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Mouse atonal homolog 1 directs intestinal progenitors to secretory cell rather than absorptive cell fate

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ABSTRACT

The Notch-regulated transcription factor mouse atonal homolog 1 (Math1) is required for the development of intestinal secretory cells, as demonstrated by the loss of goblet, endocrine and Paneth cell types in null mice. However, it was unknown whether Math1 is sufficient to induce the program of secretory cell differentiation. To examine the function of Math1 in the differentiation of intestinal epithelial cells, intestinal morphology and epithelial and mesenchymal cell fate were examined by histological staining and marker gene expression in transgenic mice expressing a villin-regulated Math1 transgene. Late prenatal transgenic founders exhibited a gross cellular transformation into a secretory epithelium. The expansion of secretory cells coupled with the almost complete loss of absorptive enterocytes suggested reprogramming of a bipotential progenitor cell. Moreover, Math1 expression inhibited epithelial cell proliferation, as demonstrated by a marked reduction in Ki67 positive cells and blunted villi. Unexpectedly, the transgenic mesenchyme was greatly expanded with increased proliferation. Several mesenchymal cell types were amplified, including smooth muscle and neurons, with maintenance of basic radial patterning. Since transgenic Math1 expression was restricted to the epithelium, these findings suggest that epithelial-mesenchymal signaling is altered by the cellular changes induced by Math1. Thus, Math1 is a key effector directing multipotential precursors to adopt secretory and not absorptive cell fate.

Introduction

The intestine is composed of a simple columnar epithelium overlying a complex multi-layered mesenchyme. The tissue is organized into villi that protrude into the lumen and crypts that invaginate into the mesenchyme. Stem and progenitor cells in the crypts replenish the epithelium at a rapid rate throughout the lifespan of the organism. The multipotential progenitor cells differentiate into two general cell lineages. The absorptive (columnar) cell lineage forms enterocytes, which are responsible for absorption of nutrients and constitute the majority of the epithelium. The secretory (granulocytic) cell lineage is composed of goblet, endocrine and Paneth cells, which secrete mucus, hormones and antimicrobial peptides, respectively. Epithelial cytodifferentiation first occurs during fetal development; the various mature cell types are established by birth, with the exception of Paneth cells, which are formed postnatally with the emergence of crypts. Although the mechanisms regulating epithelial cell fate choice have not been fully elucidated, multiple signaling pathways, including Notch, Wnt and Hedgehog, have been shown to influence progenitor cell proliferation and epithelial cell specification (Scoville et al., 2008; van den Brink, 2007).

Animals with perturbations in intestinal Notch signaling remodel their epithelium in a manner suggesting that activation of this pathway directs multipotential precursors toward an enterocyte fate at the expense of secretory fates (Crosnier et al., 2005; Fre et al., 2005; Milano et al., 2004; Riccio et al., 2008; Stanger et al., 2005; van Es et al., 2005; Wong et al., 2004). For example, transgenic mice expressing a constitutively active form of the Notch 1 receptor (NotchICD) in the intestinal epithelium exhibited a loss of secretory lineage cells, including goblet and endocrine cells as well as Paneth cell markers (Fre et al., 2005; Stanger et al., 2005). Conversely, blockade of Notch signaling in rodent intestine with gamma secretase inhibitors or by deletion of either the Notch pathway transcription factor CSL/RBP-J or both Notch1 and Notch2 receptors promoted excessive differentiation of secretory cell types (Milano et al., 2004; Riccio et al., 2008; van Es et al., 2005; Wong et al., 2004). A similar phenotype was observed in zebrafish with disrupted Notch signaling (Crosnier et al., 2005), suggesting that Notch is a fundamental pathway regulating the specification of absorptive versus secretory cells in the vertebrate gut.

A cascade of Notch-regulated basic-helix-loop-helix (bHLH) transcription factors have been identified as critical effectors directing intestinal epithelial cell fate decisions. In particular the bHLH transcriptional repressor Hairy and enhancer-of-split 1 (Hes1) has been determined to regulate the choice between absorptive and

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secretory cell differentiation. Hes1 is known to be a transcriptional target of Notch signaling, with expression localized to the epithelial progenitor zone of the intestine (Jarriault et al., 1998; Schroder and Gossler, 2002). Importantly, mice with a Hes1 loss-of-function mutation exhibited a phenotype similar to models of Notch disruption, with excessive differentiation of secretory cell types, suggesting that Notch signaling primarily exerts its influence on intestinal progenitor cell fate choice through Hes1 (Jensen et al., 2000). In turn, Hes1 acts, at least in part, by repressing transcription of the bHLH activator atonal homolog 1 (Atoh1) (Akazawa et al., 1995). Accordingly, Notch disruption results in decreased expression of Hes1 and increased expression of Atoh1 (Jensen et al., 2000; van Es et al., 2005). Mouse Atoh1 (Math1) loss-of-function mutants have a phenotype similar to Notch gain-of-function models, with loss of goblet, endocrine and Paneth cells, demonstrating that Math1 is required for intestinal secretory cell development (Shroyer et al., 2007; Yang et al., 2001). Additional bHLH transcription factors have been shown to function downstream of Math1, including Neurogenin 3 (Neurog3), which is instructive for endocrine cell development, as demonstrated by loss of enteroendocrine cells in null mice (Jenny et al., 2002) and enhanced endocrine cell development in transgenic mice expressing Neurog3 in the developing intestinal epithelium (Lopez-Diaz et al., 2007).

Since Math1 expression is required for intestinal secretory cell development, it has been hypothesized to be the key effector regulated by Notch signaling that controls the bimodal switch between absorptive and secretory lineages. However, since Math1 is expressed in mature secretory cells in addition to cells in the progenitor zone (Pinto et al., 2003; Yang et al., 2001), it is not clear whether Math1 functions to initiate the secretory cell program of differentiation or to maintain the differentiated phenotype. In this study we tested whether Math1 expression promotes the program of secretory cell development with a transgenic mouse model that overexpressed Math1 in the developing intestinal epithelium.

Materials and methods

Generation of Vil-Math1 transgenic mice

The Vil-Math1 transgene contained the mouse cDNA under the control of the mouse villin enhancer/promoter. The transgene was prepared by isolating Math1 from pCS2 + Math1 (Farah et al., 2000) after *Cla*I/*Kpn*I digestion and shuttling into pBluescript SK (+/−) (Stratagene) before cloning into the villin expression plasmid pBSII-p12.4KVillΔATG (Madison et al., 2002). Following verification by sequencing, the 15 kb transgene was excised with *Pme*I and microinjected into F2 zygotes from C57BL/6 X SJL parents by the University of Michigan Transgenic Animal Model Core. Potential founders were harvested at E18.5 and screened by polymerase chain reaction (PCR) amplification of a 270 bp product using the following primers: V1S 5'-GTAACAGGCACTAAGGGAGCCAATGTAGAC; CM, 5'-TTACCTCAGCC-CACTCTTCTGCATGCAGCA. Mouse use was approved by the University of Michigan Committee on Use and Care of Animals.

Tissue morphology and immunohistochemistry

Intestines were dissected from potential E18.5 Vil-Math1 transgenic founders, and the proximal region (one cm distal to the pylorus), distal region (one cm distal to the midpoint), and colon (one cm distal to the cecum) were paraffin embedded after fixing overnight in 4% paraformaldehyde. Adjoining intestinal segments were processed for RNA. Sections (5 μm) were stained with H&E to assess cellular morphology and Periodic-acid Schiff (PAS)/Alcian blue (Newcomer Supply) to visualize mucin-containing goblet cells. Staining to visualize enterocytes was performed with the Alkaline Phosphatase Substrate Kit I (Vector Laboratories). For immunostaining, the following primary antibodies were used: rabbit anti-chromogranin A (1:500; 94188/5 gift from J. F. Rehfeld), rabbit anti-Math1 (1:50; gift from J.E. Johnson), rabbit anti-Muc2 (1:500; Santa Cruz), rabbit anti-Ki67 (1:500; Novacastra), mouse anti-α SMA conjugated to Cy-3 (1:500; Sigma), rabbit anti-desmin (1:500; Abcam), and rabbit anti-neurofilament (1:500; Zymed), followed by appropriate secondary antibodies conjugated to Cy2 or Cy3 (1:400; Jackson ImmunoResearch Laboratories), AlexaFluor488 (1:500; Invitrogen) or biotin (1:200; Vector Laboratories), as described (Lopez-Diaz et al., 2006). Staining for Math1 and co-staining for desmin and Ki-67 (1:100; BD Pharmigen mouse monoclonal) used Trilogy antigen retrieval (Cell Marque). For Math1 immunostaining, primary antibody was applied for 3 days at 4 °C. Staining for Neurog3 (1:4000, F25A1B3 concentrated mouse monoclonal, Developmental Studies Hybridoma Band, University of Iowa) used tyramide signal amplification (TSA kit #2, Molecular Probes-Invitrogen), as previously described (Lopez-Diaz et al., 2007). TUNEL staining was performed as previously described (Jain et al., 2008). Microscopy was performed with either a Nikon E800 or Olympus BX-51 equipped with a SPOT or Olympus DP70 digital camera, respectively, or a Zeiss LSM 510 confocal microscope.

Quantitation of mRNA abundance

Gene expression was measured by quantitative reverse transcription-PCR (qRT-PCR) analysis of transgenic (Tg) founders and nontransgenic (Ntg) littermate controls using the distal intestinal segment unless otherwise noted. RNA was isolated, DNase-treated and purified using the RNeasy Mini kit (Qiagen). RT reactions (50 μl) used 1 μg RNA and the Iscript cDNA synthesis kit (Bio-Rad), as recommended by the manufacturer. qRT-PCR was performed as described (Jain et al., 2006) with SYBR green dye and the primers listed in Supplementary Table 1. Expression levels were determined for individual embryos with triplicate assays per sample and normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase (Gapdh), which remained the same in Tg and Ntg controls.

Morphometric analysis

Image J (1.34 s by Wayne Rasband, NIH, USA, http://rsb.info.nih.gov/ij/) was used to calculate epithelial or total tissue area from 7 to 20 contiguous field views of an intestinal region (representing the whole tissue segment), except for goblet cell analysis which included 3 contiguous field views. For specific epithelial cell populations, the number of positive cells was counted and data were expressed as number of positive cells/area of epithelium (μm^2). To determine proportional tissue areas, the measured epithelial area (μm^2) was divided by the measured total tissue area (μm^2) to calculate the epithelial component, while the remainder was attributed to mesenchyme.

Statistics

Quantitative data were presented as mean ± SEM and analyzed by ANOVA followed by a Dunnett post test to compare data from each Vil-Math1 Tg founder to Ntg littermate controls. $P<0.05$ was considered significant.

Results

Increased secretory cell development in Vil-Math1 transgenics

To test the relationship between Math1 and secretory cell differentiation, we used the mouse villin promoter (Madison et al., 2002) to target Math1 expression in transgenic mice to all intestinal epithelial cells, including stem and progenitor cells (Fig. 1). Stable Vil-

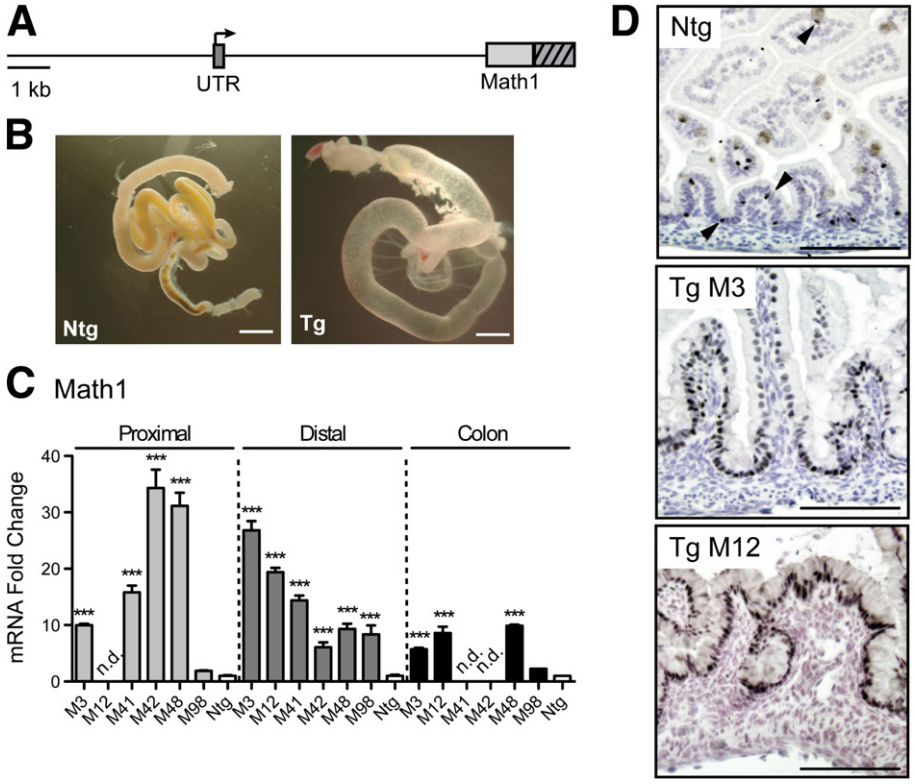

Fig. 1. Abnormal intestinal morphology in Vil-Math1 E18.5 transgenic mice. (A) The Vil-Math1 transgene contained mouse villin sequences, including 5' flanking sequence, the first untranslated exon (UTR) and intron, to regulate expression of the mouse Math1 cDNA, and SV40 sequences (hatched) to provide a polyA site. (B) Transgenic (Tg) intestines were fluid-filled and translucent (observed in M12, M41, and M48) compared to Nontransgenic (Ntg) controls. Bars: 2 mm. (C) Total Math1 mRNA (transgene plus endogenous) was measured by quantitative reverse transcriptase-PCR (qRT-PCR) analysis of proximal intestine, distal intestine, and colon RNA. Six Vil-Math1 founders exhibited significantly higher Math1 mRNA levels in at least one region. Values were normalized to Gapdh expression and reported as fold-change relative to the corresponding region of Ntg littermate controls (***$P<0.001$). n.d., not determined. (D) Immunostaining for Math1 in proximal intestine of Ntg and two representative transgenic founders. Math1 is normally expressed in nuclei of secretory progenitor cells and mature secretory cells (arrowheads); transgenics had widespread expression of Math1 throughout the epithelium. Hematoxylin nuclear stain. Bars: 100 μm.

Math1 transgenic lines were unable to be generated due to the lack of recovery of founder mice with effective transgene expression. Thus we analyzed prenatal transgenic founders to avoid the lethality resulting from the dramatic cellular changes induced by Math1. Interestingly, the intestines of Vil-Math1 transgenics were distended, translucent and fluid-filled (Fig. 1B), possibly due to decreased fluid absorption or increased secretion resulting from the epithelial cell remodeling caused by Math1 expression (described below). Total Math1 mRNA was measured in proximal small intestine, distal small intestine, and colon intestinal segments and six Vil-Math1 founder embryos with increased Math1 were analyzed. Increased Math1 mRNA was generally observed throughout the gut of Vil-Math1 mice with increases up to 34-fold in the proximal intestine, 27-fold in the distal intestine, and 10-fold in the colon (Fig. 1C). Normally, intestinal

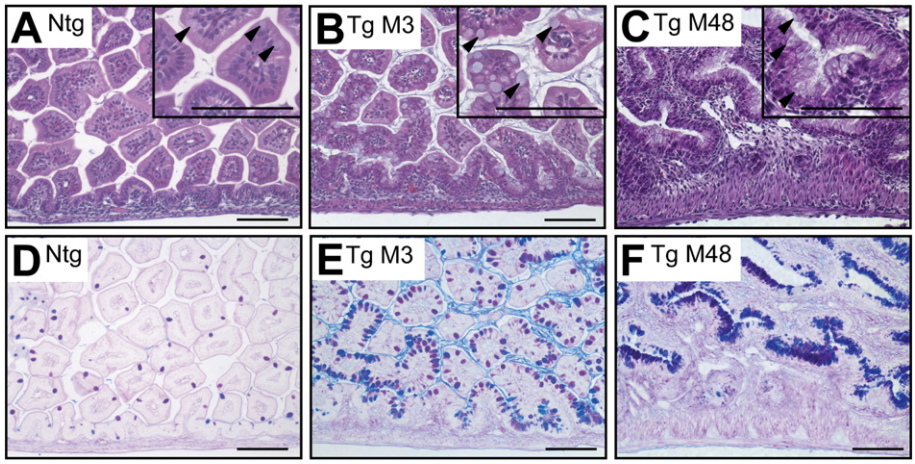

Fig. 2. Math1 promotes the development of goblet cells throughout the intestine. Histological analysis of Ntg (A, D) and Tg (B, C, E, F) intestines from E18.5 founders. Transgenics M3 and M48 are shown to demonstrate the range in phenotypes. (A–C) H&E staining of proximal intestine showed increased numbers of goblet-like cells in Tg mice, apparent in the higher magnification insets (arrowheads), as well as mesenchymal tissue expansion. (D–F) PAS/Alcian blue staining confirmed increased goblet cells in the proximal intestine of Tg mice. Bars: 100 μm.

Math1 protein is expressed in the nuclei of secretory progenitor cells and in mature secretory cells (Pinto et al., 2003; Yang et al., 2001) (Fig. 1D). Transgenic founders exhibited increased numbers of Math1-positive epithelial cells, including both cells in the intervillus zone and on the villi, consistent with the reported expression of the villin promoter (Madison et al., 2002) (Fig. 1D).

Histological analysis demonstrated a complex, disorganized morphology in transgenic intestine with stunted villi, hypocellular epithelium and expanded mesenchyme (Fig. 2). Analysis of the cellular changes induced by Math1 included three separate regions, proximal and distal small intestine, and colon, to account for the normal regional variation in secretory cell numbers along the anterior–posterior gut axis. Analysis of Ntg controls demonstrated that Math1 expression increases in the posterior intestine in strong correspondence to goblet cell numbers (Supplementary Fig. 1). Thus, analysis of Tg phenotypes strictly compared outcomes with the corresponding region in Ntg controls. H&E staining showed increased numbers of goblet-like cells throughout the gut, which was confirmed by staining with PAS/Alcian blue (Fig. 2 and Supplementary Fig. 2). The epithelium of some Vil-Math1 transgenics appeared to be almost completely transformed to the goblet cell lineage (e.g. Fig. 2F). Morphometric analysis demonstrated significant increases in goblet cell number along the entire length of the small intestine and colon (Supplementary Fig. 2G-I). For example, Tg M12 exhibited greater than 10-fold increases in goblet cell numbers in both proximal and distal small intestine, and a smaller, yet significant, increase in colon.

Next we examined endocrine cells, another cell type belonging to the intestinal secretory lineage. Similar to the goblet cell findings, a general expansion of endocrine cells was observed in all three regions of the Vil-Math1 intestine. Immunostaining for the pan-endocrine marker chromogranin A (CgA) revealed 2- to 8-fold increased endocrine cell numbers in Vil-Math1 transgenics compared to Ntg littermates (Fig. 3A–D). Normally, endocrine cells are distributed in the epithelium as single, scattered cells surrounded by enterocytes. In contrast, some CgA-positive cells were found in juxtaposition in the transgenics (Fig. 3C, inset). Goblet cell juxtaposition was also commonly observed in the transgenic intestine (e.g. Fig. 2F). The observation of enteroendocrine and goblet cells clustering in the Vil-Math transgenic mice suggested that increased Math1 expression disturbed the process of epithelial cell distribution, which is thought to be orchestrated through lateral inhibition regulated by Notch signaling (Apelqvist et al., 1999; Bjerknes and Cheng, 2005).

The endocrine cell expansion encompassed both mature endocrine cells and endocrine progenitor cells, which are marked by the transcription factor Neurog3 (Bjerknes and Cheng, 2006; Gradwohl et al., 2000; Jenny et al., 2002; Lopez-Diaz et al., 2007). In Ntg controls, Neurog3-positive cells were observed as very rare single cells located in the intervillus progenitor cell zone (Fig. 3F). Increased numbers of Neurog3-positive cells were observed in Vil-Math1 transgenic founders, with transgenic founders having 2- to 102-fold increases in cell numbers (Fig. 3I). These cells were often observed on the villus, displaced from their normal location in the intervillus zone (Fig. 3F–H). Correlating with the increased numbers of Neurog3-positive cells, Neurog3 mRNA levels were increased up to 40-fold in Vil-Math1 Tg founders (Fig. 3J). Tg M3, M41, and M98, which exhibited the greatest increase in Neurog3 mRNA levels, also had the highest number of Neurog3-positive staining cells (Fig. 3I, J).

Analysis of mRNA abundance for the hormone cholecystokinin (CCK) showed higher levels in transgenic founders, suggesting increased differentiation of mature, hormone-expressing endocrine cells (Fig. 3E). Co-immunostaining for CCK and serotonin showed increased numbers of cells expressing a single hormone product (a pattern characteristic of mature endocrine cells) and did not reveal cells expressing multiple hormones (data not shown). Analysis of CCK and glucagon mRNA abundance along the length of the intestine showed that hormone expression was increased in all three regions of the Vil-Math1 intestines (Supplementary Fig. 3). Furthermore, expression patterns for these two hormones generally followed the normal regional patterning, with greater expression observed in small intestine than in colon. These data suggest that forced Math1 expression in the developing intestinal epithelium

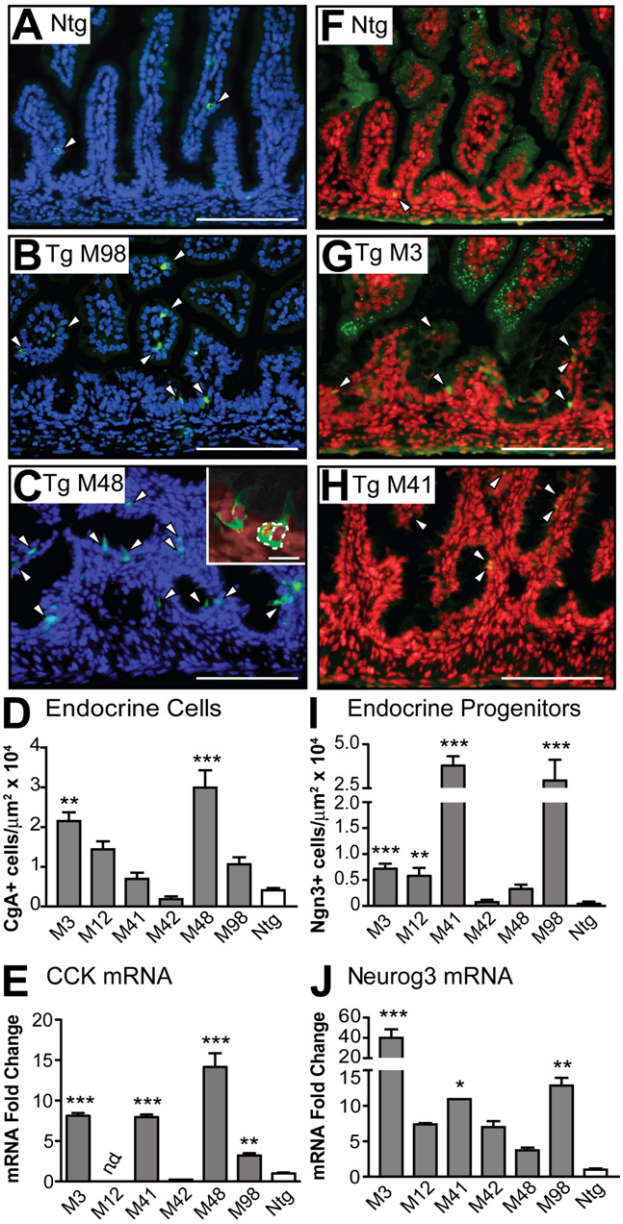

Fig. 3. Math1 stimulates the endocrine cell differentiation program. (A–C) Immunostaining for chromogranin A (CgA; green) showed increased endocrine cell numbers (arrowheads) in proximal Tg intestines, which was associated with loss of lateral inhibition (C, inset shows confocal image of 2 cell doublets with one doublet outlined). DAPI (blue/red) nuclear stain. Bars: 100 μm; 10 μm (inset). (D) Quantification of endocrine cells from CgA-stained sections. Values were normalized to epithelial area (μm^2) and statistical significance was determined by comparison to Ntg (**$P<0.01$; ***$P<0.001$). (E) qRT-PCR analysis of CCK mRNA abundance in proximal intestine. Values were normalized to Gapdh expression and reported as fold-change relative to Ntg (**$P<0.01$; ***$P<0.001$). (F–H) Immunostaining proximal intestines for Neurogenin 3 (Neurog3; green nuclear stain) demonstrated increased numbers of Neurog3-positive cells (arrowheads) in Vil-Math1 transgenics, which were often mislocated outside of the intervillus zone. DAPI (pseudocolored red) nuclear stain. Bars: 100 μm. (I) Quantification of endocrine progenitor cells from Neurog3-stained sections. Values were normalized to epithelial area (μm^2) and statistical significance was determined by comparison to Ntg (**$P<0.01$; ***$P<0.001$). (J) qRT-PCR analysis of Neurog3 mRNA abundance in distal intestine. Values were normalized to Gapdh expression and reported as fold-change relative to Ntg (*$P<0.05$; **$P<0.01$; ***$P<0.001$).

increased the formation of Neurog3-positive endocrine progenitor cells that further differentiated to form expanded numbers of mature endocrine cells. Moreover, the regulatory network that influences differentiation of endocrine cells to specific hormone-producing subtypes was conserved.

Paneth cells, the third secretory cell type, are not mature at E18.5; however, expression of Paneth cell anti-microbial genes is observed in the prenatal intestine. Staining distal intestine from control mice for the Paneth cell markers lysozyme (Fig. 4A) and MMP7 (not shown) showed weakly immunopositive cells scattered on the villus epithelium, a pattern that has been previously described in perinatal intestine (Darmoul et al., 1997). In contrast, Vil-Math1 transgenic founders had increased numbers of intensely-stained cells, which were commonly found in or near the intervillus zone (Fig. 4B and C). In accordance with the expansion of Paneth-like cells, both lysozyme and cryptdin mRNA levels were increased in the distal intestines of Vil-Math1 transgenics, with cryptdin levels up to 60-fold greater than in Ntg controls (Fig. 4F and G). Unexpectedly, lysozyme staining, which is never observed in the Ntg colon, was widespread in Vil-Math1 Tg colon (Fig. 4D and E) together with increased levels of colonic cryptdin mRNA (Fig. 4G), suggesting that increased Math1 expression in the colon shifted the normal pattern of secretory cell differentiation to include Paneth cell genes. Together, these findings demonstrate that Math1 is sufficient to trigger the program of intestinal differentiation to goblet, endocrine and Paneth cells throughout the intestine and colon.

Loss of enterocytes in Vil-Math1 transgenics

We tested the absorptive cell lineage in the Vil-Math1 transgenics to determine if the increase in secretory cells was associated with a corresponding reduction in enterocyte differentiation. A striking decrease in enterocytes was demonstrated in both proximal and distal small intestine by staining for the brush border enzyme alkaline phosphatase (Fig. 5). The Vil-Math1 transgenics exhibited decreased intensity of staining with an almost complete loss of staining in the most severely affected transgenics. Transgenics with the greatest increase in secretory cell number exhibited the most dramatic loss of enterocytes. Indeed, analysis of epithelial cells with various lineage-specific stains demonstrated that the cells in these transgenic founders are largely accounted for by secretory cells (Fig. 1–3), although we cannot rule out the potential presence of a small number of committed enterocyte progenitor cells. Thus, excessive differentiation of the secretory lineage occurs to the detriment of the absorptive lineage in Vil-Math1 mice. Analysis of the enterocyte brush border enzyme lactase showed a dramatic reduction in mRNA abundance, with greater than 2000-fold decreases, consistent with the observed loss of enterocytes (Fig. 5G). Similar reductions in mRNA abundance were seen for the enterocyte marker intestinal fatty acid binding protein (data not shown). These results suggest that Math1 is capable of directing multipotential progenitor cells to adopt the secretory program of differentiation and not absorptive cell fate.

Reduced and displaced epithelial cell proliferation in Vil-Math1 transgenics

The epithelium in Vil-Math1 transgenics comprised a significantly smaller proportion of the intestinal tissue area than that of nontransgenics, consistent with the hypocellular appearance of the epithelium (Fig. 2 and Supplementary Fig. 4). This phenotype led us to examine whether Vil-Math1 transgenics had alterations in cellular

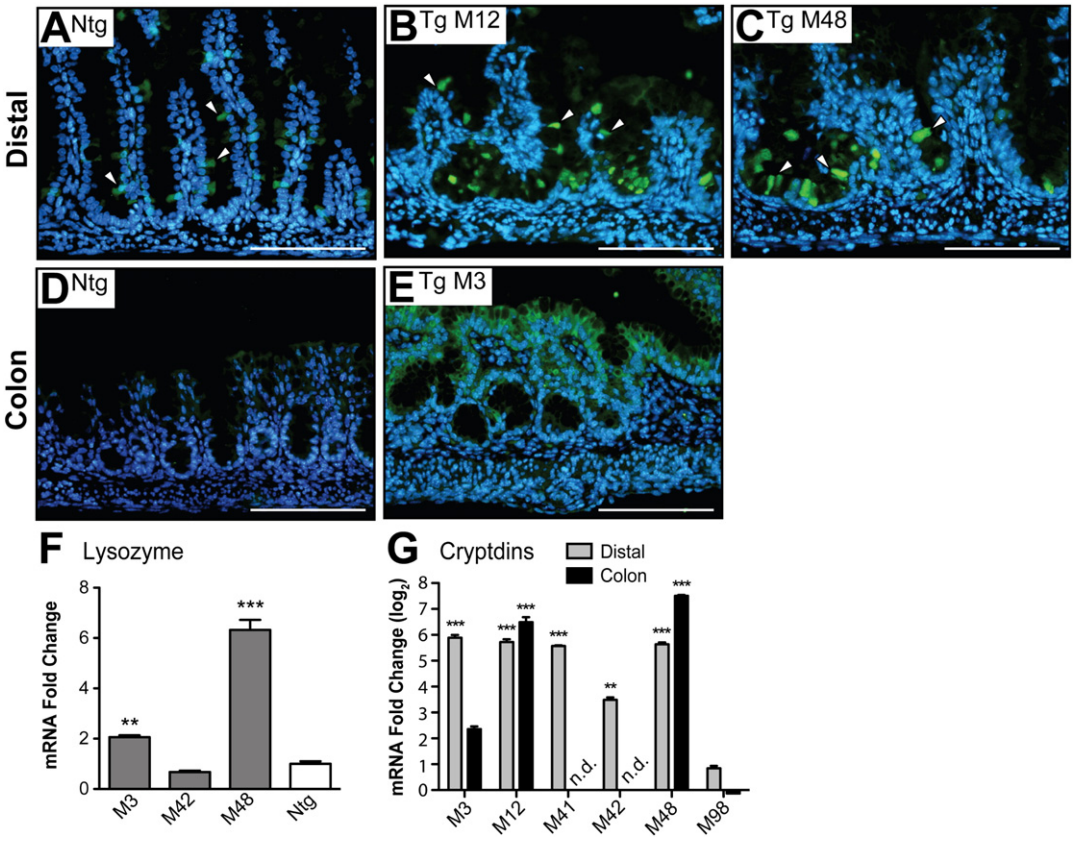

Fig. 4. Emergence of a Paneth-like cell in the intervillus zones and colons of Vil-Math1 transgenic intestine. (A–E) Immunostaining for lysozyme (green) showed faintly-staining cells scattered in Ntg distal intestine (A) and no positive-staining in the Ntg colon (D). Tg founders had increased numbers of lysozyme-positive cells predominantly in the intervillus zone of the distal intestine. Arrowheads denote some lysozyme-positive cells (B, C). Lysozyme-positive cells were also apparent in the Tg colon (E). DAPI (blue) nuclear stain. Bars: 100 μm. (F, G) qRT-PCR analysis of lysozyme mRNA abundance in distal intestine (F) and cryptdin (G) mRNA abundance in distal intestine and colon. Values were normalized to Gapdh expression and reported as fold-change relative to Ntg (**$P<0.01$; ***$P<0.001$).

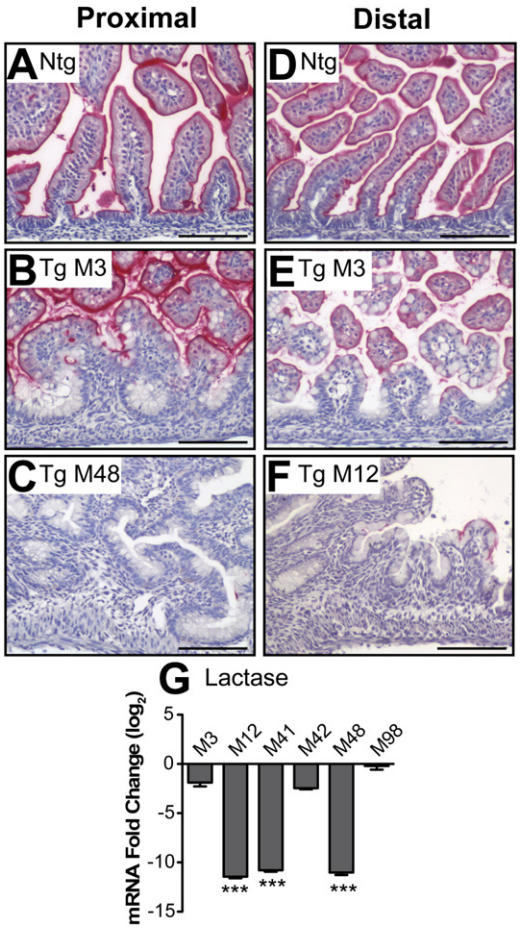

Fig. 5. Loss of the absorptive lineage in E18.5 Vil-Math1 transgenics. Ntg (A, D) and Tg (B, C, E, F) proximal (A–C) and distal (D–F) intestine sections were stained for the enterocyte brush border enzyme alkaline phosphatase (red) and counterstained with hematoxylin (blue). Bars: 100 μm. (D) Lactase mRNA abundance was measured by qRT-PCR analysis of distal intestine. Values were normalized to Gapdh expression and reported as fold-change relative to Ntg (***$P<0.001$).

proliferation or apoptosis. Analysis of apoptosis by TUNEL staining showed no change in apoptotic rate (data not shown). However, striking changes in cellular proliferation were observed. Normally, proliferating cells are restricted to the intervillus zone of the E18.5 intestinal epithelium as well as scattered cells in the mesenchyme, as shown by immunostaining for the Ki67 antigen (Fig. 6A). In contrast, epithelial proliferation in Vil-Math1 transgenics was displaced and significantly decreased (Fig. 6B–D). An overall 2-fold reduction in epithelial proliferation in Vil-Math1 transgenic mice was shown by morphometric analysis of Ki67-positive cells (Fig. 6E). All 6 transgenic founders exhibited displacement of Ki67-positive cells from the intervillus zone to the villus (Fig. 6B–D and data not shown) suggesting that the progenitor cell "niche" was displaced towards the villus tips. Our finding that Neurog3-positive cells were also displaced to the villus, strengthens this conclusion (Fig. 3). Moreover, in contrast to Ntg controls, Vil-Math1 transgenics had many cells in the intervillus zone that stained with PAS/Alcian blue or lysozyme, suggesting that differentiated cells occupied the intervillus zone and indicating that stem and progenitor cell populations might be altered in transgenic founders (Figs. 2 and 4). Thus, we examined mRNA abundance of the crypt base columnar (CBC) stem cell marker Lgr5 and the transit amplifying (TA) progenitor cell marker Prominin1 (Prom1) (Fig. 6F). Lgr5 was observed to be decreased in transgenic founders while Prom1 was increased (Fig. 6F). In adult intestine, differentiating Lgr5-positive CBC stem cells first form Prom1-positive TA progenitor cells that further differentiate to a mature epithelial cell type (Barker et al., 2007; Snippert et al., 2009). Although expression of these markers has not been fully described in perinatal intestine, our data suggest that forced expression of Math1 promotes precocious differentiation of Lgr5-positive stem cells, leading to increased TA progenitor cells.

Mesenchymal remodeling in Vil-Math1 transgenics

Signaling between the intestinal epithelium and mesenchyme is thought to play a critical role in the regulation of intestinal differentiation and proliferation (Crosnier et al., 2006). Although Math1 transgene expression was limited to the epithelium (Fig. 1D), we observed marked changes in the mesenchyme. In contrast to the overall reduction in epithelial cell proliferation, there was a robust increase in Ki67-positive cells in the mesenchyme of Vil-Math1 transgenics (Fig. 6A–D, arrowheads), accounting for the thickened mesenchyme layer seen in H&E stained sections (Fig. 2). Mesenchymal expansion was observed in the villus core regions as well as the submucosa (Fig. 6 and data not shown).

The mesenchymal expansion was substantiated by increased expression of the general mesenchyme marker vimentin, with 2- to 4-fold increased expression (Fig. 7A). The cellular basis for the expansion was determined by immunohistochemical analysis. Co-staining with antibodies to α-smooth muscle actin (SMA) and desmin enabled identification of three different mesenchymal cell types: myofibroblasts (SMA-positive), myoblasts (desmin-positive) and differentiated smooth muscle (SMA- and desmin-positive) (Fig. 7C–E). Vil-Math1 transgenics exhibited an expansion of both SMA-positive myofibroblasts and desmin-positive smooth muscle precursors. In addition, there was a substantial increase in double-stained, differentiated smooth muscle. The myocyte expansion was verified by analysis of SM22α, a differentiated smooth muscle marker (Fig. 7B). SM22α expression was increased as much as 3.7-fold in Vil-Math1 transgenics, similar to the overall expansion in mesenchyme shown by increased vimentin expression (Fig. 7A and B). Immunostaining for the neuronal marker neurofilament demonstrated that enteric neurons were also expanded in the transgenic mesenchyme (Fig. 7F–H). Although Vil-Math1 transgenics exhibited increased numbers of numerous mesenchymal cell types, the basic radial patterning was maintained, with myofibroblasts more closely associated with the epithelium and enteric neurons situated between the circular and longitudinal smooth muscle. These data suggested that the epithelial cell changes induced by forced Math1 expression altered signaling to the mesenchyme to affect proliferation and differentiation, with resulting expansion of several mature cell types.

Discussion

This study has established that Math1 is sufficient to induce the program of secretory cell development in the mouse intestine. Transgenic expression of Math1 resulted in almost complete transformation of the intestine into a secretory epithelium. The loss of enterocytes and expansion of the secretory cell lineage suggested that Math1 regulates the cell fate choice of a bipotential progenitor. The importance of Math1 for intestinal lineage determination had previously been suggested from the loss of secretory cell types in Math1-deficient mice (Shroyer et al., 2007; Yang et al., 2001). Since Math1 is expressed in mature secretory cells as well as in cells in the progenitor zone (Pinto et al., 2003; Yang et al., 2001), the question remained whether its primary role was maintenance of the differentiated secretory cell phenotype or induction of differentiation. Our study showed that Math1 expression can override the normal developmental program to induce secretory cell differentiation, suggesting that a primary role of this transcription factor is determination of cell fate choice. Interestingly, expression of Math1 stimulated apparently normal terminal differentiation processes since

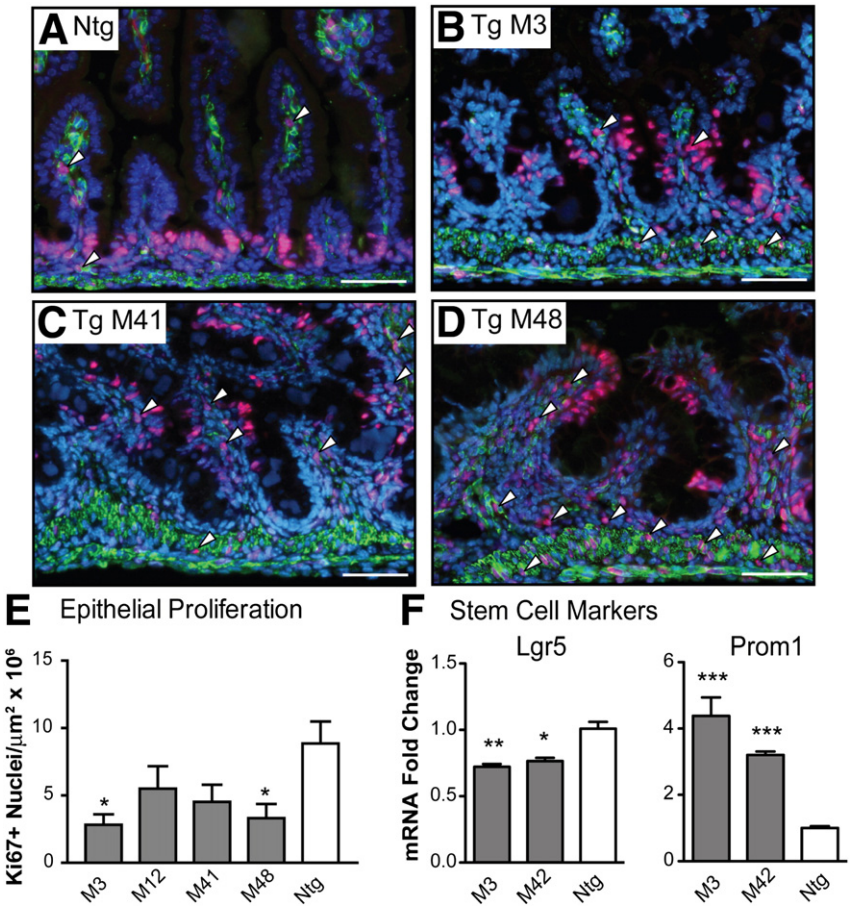

Fig. 6. Altered epithelial and mesenchymal cell proliferation in E18.5 Vil-Math1 intestine. (A–D) Proliferation was assessed in Ntg (A) and Tg (B–D) proximal intestine by Ki67 immunostaining (red) co-immunostained with desmin (green) to mark mesenchymal cells and DAPI (blue) nuclear stain. Arrowheads identify some proliferating cells in the mesenchyme. Bars: 50 μm. (E) Quantification of epithelial cell proliferation by morphometric analysis of Ki67 immunostaining of proximal intestine. Data are presented as Ki67-positive nuclei per epithelial area (μm^2) (*P<0.05 compared to Ntg). (F) qRT-PCR analysis of the stem cell markers Lgr5 and Prom1 in distal small intestine. Values were normalized to Gapdh expression and reported as fold-change relative to Ntg (*P<0.05; **P<0.01; ***P<0.001).

we observed expression of differentiation markers specific for each secretory cell type, including Muc2 (goblet cells), hormones (enteroendocrine cells), and cryptdins (Paneth cells). Accordingly, the expression of transcription factors downstream of Math1 that are known to be important for specific secretory cell types were increased robustly in Vil-Math1 transgenic mice, including Neurog3 (Fig. 3) (Jenny et al., 2002; Lopez-Diaz et al., 2007) and Gfi1 (data not shown) (Shroyer et al., 2005).

Notch signaling has been shown to play a primary role in the regulation of cell lineage determination in the intestinal epithelium. Our study and others point to Notch regulation of Math1 transcription as the critical determinant of cell fate. Treatment with γ-secretase inhibitors to block Notch signaling has been shown to induce intestinal phenotypes similar to those evident in the Vil-Math1 transgenic mice (Milano et al., 2004; Searfoss et al., 2003; van Es et al., 2005). Similarly, genetic models with disrupted Notch signaling exhibited a generalized secretory cell expansion together with loss of enterocytes (Crosnier et al., 2005; Jensen et al., 2000; Riccio et al., 2008; van Es et al., 2005). Math1 expression was increased in these models of Notch disruption, consistent with our finding that Math1 expression controls absorptive versus secretory cell fate. Accordingly, genetic models with increased Notch signaling exhibited decreased Math1 and loss of intestinal secretory cell types (Fre et al., 2005; Stanger et al., 2005). To determine if increased Math1 mRNA had an effect on the Notch signaling pathway in the Vil-Math1 transgenics, we analyzed expression of the Notch target gene Hes1. No change in Hes1 mRNA levels were observed in Vil-Math1 transgenic founders (Supplementary Fig. 5), suggesting that the epithelial changes that we observed in our studies were due to increased levels of Math1 and not due to inhibition of Notch signaling. Together these studies and our data point to Math1 as the key effector regulating the choice of secretory versus absorptive cell fate choice in the intestine.

Transgenic Math1 expression affected intestinal proliferation in complex ways. We observed an overall reduction in epithelial cell proliferation, which suggested that Math1 stimulated differentiation and withdrawal from the cell cycle. Loss of Notch signaling in the intestine, and thus increased Math1, had previously been shown to convert proliferating crypt progenitors into post-mitotic cells with derepression of the cyclin-dependent kinase inhibitors $p27^{Kip1}$ and $p57^{Kip2}$ (Riccio et al., 2008). Increased cell-cycle inhibitor expression was proposed to result from loss of Hes1 repressor action on the promoters of these genes (Riccio et al., 2008). However, our observations suggest that increased Math1 may also contribute to the Notch affect on proliferation. Although Vil-Math1 transgenics exhibited decreased epithelial cell proliferation, intestinal Hes1 mRNA expression was not changed, suggesting that the reduced proliferation and increased differentiation of progenitors was likely due to changes in Math1 and not Hes1. Finally, Math1 was observed to affect the expression of intestinal stem cell markers, with decreased expression of Lgr5 and increased expression of Prom1, consistent with Math1 promoting the differentiation of Lgr5-positive crypt base columnar stem cells to Prom1-positive committed progenitor cells.

In addition to the profound changes to the epithelium, there were marked changes to the mesenchyme in Vil-Math1 transgenic mice,

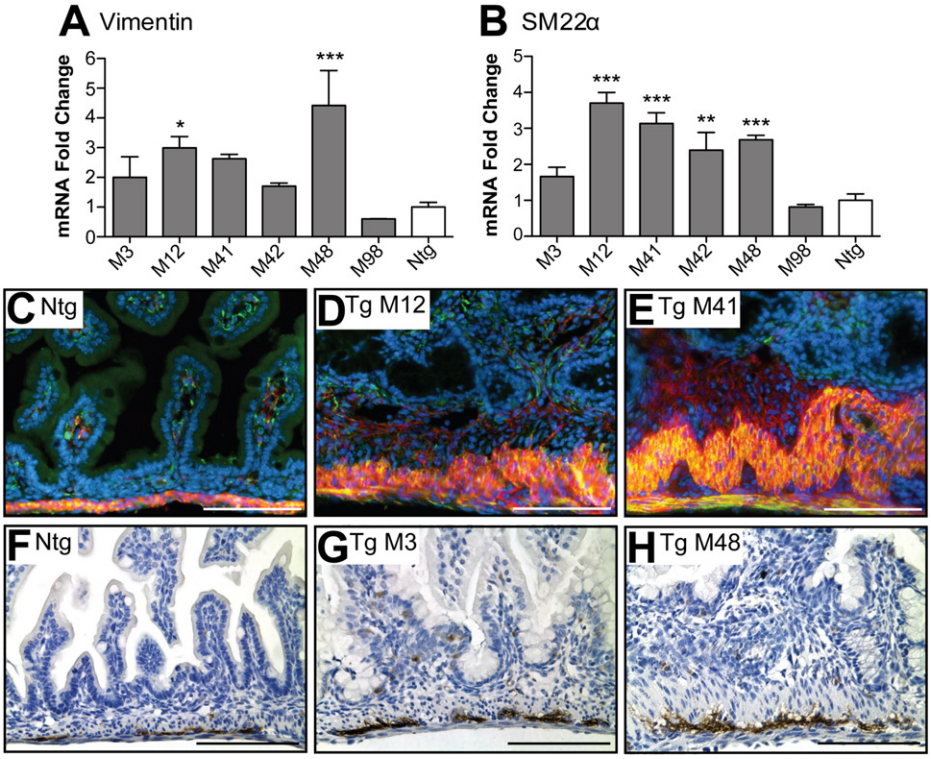

Fig. 7. Remodeled mesenchyme in E18.5 Vil-Math1 intestine. (A, B) qRT-PCR analysis of vimentin (A) and SM22α (B) mRNA abundance was performed with distal small intestine RNA. Values were normalized to Gapdh expression and reported as fold-change relative to Ntg (*P<0.05; **P<0.01; ***P<0.001). (C–E) Co-immunostaining of proximal intestine sections with α-smooth muscle actin (red) and desmin (green) with DAPI (blue) nuclear stain. Differentiated smooth muscle is yellow due to co-expression of these two markers. (F–G) Immunostaining for the neuronal marker neurofilament (brown), with hematoxylin counterstain (blue) shows expansion of the enteric nervous system in Vil-Math1 transgenics. Bars: 100 μm.

with a general cellular expansion and increased proliferation observed in both submucosa and villus cores. Since Math1 expression in our transgenic model was limited to epithelial cells, the mesenchymal changes were indirect, suggesting that the epithelial cell changes altered signaling to control the differentiation of the mesenchyme. The epithelial cell changes were multifaceted, thus signaling changes could result from changes to the intervillus progenitor zone, the loss of enterocytes, and/or expansion of secretory cell types. It is likely that multiple signaling pathways were affected by these cellular changes, including Wnt and Hedgehog.

In conclusion we have demonstrated that Math1 is a key transcriptional determinant of cell fate choice in the intestine. Proper Math1 levels are required for the normal patterning of epithelial cell proliferation as well as the distribution of secretory versus absorptive cells. The associated expansion of secretory cell types and loss of enterocytes observed when Math1 levels were increased suggests that this transcription factor directs the cell fate choice of a bipotential progenitor. Furthermore, the epithelial cell changes induced by increasing Math1 had consequences for the mesenchyme, presumably due to alterations in epithelial-mesenchymal crosstalk. Although radial patterning was essentially preserved, the mesenchyme of Vil-Math1 transgenic mice was grossly expanded, with increased proliferation and differentiation of several mesenchymal cell types. Thus, Math1 expression is critical for coordinated differentiation and morphogenesis of both epithelium and mesenchyme.

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Author contributions

Kelli L VanDussen: study concept and design; acquisition of data; analysis and interpretation of data; drafting of the manuscript; critical revision of the manuscript for important intellectual content; statistical analysis. Linda C Samuelson: study concept and design; analysis and interpretation of data; drafting of the manuscript; critical revision of the manuscript for important intellectual content; obtained funding; study supervision.

Disclosures

The authors have no conflicts to disclose.

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