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NHERF1/EBP50 Controls Morphogenesis of 3D Colonic Glands by Stabilizing PTEN and Ezrin-Radixin-Moesin Proteins at the Apical Membrane[1,2]

Maria-Magdalena Georgescu*,†, Gilbert Cote‡, Nitin Kumar Agarwal† and Charles L. White III*

*Department of Pathology, The University of Texas Southwestern Medical Center, Dallas, TX, USA; †Department of Neuro-Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX, USA; ‡Department of Endocrine Neoplasia, The University of Texas MD Anderson Cancer Center, Houston, TX, USA

Abstract

Na^+/H^+ exchanger 3 regulating factor 1/ezrin-radixin-moesin (ERM)–binding phosphoprotein 50 (NHERF1/EBP50), an adaptor molecule that interacts with the ERM–neurofibromatosis type 2 family of cytoskeletal proteins through its ERM-binding region and with phosphatase and tensin homolog (PTEN) and β-catenin through its PDZ domains, has been recently implicated in the progression of various human malignancies, including colorectal cancer (CRC). We report here that NHERF1 controls gland morphogenesis, as demonstrated in three-dimensional (3D) human intestinal glands developing from a single nonpolarized cell. Starting from the early two-cell developmental stage, NHERF1 concentrates at the cellular interface in a central membrane disc that marks the apical pole delimiting the forming lumen. NHERF1 depletion leads to severe disruption of the apical-basal polarity, with formation of enlarged and distorted cell spheroids devoid of a central lumen. This characteristic and the increased number of mitoses in NHERF1-depleted spheroids, including multipolar ones, mimic high-grade dysplasia lesions observed in CRC progression. NHERF1 ERM-binding or PDZ-domain mutants fail to localize apically and impair gland formation most likely by outcompeting endogenous ligands, with the latter mutant completely aborting gland development. Examination of NHERF1 ligands showed that even if both ezrin and moesin colocalized with NHERF1 at the apical membrane, moesin but not ezrin depletion disrupted morphogenesis similarly to NHERF1. NHERF1 depletion resulted also in membrane displacement of PTEN and nuclear translocation of β-catenin, events contributing to polarity loss and increased proliferation. These findings reveal an essential role of NHERF1 in epithelial morphogenesis and polarity and validate this 3D system for modeling the molecular changes observed in CRC.

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Introduction

Epithelial gland morphogenesis is the process resulting in the establishment of polarized tubular or acinar structures formed by tightly connected cells arranged on a basement membrane that delimit a lumen by their apical surface. The polarized architecture of glandular epithelia allows unidirectional fluid and solute transport, which is the basis for the absorptive and secretory functions. Its alteration leads to epithelial sheet disorganization and development of initially benign glandular tumors, or adenomas, that, on further accumulation of mutations, may become malignant, or adenocarcinomas. Epithelial morphogenesis is initiated by cell adhesion molecules, such as cadherins, followed by organization of the cytoskeleton and differential sorting of

Abbreviations: 3D, three-dimensional; CIP, calf intestinal phosphatase; IF, immunofluorescence; CRC, colorectal cancer; ERM, ezrin-radixin-moesin; NF2, neurofibromatosis type 2; NHERF1/EBP50, Na^+/H^+ exchanger 3 regulating factor 1/ERM-binding phosphoprotein 50; pHH3, phospho-histone H3; PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP_3, phosphatidylinositol 3,4,5-trisphosphate; PM, plasma membrane

Address all correspondence to: Maria-Magdalena Georgescu, MD, PhD, University of Texas, Southwestern Medical Center, 5323 Harry Hines Blvd, Dallas TX 75390.
E-mail: maria-magdalena.georgescu@phhs.org

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proteins to the cortical apical and basolateral compartments [1]. Recently, the compartmentalization of lipids, especially phosphoinosides, was shown to maintain epithelial cell polarity, with phosphatidylinositol 4,5-bisphosphate (PIP_2) enriched at the apical membrane and phosphatidylinositol 3,4,5-trisphosphate (PIP_3), at the basolateral membrane [2]. PTEN tumor suppressor, a phosphoinositide phosphatase that dephosphorylates PIP_3 to PIP_2 [3] and opposes the activity of the phosphoinositol 3-OH kinase, has been implicated in maintaining this balance [4].

Na^+/H^+ exchanger 3 regulating factor 1/ezrin-radixin-moesin (ERM)–binding phosphoprotein 50 (NHERF1/EBP50) is an adaptor protein that interacts directly with PTEN [5] and is localized mainly at the apical plasma membrane (PM) in human epithelial tissues [6]. NHERF1 knockout mice have ultrastructural defects of the apical intestinal brush border membrane [7,8] and also show mammary alveolar membrane polarity disruption with lactation deficit [9]. Our mechanistic studies in cultured cells showed that NHERF1 behaves as a tumor and epithelial-to-mesenchymal transition suppressor through its effects on PTEN and β-catenin [5,10,11]. We and others have also shown that NHERF1 is involved in a series of human cancers, including adenocarcinomas of colon and breast [6,10,12]. We have now examined the involvement of NHERF1 in three-dimensional (3D) intestinal gland formation in which glands develop from single stem cells. We found that NHERF1 is required for epithelial morphogenesis by interacting with moesin and stabilizing PTEN at the apical membrane. This morphogenetic study provides mechanistic support to our previous observations of NHERF1 loss in human colorectal cancer (CRC) that occurs as an early step in adenoma progression [10].

Materials and Methods

Plasmids and Retroviral Infections

The retroviral constructs encoding Myc-tagged NHERF1 wild type and ΔSNL mutant in the pCX_b vector (blasticidin selection) and the NHERF1 short hairpin RNA (shRNA) Nos 1 and 4, ezrin short hairpin RNA (shRNA) No. 8, and moesin shRNA No. 4 used for knockdown in pSIREN-RetroQ vector (puromycin selection) have been described [11,13–15]. The Myc-tagged PDZ-domain double mutant (PDZ1-2-DM) construct in pCX_b retroviral vector, which has both PDZ-domain binding pockets disrupted by GF to AA substitutions at positions 25 to 26 (PDZ1) and 165 to 166 (PDZ2), was obtained by polymerase chain reaction mutagenesis. The retroviral constructs encoding Akt, PTEN, and PTEN-ΔPDZ (former PTEN-401) [16] were obtained by inserting in the pCX_n vector (G418 selection) the cDNAs in frame with HA or Myc tags, respectively. Transfections and retroviral infections were performed as previously described [16].

Epithelial Morphogenesis Assay

Caco-2 human CRC cells were grown in a 50/50 mixture of Dulbecco's modified Eagle's medium /Ham's F-12 nutrients supplemented with 10% FBS. Caco-2 cells were embedded in 40% vol/vol Growth Factor–Reduced Matrigel (BD Biosciences, La Jolla, CA)/growth medium to produce 3D glands as previously described [17]. Immunofluorescence (IF) staining of glands was performed 2 to 8 days postembedding. The following primary antibodies were used at 1 mg/ml overnight at 4°C: E-cadherin, ezrin, moesin, GM130 (BD Biosciences), NHERF1 (Affinity BioReagents/Thermo, Rockford, IL), laminin, β-catenin, (Sigma-Aldrich, St Louis, MO), ZO-1 (Zymed/Invitrogen, Carlsbad, CA), atypical protein kinase C (aPKC) (C-20), PTEN (A2B1), Myc (9E10) (Santa Cruz Biotechnology, Santa Cruz, CA), HA (Boehringer Mannheim/ Roche, Indianapolis, IN), and phosphohistone H3 (pHH3)–S10 (Upstate Biotechnology/Millipore, Billerica, MA). Actin cytoskeleton was stained with rhodamine-labeled phalloidin (Molecular Probes/Invitrogen, Carlsbad, CA) at 1:200. Both the secondary antibodies Alexa Fluor 568 goat anti-rabbit IgG and Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes/Invitrogen) were used at 1:200, with TO-PRO-3 iodide (Molecular Probes/Invitrogen) at 1:2000. The slides were mounted with the SlowFade Gold antifade reagent (Invitrogen). The Carl Zeiss MicroImaging, Thornwood, NY 510 confocal microscope was used to acquire images, with ×63/1.40 objective with oil immersion.

CRC Resection Specimens and Histology

Resection specimens from patients with CRC were obtained from the Gastrointestinal Tumor Bank at MD Anderson Cancer Center (Houston, TX) and contained areas of adenoma, carcinoma, and adjacent normal mucosa on the same slide [10]. The CRC specimens were deparaffinated and hydrated as previously described [11], followed by hematoxylin and eosin staining or immunohistochemistry with NHERF1 antibody (Affinity BioReagents/Thermo), at 1:200.

Protein Analysis

Cell lysis and immunoblot analysis were performed as previously described [5]. Antibodies for Western blot analysis were NHERF1, ezrin (BD Biosciences), glyceraldehyde 3-phosphate dehydrogenase; Erk, extracellular signal-regulated kinase (GAPDH) (0411), Myc (9E10), PTEN (A2B1), moesin (C15), Erk1 (C16) and Erk2 (C-14) (Santa Cruz Biotechnology), Akt (Cell Signaling Technology, Danvers, MA), and HA (Boehringer Mannheim/Roche). Calf intestinal phosphatase (CIP) assay was performed as per manufacturer's instructions (New England Biolabs, Ipswich, MA). Briefly, cells were lysed in lysis buffer (40 mM Hepes (pH 7.5), 120 mM NaCl, and 1% Triton X-100). Lysates were incubated with 10 U of CIP or no enzyme control at 37°C for 1 hour and then analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. ImageJ program (National Institutes of Health, Bethesda, MD) was used for densitometric analysis.

Results

NHERF1 Controls the Morphogenesis of Intestinal Cell Glands

NHERF1 is a 50-kDa adaptor protein comprising two tandem PDZ domains and a carboxyl (C)-terminal ERM-binding region [18,19]. It associates with the PTEN tumor suppressor protein through its PDZ1 domain [5] and with β-catenin through its PDZ2 domain [20] (Figure 1*A*).

To model the development of adenocarcinoma, we examined the effect of NHERF1, a molecule lost early in CRC progression [10], on the development of 3D glands. We have previously shown that NHERF1 exhibits apical PM localization in Caco-2 CRC cells similarly to normal colon epithelial cells [10]. Caco-2 cells have been used as a model of normal intestinal cells because they form polarized cell monolayers with well-developed apical microvilli in confluent 2D, two-dimensional cultures [21] and 3D cysts with a central lumen when grown in Matrigel [17]. These 3D cysts or glands develop from nonpolarized single cells through successive symmetric cell divisions, labeled by pHH3 (Figure 1*B*), and, progressing through two-cell, three-cell, and multicell stages, reach the mature gland stage by 8 days in culture (Figure 1*C*). As early as from the two-cell stage, polarity is apparent (see also polarity markers in Figure 2*B*), and NHERF1 strongly