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ScienceDirect
Developmental Biology 309 (2007) 298–305
DEVELOPMENTAL BIOLOGY
www.elsevier.com/developmentalbiology

Intestinal Neurogenin 3 directs differentiation of a bipotential secretory progenitor to endocrine cell rather than goblet cell fate

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Received for publication 2 March 2007; revised 12 July 2007; accepted 16 July 2007
Available online 24 July 2007

Abstract

Neurogenin 3 is essential for enteroendocrine cell development; however, it is unknown whether this transcription factor is sufficient to induce an endocrine program in the intestine or how it affects the development of other epithelial cells originating from common progenitors. In this study, the mouse villin promoter was used to drive Neurogenin 3 expression throughout the developing epithelium to measure the affect on cell fate. Although the general morphology of the intestine was unchanged, transgenic founder embryos displayed increased numbers of cells expressing the pan-endocrine marker chromogranin A. Accordingly, expression of several hormones and pro-endocrine transcription factors was increased in the transgenics suggesting that Neurogenin 3 stimulated a program of terminal enteroendocrine cell development. To test whether increased endocrine cell differentiation affected the development of other secretory cell lineages, we quantified goblet cells, the only other secretory cell formed in embryonic intestine. The Neurogenin 3-expressing transgenics had decreased numbers of goblet cells in correspondence to the increase in endocrine cells, with no change in the total secretory cell numbers. Thus, our data suggest that Neurogenin 3 can redirect the differentiation of bipotential secretory progenitors to endocrine rather than goblet cell fate.

Keywords: Transgenic mice; Intestine development; Notch signaling; Stem cell; Cell fate; Enteroendocrine cell

Introduction

Multipotential stem cells in the intestine give rise to two general cell lineages to form the epithelium. The absorptive or columnar lineage forms enterocytes, the predominant cell type, which is responsible for absorption of nutrients. The secretory or granulocytic lineage forms three distinct cell types, including goblet cells, enteroendocrine cells and Paneth cells, which are responsible for secretion of mucus, hormones and antimicrobial peptides, respectively. With the exception of Paneth cells, which do not emerge until crypt formation after birth, these various cell types are first established in fetal development during intestinal organogenesis. Cell specification first occurs around the time of morphological transformation from a pseudostratified to a columnar epithelium with the emergence of villi and then continues throughout the lifespan of the organism with constant replenishment of the epithelium from stem cells in the crypts. Notch signaling appears to play a critical role in the fate decision between the absorptive and secretory lineages. Disruption of Notch signaling in the intestine results in loss of enterocytes and increased numbers of secretory cells (Jensen et al., 2000; van Es et al., 2005). The Notch-regulated basic-helix–loop–helix (bHLH) transcription factor Math1 (mouse atonal homologue 1) is thought to be the key determinant of secretory cell development, as demonstrated by the loss of all intestinal secretory cell types in mice carrying a Math1 null mutation (Yang et al., 2001).

The mechanisms regulating the development of the various secretory cell types from multipotential precursors in the

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0012-1606/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2007.07.015

intestine are not fully understood. Mature endocrine cells arise from Neurogenin 3 (Neurog3)-expressing progenitor cells in the proliferative zone near the resident stem cell population (Bjerknes and Cheng, 2006; Jenny et al., 2002; Schonhoff et al., 2004). The importance of this bHLH transcription factor for specification of the enteroendocrine cell lineages is demonstrated by loss of all intestinal endocrine cells in mice carrying a Neurog3 null mutation (Jenny et al., 2002). A similar finding, near complete loss of enteroendocrine cells, was also recently reported for human patients with NEUROG3 gene mutations (Wang et al., 2006). Endocrine progenitors normally form numerous distinct mature cell types, each defined by its specific hormone product, including cholecystokinin (CCK), secretin, somatostatin, serotonin and many others (Hocker and Wiedenmann, 1998). Although Neurog3 is not expressed in mature endocrine cells, it is known to stimulate pro-endocrine transcription factors, such as Neurod1, Pax4, Pax6, Nkx2.2 and IA-1 (Heremans et al., 2002; Huang et al., 2000; Mellitzer et al., 2006; Smith et al., 2003; Treff et al., 2006). These and other pro-endocrine transcription factors are thought to be responsible for differentiation and maintenance of the mature endocrine populations. For example, Neurod1 is known to stimulate cell cycle withdrawal and transcription of the hormone secretin (Mutoh et al., 1998; Naya et al., 1997). Mice with a Neurod1 null mutation are missing secretin and CCK cells, although they maintain many other enteroendocrine cells, including serotonin- and glucagon-expressing cells (Mutoh et al., 1998; Naya et al., 1997). Neurog3 is also the key determining factor for endocrine cell development in the pancreas as demonstrated by the loss of pancreatic endocrine cell precursors and mature pancreatic endocrine cells in Neurog3-deficient mice (Jenny et al., 2002). Analysis of these null mice has also shown that Neurog3 is required for the development of a subset of endocrine cells in the glandular stomach (Jenny et al., 2002; Lee et al., 2002).

Ectopic expression studies in transgenic mouse models have shown that Neurog3 is sufficient to induce some aspects of pancreatic endocrine cell development. A predominant formation of glucagon-expressing cells was observed in mice carrying transgenes that activate Neurog3 expression in the early pancreatic endodermal bud (Apelqvist et al., 1999; Schwitzgebel et al., 2000). Similar results were seen in early chick embryos electroporated with a mouse Neurog3 construct, where endodermal expression stimulated cell migration to form islet-like cell clusters that express glucagon and somatostatin (Grapin-Botton et al., 2001). Thus, ectopic expression of Neurog3 appears to stimulate early pancreatic progenitors to differentiate along certain pancreatic islet cell lineages. However, whether Neurog3 is sufficient to promote endocrine cell differentiation in the intestine has not been investigated. In this study we generated transgenic mice that express Neurog3 throughout the developing intestinal epithelium. We observed a widespread induction of enteroendocrine cells. Surprisingly, the increase in endocrine cell number was countered by a decrease in the number of goblet cells, suggesting that Neurog3 affects the choice of a bipotential secretory cell to adopt endocrine rather than goblet cell fate.

Materials and methods

Generation of Vil-Neurog3 transgenic embryos

The Vil-Neurog3 transgene construct contained the mouse Neurog3 cDNA under the control of the mouse villin enhancer/promoter followed by SV40 sequences that provided a polyA$^+$ site (Fig. 1A). To engineer the transgene, a DNA fragment containing the 646 bp Neurog3 coding sequence was isolated from pCS2+ mNeurog3 (Vojtek et al., 2003) after *Bam*HI/*Xba*I digestion and shuttled into pBluescript SK before cloning into the villin expression plasmid pBSII-12.4KbVill/ΔATG (Madison et al., 2002) with *Cla*I/*Sac*II digestion. Following verification of the construct by sequencing, the 14.0 kb transgene was excised from the vector with *Pme*I and microinjected into F2 zygotes from C57BL/6×SJL parents by the University of Michigan Transgenic Animal Model Core. Potential transgenic founders were harvested at E18.5 and screened for the transgene by PCR amplification of genomic DNA (298 bp product) (Lopez-Diaz et al., 2006) using the following primers: V1S 5′ GTAACAGGCACTAAGGGAGCCAATGTAGAC and Neurog3PCR 5′ ACACTTGGATGGTGAGCGCATCCAAGGGAT.

Tissue morphology and immunohistochemistry

Intestine was dissected from potential E18.5 Vil-Neurog3 transgenic founders, and the proximal region (one cm distal to the pylorus) was paraffin embedded after fixing overnight in 4% paraformaldehyde. Sections (4 μm) were stained with H&E, alcian blue and PAS (Newcomer Supply Company) and alkaline phosphatase (Red Alkaline Phosphatase Kit, Vector Laboratories) or immunostained using the following primary antibodies: rabbit anti-CgA (1:500; 94188/5 gift from J.F. Rehfeld), goat anti-5HT (serotonin) (1:500, Immunostar #20079) and rabbit anti-CCK (1:1000, Chemicon #AB1972) followed by appropriate secondary antibodies conjugated to Cy2 or Cy3 (1:500, Jackson ImmunoResearch Laboratories). After deparaffinization and rehydration, sections were subjected to antigen retrieval with Antigen Unmasking Solution (Vector Labs) at 100 °C for 10 min, cooled, rinsed in PBS and incubated in TPBS (0.01% Triton X-100 in phosphate buffered saline (PBS)) for 5 min. Blocking was performed for 30 min at room temperature with TPBS containing 10% donkey serum and 1% bovine serum albumin (BSA). Primary antibodies were incubated overnight at 4 °C in PBS containing 1% BSA and 0.1% Triton X-100. Slides were rinsed in TPBS, blocked for a second time and incubated with secondary antibodies in 1% BSA and 0.1% Triton X-100 for 30 min, rinsed in PBS and mounted with coverslips in ProLong® Gold (Molecular Probes) containing 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI). Microscopy was performed with a Nikon E800 equipped with a SPOT digital camera or a Zeiss LSM 510 confocal microscope.

Neurog3 immunostaining used a mouse monoclonal antibody (Zahn et al., 2004) (1:4000, F25A1B3 concentrated) obtained from the NICHD Developmental Studies Hybridoma Bank (University of Iowa), with tyramide signal amplification (TSA Kit #2, Molecular Probes–Invitrogen). Briefly, deparaffinized and rehydrated paraffin sections were subjected to antigen retrieval, endogenous peroxidase activity was quenched with 3% H_2O_2 for 1 h, blocked as recommended by the TSA kit and incubated overnight with Neurog3 monoclonal antibody in blocking solution at 4 °C. After rinsing with PBS, slides were incubated with HRP-conjugated goat anti-mouse IgG (1:100, provided in TSA kit) and Alexa Fluor® 488 tyramide amplification was performed according to manufacturer's recommendations.

Morphometric analysis

For each founder embryo the complete region of intact tissue on a stained paraffin section was captured in contiguous digital images (5–15 fields). A single individual blinded to genotype performed the morphometric analysis using Image J (1.34 s by Wayne Rasband, NIH, USA; http://rsb.info.nih.gov/ij/) to measure the total area of epithelial tissue contained in each composite image and count positively stained cells within the region. Data were expressed as number of positive cells/area of epithelium (μm^2).