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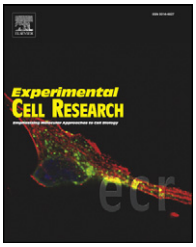

Research Article

Numb modulates intestinal epithelial cells toward goblet cell phenotype by inhibiting the Notch signaling pathway☆

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ABSTRACT

Numb was originally identified as an important cell fate determinant that is asymmetrically inherited during mitosis and controls the fate of sibling cells by inhibiting the Notch signaling pathway in neural tissue. The small intestinal epithelium originates from the division of stem cells that reside in the crypt, which further differentiate into goblet cells, absorptive cells, paneth cells, and enteroendocrine cells. However, Numb's involvement in the differentiation process of intestinal epithelium is largely unknown. In the present study, we confirm that both the Numb mRNA and protein isoforms are expressed in adult mouse intestinal mucosa. Numb protein is ubiquitously expressed throughout the crypt–villus axis of the small intestinal epithelium and is mainly localized to the cytoplasmic membrane. Down-regulation of endogenous Numb using RNA interference in cultured intestinal LS174T cells increased Notch signaling, leading to the up-regulation of Hes1 and the down-regulation of Hath1. Knockdown of Numb alleviated MUC2 protein expression and led to loss of the goblet cell phenotype in LS174Tl cells. Our results provide the first evidence that Numb, an important cell fate determinant, modulates intestinal epithelial cells towards the goblet cell phenotype by inhibiting the Notch signaling pathway.

Introduction

The small intestinal epithelium forms the villi and crypt architecture. Villi are finger-like protrusions that point toward the lumen and are lined by a single-layer of terminally differentiated cells that include enterocytes, goblet cells, and enteroendocrine cells. Crypts result from epithelial invaginations into the gut mucosa and have vigorous proliferation potential. The differentiated epithelial cells at the villi tips undergo apoptosis and are replaced every few days by progenitor stem cells that dwell in the crypts and move up the villi as they differentiate [1]. It is widely accepted that the initial differentiation of the progenitor stem cells is affected by the position of the epithelial cell along the crypt-to-villus axis and by its interactions with neighboring cells [2].

It is not very clear how progenitor stem cells in the crypt become lineage restricted. Recent studies in mice have revealed

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Abbreviations: NICD, Notch intracellular domain; PBS, phosphate buffered saline; PTB, amino-terminal phosphotyrosine-binding domain; PRR, carboxyl-terminal praline rich region

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that several key molecules (also known as cell fate determinants), including Hath1 (Notch signaling pathway) and Ascl2 (Wnt signaling pathway) [3], are crucial for the progenitor stem cells within the crypt to commit to a specific lineage. Numb is a critical cell fate determinant originally identified in *Drosophila*. *Numb* mutant embryos display an alteration of sensory organ precursor lineage choices [4]. Numb is a membrane-associated protein that asymmetrically segregates in dividing cells and determines distinct cell fates by interacting with and inhibiting Notch [5], a process that has been extensively characterized in mammalian neuroepithelial cells. However, its expression and function in mammalian intestinal tissue has not been characterized.

Numb is evolutionary conserved, and the mammalian homolog encodes four alternatively spliced transcripts, generating four different proteins. Numb contains multiple protein–protein interaction regions, including an amino-terminal phosphotyrosine-binding (PTB) domain, a carboxyl-terminal praline rich region (PRR), and Eps15 homology (EH) regions (DPF and NPF) [6]. The different isoforms result from the presence or absence of exon 3 (found within the PTB domain) and/or exon 9 (found within the PRR domain) [7]. The key factors that crosstalk with Numb within the microenvironment to trigger cell fate specification are unknown. Nevertheless, a number of pathways that control stem/progenitor cell proliferation and differentiation are known to interact with Numb. For instance, Numb's involvement in Notch or Hedgehog activation, and loss of p53 function, promotes stem cell maintenance and expansion [8–10]. Numb inhibits Notch signaling in several ways. Numb interacts with the Notch intracellular domain (NICD) and recruits the E3-ubiquitin ligase, Itch, to the membrane tethered protein, leading to polyubiquitination and degradation of NICD [11]. In addition, Numb may also control Notch intracellular trafficking by interacting with the endocytic machinery components α-adaptin and Eps15 through its DPF and NPF domain, respectively [12]. Endocytosis and sequestration of full-length membrane-tethered Notch or NICD lead to its inhibition.

Binding of Notch ligands to the Notch receptors induces proteolytic cleavage of Notch receptors by γ-secretase, causing release of the NICD. The released NICD then translocates to the nucleus resulting in an active transcriptional complex with RBP-j (CSL or CBF-1). The formation of this complex induces hairy/enhancer of split (*Hes*) expression. Hes1, a basic helix–loop–helix (bHLH) transcription factor, activates target gene expression that ultimately regulates proliferation and differentiation [13]. Furthermore, it has been shown that Math1, a basic helix–loop–helix transcription factor, is a downstream target of Hes1, and controls the initial cell fate choice made by the crypt progenitor stem cells [14]. Math1 null mice intestines fail to develop all three secretory cell lineages, including goblet cells [14].

Numb is also expressed in most adult tissues including, but not limited to, breast, lung, testis, and salivary glands [15–18], suggesting a function for Numb outside of neurogenesis. In the present study, we explored the expression and function of Numb in intestinal tissues. We found that Numb is ubiquitously expressed throughout the crypt–villus axis of the small intestinal epithelia in adult mice. Suppression of Numb expression leads to increased Notch signaling and reduced expression of Mucin 2 in LS174T cells, suggesting Numb functions in the regulation of intestinal epithelial cells towards the goblet cell phenotype.

Materials and methods

Cell culture

Human colon cancer-derived LS174T and HT29 cells were obtained from the American Type Culture Collection (Manassas, VA USA). Both of the cell lines were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (HyClone, USA), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, USA), at 37 °C in a humidified incubator with 5% CO_2. Upon reaching 90% confluency, cells were dissociated with 0.25% trypsin and 0.02% EDTA and split.

Reverse transcription (RT)-PCR

Total mRNA was extracted from scraped mouse small intestinal tissue, LS174T, and HT29 cells using Trizol reagent (Invitrogen, Carlsbad, CA), and 2 μg of total RNA was used to prepare cDNA in a total volume of 25 μl (PrimeScript® RT-PCR Kit, Takara, Japan). PCR was then performed on 1 μl of cDNA using the following primer pairs: Human Numb forward, 5′-CGATGACCAAACCAGTGACAG-3′ and human Numb reverse, 5′-AGAGGGAGTACGTCTATGACCG-3′; mouse Numb forward, 5′-GAAGACCGATTTCCCAATAAA-3′ and mouse Numb reverse, 5′-GCAGCACCAGAAGACTGACC-3′; GAPDH forward, 5′ ACCACAGTCCATGCCATCAC 3′ and GAPDH reverse, 5′ TCCACCACCCTGTTGCTGTA-3′.

Real-time quantitative PCR

Real-time quantitative PCR was performed using the 7500 Real-Time PCR System (Applied Biosystems). Reaction mixtures (25 μl) contained 10 pmol of each primer, 12.5 μl SYBR® Premix Ex Taq™ (Takara, Japan), and cDNA. Primer sequences were 5′-ATGCCAGCT-GATATAATGGAG-3′ (forward) and 5′-TCACCTCGTTCATGCACTCG-3′ (reverse) for Human Hes1, 5′-CAGAAGCAGAGACGGCTAGCA-3′ (forward) and 5′-GGTCTCATATTTGGACAGCTTCTTGT-3′ (reverse) for Hath1, and 5′-CTGCACCAAGACCGTCCTCATG-3′ (forward) and 5′-GCAAGGACTGAACAAAGACTCAGA-3′(reverse) for Human MUC2. To quantify changes in gene expression, the $^{\Delta\Delta}$Ct method was used to calculate relative fold changes normalized against glyceraldehyde 3-phosphate dehydrogenase, as described in the manufacturer's protocol (Applied Biosystems). Each cDNA was tested in triplicate.

Immunoblots

Lysates were extracted from small intestinal tissue from 8-week-old wild-type C57BL/6J mice and cell lines homogenized in lysis buffer [1× phosphate buffered saline, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.7 mM EDTA, and 1 mM PMSF, supplemented with Protease Inhibitor Cocktail (Roche)]. Protein concentrations were determined by the BCA Protein Assay. Equal loading was run on a 10% polyacrylamide gel and transferred to PVDF membranes. Membranes were incubated with anti-Numb (1:1000, Abcam, Cambridge, UK), anti-MUC2 (1:1000, Santa Cruz Biotechnology, USA), and anti-G3PDH (1:500, Zhongshan company, Beijing, China) antibodies overnight at 4 °C in blotting solution (Tris-buffer saline containing 5% nonfat dried milk and 0.1% Tween-20). Blots were then incubated with the appropriate horseradish peroxidase-conjugated secondary