization in Muc4/SMC-transfected cells, indicating that the translocation of ErbB2 is not the result of depolarization of the cells. A model may not be complete, however, because it does not account for some observations on ErbB2 signaling. For example, neurogulin binds ErbB3 to induce its heterodimerization with ErbB2 and trigger ErbB3 phosphorylation (8). Although the ErbB3 kinase domain is impaired (9), neurogulin also stimulates ErbB2 phosphorylation. Thus, ErbB2 mechanisms of activation appear to be complex and may include factors capable of influencing availability and phosphorylation, which, in turn, could determine strength or specificity of signaling (10).

We have recently discovered a novel mechanism for activation and modulation of ErbB2 phosphorylation and signaling (11). This mechanism involves Muc4/SMC, a cell surface heterodimeric glycoprotein. Muc4/SMC is composed of two non-covalently associated subunits, ASGP-1 and ASGP-2, which arise from proteolytic processing of a single gene product (12). The mucin subunit ASGP-1 (~600 kDa) endows the molecule with anti-adhesive properties and contributes to the ability to evade immune recognition (13, 14). Subunit ASGP-2 (~120kDa) tethers the complex to the cell surface and serves as an intramembrane ligand for the receptor tyrosine kinase ErbB2 via an epidermal growth factor-like domain (11). This interaction induces phosphorylation of ErbB2 in the absence of a soluble ligand and potentiates the phosphorylation of the ErbB2-ErbB3 heterodimer in the presence of the ErbB3-soluble ligand neurogulin. The Muc4/SMC-ErbB2 complex was first observed in highly metastatic rat ascites 13762 mammary adenocarcinoma cells, wherein the receptor and several of its associated intracellular signaling proteins appeared constitutively tyrosine-phosphorylated (15). The Muc4/SMC-ErbB2 in-

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teraction has been demonstrated in several systems, including normal lactating mammary gland, ascites tumors, isolated rat mammary epithelial cells, Muc4/SMC-transfected MCF-7 breast cancer cells, and a baculovirus-insect cell expression system (16). Muc4/SMC is constitutively expressed in many epithelial tissues wherein it is apically located and serves mainly a protective function (16). Its expression is tightly regulated in the mammary gland and the female reproductive tract (17, 18), and its expression at specific times during epithelial differentiation in certain organs suggests a role in developmental processes (16). In some carcinomas, the regulatory mechanisms have been suppressed, and Muc4/SMC is highly over-expressed (16).

One question of importance to forming a Muc4/SMC-ErbB2 complex concerns the localization of the molecules. Muc4/SMC is localized apically in polarized epithelial cells, whereas ErbB2 is often considered to be a basolateral protein (19, 20). However, we have recently demonstrated both apical and basolateral localizations of ErbB2, even in the same epithelium (21). From these results we have suggested that Muc4/SMC may contribute to determining the localization of ErbB2 and, thus, its signaling capabilities in polarized epithelia. To address this question, we examined the localization of these proteins in polarized human colon carcinoma CACO-2 cells. When grown in culture, these epithelial cells form polarized monolayers that establish a barrier to the passage of most small molecules and ions. The extracellular spaces are sealed by tight junctions, whose structural integrity is essential to the functional integrity of the tissue. A specific contributor to this structural integrity is E-cadherin, a transmembrane cell-cell adhesion protein located at the lateral junctions and usually concentrated in adhesion belts just below the tight junctions, which connect to the actin cytoskeleton of the cells (22). Our results demonstrate in CACO-2 cells that ErbB2 is localized to the lateral membrane, which is primarily co-localized with cadherin. However, when Muc4/SMC is expressed in these cells, the ErbB2 is translocated to the apical domain.

**MATERIALS AND METHODS**

**Cell Culture**—CACO-2 cells were obtained from the American Type Culture Collection (Manassas, VA). They were maintained in Dulbecco’s modified Eagle’s medium–F12 supplemented with 10% fetal bovine serum and 1× sodium pyruvate (Invitrogen) at 5% CO2 and 37 °C. The cell stocks were kept in 25-cm² tissue culture flasks and collected after dissociation with 0.25% trypsin and 2 mM EDTA for 15 min. For immunofluorescence experiments, the cells were plated on 12-mm round coverslips (Fisher Scientific) or 6-mm Transwell-Clear™ filters (Corning Costar) at high density (~5 × 10³ cells/cm²) in order to obtain confluency in 2–3 days. For biotinylation experiments the cells were plated on 24-mm Transwell-Clear™ filters (Corning Costar).

**Transient Transfections**—Forty-eight hours prior to immunofluorescence or biotinylation experiments, CACO-2 cells (70% confluent) were transiently transfected with Muc4/SMC using FuGENE 6 (Roche Diagnostics) according to the manufacturer’s instructions. For each experiment, plates of CACO-2 cells were prepared; half of them were transfected, and the other half were untransfected.

**Antibodies and Reagents**—The primary antibodies used in this study were against the following antigens: Muc4, ErbB2, E-cadherin, and Na⁺/K⁺-ATPase. Monoclonal antibody 4D12, which was developed in our laboratory, was used to detect Muc4 (27). This antibody was also used in its biotinylated form. To study ErbB2, we used four monoclonal antibodies from Lab Vision (Fremont, CA), namely NeoMarkers 2 (clone 9G6.10) and NeoMarkers 10 (clone L87 + e2–4001), which react against the extracellular domain of ErbB2, NeoMarkers 17 (clone e2–4001 + 3B5), and NeoMarkers 1, which reacts against the phosphorylated tyrosine at position 1248. The polyclonal antibody against ErbB2 from DakoCytomation ( Carpinteria, CA) was also used. E-cadherin was studied with the following monoclonal antibodies from Transcription Laboratories and BD Biosciences: E-cadherin antibody clone 34, Ecadherin antibody clone 36, and the FITC-conjugated E-cadherin antibody. The Na⁺/K⁺-ATPase was detected with a monoclonal antibody...
against this protein from Upstate Laboratories (clone c464.6) and an antibody kindly provided by Dr. W. James Nelson. All secondary antibodies were affinity-purified and did not cross-react with immunoglobulins of species other than their specific target. Absence of cross-reactivity was determined by agar diffusion assay before co-localization experiments. Peroxidase-conjugated secondary antibodies were obtained from Pierce and Sigma-Aldrich. Alexa Fluor® 488 and Texas Red®-conjugated secondary antibodies were used as specified by the manufacturer (Molecular Probes, Eugene, OR).

**Immunofluorescence—**CACO-2 cells grown to confluency on 12-mm round coverslips (Fisher Scientific) or 6-mm Transwell-Clear™ filters (Corning Costar) were processed for immunofluorescence studies 48 h after transient transfection with Muc4/SMC. The cells were fixed with 4% paraformaldehyde for 20 min at room temperature. After rinsing, the cells were permeabilized with 0.2% Triton-X100 for 5 min or 0.1% saponin throughout the procedure. Permeabilization was used in all cases except with anti-ErbB2 NeoMarkers 2 (LabVision, Fremont, CA). The permeabilization was followed by rinsing and quenching of the aldehyde groups in 50 mM NH₄Cl, after which the cells were incubated with primary antibody for 1 h at room temperature. The primary antibody was diluted in 1% bovine serum albumin; in co-localization experiments, 0.1% immunoglobulin G of the same species as the secondary antibody was used instead of 1% bovine serum albumin for rinsing steps and dilution of the primary antibody. Once this first incubation was completed, the cells were rinsed and then incubated with the secondary antibody conjugated to the fluorescent dye (Alexa Fluor® 488 or Texas Red® from Molecular Probes) for 1 h at room temperature in the dark. The cells were then mounted in 10% polyvinyl alcohol, 30% glycerol, 1% n-propyl gallate, and Slow Fade TM (Molecular Probes) at a dilution of 5:1. The preparations were first observed in a Leitz DM RB microscope (Leica Instruments GmbH, Wetzlar, Germany) equipped with a Leica Orthomat E microphotography system using a 63× (1.4 NA) infinity-corrected objective. Laser confocal microscopy was performed with an LSM 510 microscope from Zeiss (Carl Zeiss GmbH, Germany) equipped with two laser sources and the option of up to three channels. Cell monolayers stained with FITC, Alexa Fluor® 488, and Texas Red® were analyzed using a 63× oil immersion objective. The images were collected using the LSM 510 software (Carl Zeiss, GmbH, Germany), and each confocal section was obtained as the average of four frames.

**Polarity Assays—**Cell monolayers grown to confluency on 24-mm Transwell-Clear™ filters (Corning Costar) were biotinylated on the apical or basolateral surfaces 48 h after transient transfection with Muc4/SMC. After rinsing, the surface proteins of the cells were biotinylated at 4 °C using a cell membrane-impermeable biotin derivative, sulfo-NHS-biotin (Pierce). For proteins on the apical surface, the monolayer was exposed to the biotinylation agent for 15 min; for the basolateral surface, it was exposed for 40 min. After standard rinsing and quenching of the aldehyde groups in 50 mM NH₄Cl, the cells were lysed with radioimmunoprecipitation assay buffer (50 mM Tris-Cl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml each aprotinin, leupeptin, and pepstatin, 1 mM Na₃VO₄, and 1 mM NaF). The cells were gently scraped from the filter with a rubber policeman, sonicated on ice for 30 s, and centrifuged at 15000 × g for 10 min at 4 °C. Biotinylated proteins were affinity purified in batch mode overnight at 4 °C with streptavidin-conjugated agarose beads (Pierce). The biotinylated proteins were eluted from the beads by 1 mM Tris and 2% SDS and subjected subsequently to trichloroacetic acid precipitation. After rinsing the pellet with acetone, it was resuspended in 1 mM Tris buffer, pH 7.

**PAGE and Immunoblot—**The preparations of biotinylated proteins were obtained as described above. Unbiotinylated CACO-2 cell preparations used as negative or positive controls for primary antibodies were processed in the same way minus the biotinylation steps. The samples were run in SDS-PAGE and then blotted onto nitrocellulose sheets (27). The signal of primary monoclonal or polyclonal antibodies was detected using secondary affinity-purified goat anti-mouse or anti-rabbit immunoglobulins coupled to peroxidase and a chemiluminescent system (Pierce) and exposed on x-ray film (Kodak). The intensity of the bands was estimated by digitizing the image (Scion Image) from x-ray film. After subtracting the background, all band intensities were compared against a control.
Muc4 Translocation of ErbB2 in Polarized Cells

**RESULTS**

**ErbB2 Is Co-localized with Cadherin, a Lateral Junctional Protein—CACO-2 epithelial cells express ErbB2 at a level detectable by immunofluorescence (Fig. 1).** To determine the localization of ErbB2 in these cells, monolayers of the CACO-2 cells were stained with different antibodies for ErbB2 (21). Confocal microscopy suggested that the ErbB2 was located primarily in the lateral surfaces of these cells. To verify this localization, we co-stained cells with anti-ErbB2 NeoMarkers 2 monoclonal antibody (Lab Vision), followed by a secondary antibody conjugated to Texas Red® and the anti-cadherin FITC-conjugated antibody from BD Biosciences. As shown in the two-color merged confocal images in the x-y and x-z planes in Fig. 1, ErbB2 and cadherin are substantially co-localized (Fig. 1, A and B). This observation was confirmed by repeating the same immunofluorescence experiment using a different set of antibodies, i.e., the polyclonal anti-ErbB2 from DakoCytomation and a monoclonal antibody against cadherin from Transduction Laboratories (data not shown). These observations are consistent with previous studies showing localization of ErbB2 at intercellular junctions of epithelial cells (23).

**Muc4 Translocates ErbB2 to the Apical Surface Where It Is Co-localized with Muc4—Immunoblotting (Fig. 2A) and immunofluorescence (data not shown) assays on CACO-2 cells indicate undetectable endogenous expression of Muc4/SMC under the conditions specified in this study. To investigate the ability of Muc4/SMC to translocate ErbB2 from the basolateral membrane to the apical surface, we transiently transfected the CACO-2 cells with Muc4/SMC. Transient transfection allowed us to observe transfected cells in a population of untransfected cells (Fig. 2). Cells transfected with Muc4/SMC show co-localization of ErbB2 with the Muc4/SMC in two-color confocal merged images (Fig. 2, B and C); the translocation to the apical surface is particularly dramatic in the x-z plane (Fig. 2C, arrow).** The surrounding untransfected cells do not show the re-localization of ErbB2 (Fig. 2, B and C). The apical localization of the ErbB2 is also observed by staining the cells with a monoclonal anti-ErbB2 antibody against the ErbB2 extracellular domain added to the apical side of the cell layers (Fig. 3).

**This antibody stains basolateral ErbB2 in permeabilized layers of untransfected CACO-2 cells but fails to stain nonpermeabilized, untransfected cell layers (Fig. 3A). In contrast, the antibody heavily stains the cell surfaces from the apical side of the Muc4/SMC-transfected cells in the cell layers but not the untransfected cells in the culture (Fig. 3B; compare the distributions of staining of ErbB2 and Muc4 with the cells observed by phase contrast).**

**The location of ErbB2 was further addressed by biotinylation experiments (Fig. 5).** CACO-2 cell layers were treated with a nonpermeable biotinylating reagent from either the apical or basolateral side. The cells were then solubilized for affinity purification of the biotinylated cell surface proteins with streptavidin-conjugated agarose. Immunoblotting of the streptavidin precipitates with anti-ErbB2 monoclonal antibody NeoMarkers 17 (Lab Vision) demonstrated the surface to which the ErbB2 was exposed. This antibody was selected for its specificity and low background after trials with monoclonal antibodies 2, 8, and 10 from NeoMarkers and the polyclonal antibody from DakoCytomation. A cell lysate of A375 human melanoma cells known to express ErbB2 was run as a positive control to confirm reactivity of the antibodies. As a negative control, unbiotinylated CACO-2 cells were processed in the same manner as biotinylated cells. As shown in Fig. 4, ErbB2 is present at the apical surface in the cells transfected with Muc4/SMC but absent in untransfected cells. In contrast, as expected for transiently transfected cells, ErbB2 is present at the basolateral surfaces in both transfected and untransfected cells.

**One trivial explanation for these results is that transfection with Muc4/SMC results in a loss of cell polarization. To address this possibility, Na⁺/K⁺-ATPase, a basolateral marker, was examined by biotinylation from the apical and the basolateral surfaces. As shown in Fig. 4, no Na⁺/K⁺-ATPase was detected at the apical surfaces of either transfected or untransfected cells, but it was readily detected at the basolateral surfaces of both, indicating that loss of cell polarity has not occurred.**

**Phosphorylated ErbB2 Is Detected Predominantly in Muc4/SMC-transfected Cells—Our previous studies have shown that Muc4/SMC binding to ErbB2 leads to phosphorylation of the**

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**Table I**

**ErbB2 localization in Muc4/SMC-expressing epithelia**

The abbreviations used in this table are: M, membrane; S, soluble; IHC, immunohistochemistry; co-IP, co-immunoprecipitation; cIF, confocal immunofluorescence.

| Tissue   | Muc4 (membrane/soluble) | ErbB2 localization | Co-localization/method |
|----------|-------------------------|---------------------|------------------------|
| Mammary  | M, S                    | Apical, lateral     | Yes, IHC, co-IP        |
| Uterus   | M, S                    | Apical, basolateral | Yes, IHC, co-IP        |
| Oviduct  | M, S                    | Apical, basolateral | Yes, IHC, co-IP        |
| Lacrimal gland (28) | M, S | Membrane            | Yes, IHC, cIF, co-IP   |
| Colon    | S                      | Basolateral         | No, IHC                |
| Cervix   | M, S                    | Membrane            | Yes, when expressed in same cells in stratified epithelium, IHC, co-IP |

* Numbers in parentheses are references.

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ErbB2 on tyrosine 1248, detected by a specific anti-phospho-ErbB2. To determine whether ErbB2 in the transfected CACO-2 is in the phosphorylated form, Muc4/SMC-transfected cells as well as untransfected cells were probed with a monoclonal antibody against ErbB2 phosphorylated at position 1248. The signal from this antibody was observed predominantly in cells transfected with Muc4/SMC, compared with untransfected cells, which showed no signal (Fig. 5). Analysis of the slices of the z-stacks of transfected and untransfected cells indicate that phosphorylated ErbB2 is substantially co-localized with Muc4 at the apical membrane in the transfected cells (Fig. 5, merge). These results confirm our previous studies, which indicate that formation of the Muc4/SMC-ErbB2 complex leads to specific phosphorylation of the ErbB2 (26).

**ErbB2 Localization in Muc4/SMC-expressing Epithelia**—One question that arises concerning the ability of Muc4/SMC to affect ErbB2 localization is its generality. We have examined a number of epithelia expressing Muc4/SMC to determine the localization of ErbB2. The general picture is that ErbB2 is apically localized, although not exclusively, in simple epithelia in which Muc4/SMC is present in its membrane form and is apical. Examples include the mammary gland, uterus, and oviduct (Table I). An unusual exception is the lacrimal gland (Table I), wherein the membrane Muc4/SMC is not predominantly apical as it is in other simple epithelia. However, ErbB2 is co-localized with the membrane Muc4/SMC but not with a soluble form of Muc4/SMC present in secretory granules (28). A second exception is the colon (Table I), in which Muc4/SMC is predominantly in a soluble form in secretion granules in goblet cells (27). Finally, stratified epithelia provide an obvious exception, because Muc4/SMC and ErbB2 will not necessarily be expressed in the same cells in these complex epithelia.

**DISCUSSION**

Cell signaling mechanisms depend not only on the components of signaling pathways but also on their localization within the cell (24). ErbB receptors are usually considered to be basolateral components of epithelial cells, because their soluble ligands are produced by stromal cells or come through the blood stream and, thus, are accessible to basolateral surfaces. Signaling through heterodimerization mechanisms requires that both ErbBs destined to form a heterodimer are accessible to each other. Because ErbB2 is the key element in most heterodimerization schemes (1–4), its localization is critical to ErbB signaling. However, the localization of ErbB2 in polarized epithelia is far from clear. Both basolateral (19, 20) and apical (25) localizations have been described. Thus, it is important to understand how ErbB2 localization is determined. Recent studies have presented conflicting pictures, implicating the PDZ domain-containing protein ERBIN (19) and specific ErbB2 juxtamembrane sequences (20). However, regardless of which of these is correct in cells not expressing Muc4/SMC, our results suggest that the mucin can override these mechanisms and direct ErbB2 to the apical surface, as shown in the model in Fig. 6A. How Muc4/SMC is able to change the ErbB2 localization is yet unclear, though there are two likely possibilities. Our previous results have indicated that Muc4/SMC forms an intracellular complex with ErbB2 and that complex formation leads to phosphorylation of Tyr-1248 of the ErbB2 (11, 25). The present studies indicate that this phosphorylated form occurs...
in the Muc4/SMC-transfected cells, but not in untransfected cells, and is co-localized with the Muc4/SMC. Phosphorylation could block the interaction of ErbB2 with factors directing it to the basolateral membrane, as shown for ERBIN (19). Alternatively, Muc4/SMC could "drug" ErbB2 to the apical surface by overriding its basolateral signals. Preliminary studies on the formation of the Muc4/SMC-ErbB2 complex in the baculovirus expression system indicate that it is formed very early, before reaching the cell surface.

Regardless of the mechanism involved, localization of ErbB2 to the apical surface of polarized cells has important implications for cell behavior. Apical localization will sequester the ErbB2 from ligands with only basolateral access and possibly from other ErbBs. Such localization may also restrict the intracellular pathways to which ErbB2 can contribute. Removing ErbB2 from junctional complexes will limit its ability to phosphorylate junctional components and disrupt cell-cell adhesion mechanisms. To the contrary, overexpression of ErbB2 may override the Muc4/SMC localization mechanism, increase ErbB2 association with cell junctions, and facilitate their disruption by neoplastic transformation.

One interesting possibility for the function of the Muc4/SMC localization of ErbB2 is that apical ErbB2 provides a sensor of epithelial cell integrity. Loss of phosphorylation would expose the ErbB2 to basolateral components, potentially activating additional signaling pathways that could stimulate responses necessary for initiating repair mechanisms. This loss could occur as a result of epithelial damage that destroyed cell-cell interactions and caused the cells to become rounded. We have shown previously that neuregulin stimulation of cells containing the Muc4/SMC-ErbB2 complex leads to potentiation of the phosphorylation of both ErbB2 and ErbB3, presumably from the formation of a Muc4/SMC-ErbB2-ErbB3-neuregulin "quad" complex. The hyperphosphorylation of these receptors should lead to initiation and/or activation of additional downstream signaling pathways. Thus, loss of cell polarization due to injury could convert the epithelial cells from a differentiated state, with Muc4/SMC-ErbB2 at the apical surface, to a proliferative state, with the Muc4/SMC-ErbB2-ErbB3-neuregulin quad complex distributed around the cell surface (Fig. 6B). Based on this model, we propose that ErbB2 acts as a regulatory switch in epithelial cells between differentiation and proliferation, with Muc4/SMC acting as a modulator of that switch. Switching to the proliferative state will position the epithelial cells to begin processes necessary to repair the damage.

Similarly, loss of polarization can occur as a consequence of neoplastic transformation. Thus, we envision that tumor cells simply highjack the epithelial sensor/repair mechanism to promote proliferation and progression.

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