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Epithelial Membrane Protein 2 and β1 integrin signaling regulate APC-mediated processes

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ABSTRACT

Adenomatous Polyposis Coli (APC) plays a critical role in cell motility, maintenance of apical-basal polarity, and epithelial morphogenesis. We previously demonstrated that APC loss in Madin Darby Canine Kidney (MDCK) cells increases cyst size and inverts polarity independent of Wnt signaling, and upregulates the tetraspan protein, Epithelial Membrane Protein 2 (EMP2). Herein, we show that APC loss increases β1 integrin expression and migration of MDCK cells. Through 3D *in vitro* model systems and 2D migration analysis, we have depicted the molecular mechanism(s) by which APC influences polarity and cell motility. EMP2 knockdown in APC shRNA cells revealed that APC regulates apical-basal polarity and cyst size through EMP2. Chemical inhibition of β1 integrin and its signaling components, FAK and Src, indicated that APC controls cyst size and migration, but not polarity, through β1 integrin and its downstream targets. Combined, the current studies have identified two distinct and novel mechanisms required for APC to regulate polarity, cyst size, and cell migration independent of Wnt signaling.

1. Introduction

Epithelial cells normally form a uniform layer maintained through the regulation of cell-cell interactions and apical-basal polarity [4] by several complexes including cell junctions and polarity proteins such as Par6, atypical Protein Kinase C (aPKC), Scribble (Scrib), and Discs large (Dlg) [31,71]. The loss of these complexes, and subsequently apical-basal polarity, disrupts normal developmental processes including epithelial structure, migration, and intracellular signaling [41], predisposing cells to tumorigenesis ([9,63] and reviewed in [37,44]. Several studies have shown that the loss of junctional complexes and polarity complexes leads to disrupted morphogenesis and polarity. Down regulation of Junctional Adhesion Molecule A (Jam-A), Par6, aPKC, or lethal giant larvae (Lgl) in Madin Darby Canine Kidney (MDCK) cysts prevented lumen formation and disrupted apical-basal polarity [15,2,25,28,58,73]. Similar phenotypes are seen in acini formed by MCF-10A mammary epithelial cells with the loss of Scrib [77], and the mislocalization of Scrib from cell junctions promotes mammary tumorigenesis through PTEN and Akt signaling [14]. Furthermore, studies have shown that changes in mammary cell adhesion, signaling, and polarity can lead to development of breast cancer ([14,46] and reviewed in [4,10]). For example, loss of the polarity complex protein Par3 in conjunction with activation of Notch signaling or the expression of the oncogene h-RAS increases mammary tumor growth [45]. These studies stress the importance of regulating cellular processes like cell polarity, adhesion, and motility to prevent tumorigenesis.

It has recently been appreciated that tumor suppressors play an important role in regulating polarity to maintain tissue structure. One example of this is Liver Kinase B1 (LKB1), a tumor suppressor known to regulate cell polarity proteins and complexes, such as the Par proteins ([19] and reviewed in [41]). In breast epithelial cells, LKB1 loss increased cell migration and invasion in 2D, and disrupted morphology and polarity in 3D culture [35]. Similarly, loss of a novel tumor suppressor ductal epithelium associated ring chromosome 1 (DEAR1) in human mammary epithelial cells resulted in acini with disrupted polarity and filled lumens [11]. Studies from our laboratory provide evidence for a role for Adenomatous Polyposis Coli (APC) in regulating apical-basal polarity and morphogenesis as mammary glands of *Apc*-mutant mice exhibit alterations in polarity and epithelial

Abbreviations: APC, Adenomatous Polyposis Coli; AP-1, activator protein 1; aPKC, atypical Protein Kinase C; CREB1, cAMP responsive element binding protein 1; DEAR1, Ductal Epithelium Associated Ring Chromosome 1; Dlg, Discs large; EMP2, Epithelial Membrane Protein 2; FAK, Focal Adhesion Kinase; Jam-A, Junctional adhesion molecule A; Lgl, Lethal giant larvae; LKB1, Liver Kinase B1; MDCK, Madin Darby Canine Kidney; PAX2, paired box gene 2; Scrib, Scribble; STAT3, signal transducer and activator of transcription 3; Yki, Yorkie; ZO-1, tight junction protein 1

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structure during pregnancy and lactation [54], and the loss of APC in MDCK cells resulted in larger cysts with filled lumens and the mislocalization of the apical marker gp135 to the basal membrane [33]. Furthermore, heterozygous mutation of *Apc* altered polarity and tissue structure in human familial adenomatous polyposis (FAP) colonic tissue and mouse kidney and colonic tissue [33]. The maintenance of apical-basal polarity by tumor suppressors contributes to their role in preventing disease progression. For example, ErbB-2 mediated mammary tumorigenesis was accelerated upon loss of the 14-3-3σ tumor suppressor resulting in tumor cell lines with disrupted junctional complexes and mislocalization of Par3 [36]. Together these studies suggest that maintenance of apical-basal polarity is a critical function by which APC suppresses tumor development.

Although APC is most known for its role in the regulation of Wnt/β-catenin signaling, it has many Wnt-independent functions including regulating apical-basal polarity, microtubule and actin networks, and cell migration (reviewed in [49,55]). Consistent with previous results showing Wnt-independent functions of APC [54,56,7], we recently demonstrated that APC regulates polarity and cyst size through a Wnt-independent mechanism as TCF/LEF transcriptional activity was not increased upon APC loss and stabilized β-catenin did not increase cyst size or mislocalize gp135 in MDCK cysts [33]. Furthermore, the reintroduction of the β-catenin binding domain of APC was unable to restore cyst size and apical polarity in MDCK cysts with APC loss [33]. Interestingly, a C-terminal fragment of APC, important for binding polarity complexes, decreased cyst size and reversed the polarity phenotypes caused by APC loss [33]. The C-terminus of APC also contains binding domains for actin, microtubules, and associated cytoskeletal proteins suggesting a role for APC in cell motility. This role has been supported by studies in which *Apc*-mutant mice displayed altered enterocyte migration in the intestine [38,59,72], mammary tumor cell migration was controlled by APC localization at cell protrusions [50], and the C-terminus of APC promoted the apical extrusion of human bronchial cells [40]. Furthermore truncation mutations in APC in MDCK cells and colorectal tumors cells increased cell migration [26,27].

We identified that APC knockdown in MDCK cells does not activate the Wnt/β-catenin pathway, but results in elevated levels of Epithelial Membrane Protein 2 (EMP2) [33]. Previous studies demonstrated that overexpression of EMP2 increases endometrial cell migration in wound healing assays [17], upregulates expression of β1 integrin, and activates Focal Adhesion Kinase (FAK)/Src signaling [16,17]. β1 integrin has been shown to regulate polarity and cyst size in MDCK cells [74]. Furthermore FAK and Src have been identified as downstream targets of β1 integrin ([8,53] and reviewed in [23,66]). Given the interaction of EMP2 and β1 integrin with FAK and the role of FAK/Src signaling in migration and tumorigenesis, FAK and Src are promising candidates as downstream effectors of APC. Several studies have shown that FAK has a role in regulating cell motility. APC interacts with FAK at the leading edge of migrating cells [43]. Additionally, phosphorylated FAK and Src are increased in mammary tumors from *Apc*-mutant mice [56], and correlate with enhanced cell migration of breast cancer cells in wound healing assays [13]. These data provide a possible mechanism by which APC mediates cyst size, polarity, and migration through upregulation of EMP2 and subsequent activation of β1 integrin and downstream signaling.

The current studies test the hypothesis that APC mediates epithelial polarity, cyst size, and migration through EMP2 and β1 integrin signaling. Given the known interactions between EMP2, β1 integrin, FAK and Src, and their roles in regulating cell growth, adhesion, motility, and tumorigenesis these pathways present a possible mechanism by which APC regulates these processes. The current studies show for the first time that EMP2 regulates cyst size and apical-basal polarity, while β1 integrin signaling mediates cyst size and migration, but not apical-basal polarity. Combined, we have identified two novel molecular mechanisms by which APC influences epithelial morphogenesis, polarity, and cell motility.

2. Materials and methods

2.1. Cell culture

Madin Darby Canine Kidney (MDCK) cells were obtained from K. Matlin (University of Chicago), tested for contamination, and maintained in MEM media with 5% FBS, 2 mM L-glutamine, 10 mM HEPES, and 1% Penicillin/Streptomycin. ctl shRNA and APC shRNA cells were established previously [33], and were grown in MEM media with 5% FBS, 2 mM L-glutamine, 10 mM HEPES, 1% Penicillin/Streptomycin, and 2 μg/ml Puromycin. EMP2 was knocked down in APC shRNA cells using ribozymes targeting EMP2 (a kind gift from M. Wadehra, UCLA) [67], and cells were selected using 600 μg/ml G418. Inhibition was confirmed using rt-PCR as described below.

2.2. Morphological assay

For 3D cultures, 5,000 cells per well were overlaid in single cell suspension of 2% Matrigel media over a solid bed of 50 μl Matrigel (BD Biosciences; San Jose, CA, USA) in an 8 well chamber slide (protocol modified from Debnath et al., 2003). Cysts were grown for 7 days as previously described [33]. Cysts were imaged on an EVOS fl microscope at room temperature with 20x objective and Sony ICX285AL CCD camera, size was assessed using ImageJ software, and significance was determined using a one-way ANOVA. Adobe Photoshop and Illustrator were used to generate figures. For inhibitor treatments, 8 μg/ml AIIB2 (Developmental Studies Hybridoma Bank; Iowa City, Iowa, USA) [75] was applied at Day 1, and 50 μM PP2 (Sigma-Aldrich; St. Louis, MO, USA) or 2 μM PF-04554878 (Cayman; Ann Arbor, MI, USA) were applied at Day 4. A two-way ANOVA was used to determine statistical significance.

2.3. Immunofluorescence (IF)

MDCK cysts were fixed in 4% paraformaldehyde at days specified. Cysts were permeabilized with 0.5% Triton-X-100, blocked in IF buffer (0.1% BSA, 0.2% Triton X-100, 0.05% Tween 20, and 0.1% goat serum in PBS) and incubated with anti-gp135 (1:100; Developmental Studies Hybridoma Bank) [39] diluted in IF buffer overnight at room temperature. To visualize the staining, cysts were incubated in Alexa-conjugated secondary antibodies diluted in IF buffer (1:1000; Invitrogen A21422, lot:1180091; Carlsbad, CA, USA) [3] for 1 h at room temperature. Actin was visualized using Alexa conjugated phalloidin (1:200; Invitrogen A-12379, lot: 1217967) [6]. Slides were mounted with Fluoromount G, Hoescht dye, and TOPRO-3, and imaged on a Zeiss 710 confocal at room temperature with Pin Apo 63x objective. Images were analyzed with ZEN software and figures were generated with Adobe Photoshop and Illustrator. At least 100 cysts were classified as either normal, exhibiting apical localization of gp135, or abnormal, exhibiting basal or mixed localization of gp135 and statistical analysis was done using Fisher's exact test.

2.4. Migration assay

1×10^5 cells were plated per well in a 6 well plate. Once cells reached confluence, cells were treated with 2.5 μg/ml mitomycin (Sigma-Aldrich) to inhibit proliferation and scratched with a 1–10 μl pipette tip. For inhibitor treatments, cells were treated with 2.5 μg/ml mitomycin and 6.25 μM PP2, 8 μg/ml AIIB2, or 2 μM PF-04554878. Wounds were imaged at 0, 24, and 48 h post scratching with phase-contrast microscope (Evos fl) at room temperature with 20x objective and Sony ICX285AL CCD camera. Analysis was completed using TScratch software (developed by the Koumoutsakos group (CSE Lab), at ETH Zürich [18] and significance was determined using a one-way