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The Ets-Domain Transcription Factor Spdef Promotes Maturation of Goblet and Paneth Cells in the Intestinal Epithelium

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BACKGROUND & AIMS: Stem cells within the intestinal epithelium generate daughter cells that undergo lineage commitment and maturation through the combined action of the Wnt and Notch signaling cascades. Both pathways, in turn, regulate transcription factor networks that further define differentiation toward either enterocytes or 1 of 3 secretory cell lineages (Paneth, goblet, or enteroendocrine cells). In this study, we investigated the role of the Wnt-responsive, Ets-domain transcription factor Spdef in the differentiation of goblet and Paneth cells. **METHODS:** The in vivo function of Spdef was examined by disrupting the *Spdef* gene in mice (*Spdef*$^{-/-}$ mice) and analyzing the intestinal phenotype using a range of histologic techniques and DNA microarray profiling. **RESULTS:** In accordance with expression data, we found that loss of *Spdef* severely impaired the maturation of goblet and Paneth cells and, conversely, led to an accumulation of immature secretory progenitors. Spdef appears to positively and negatively regulate a specific subset of goblet and Paneth cell genes, including *Cryptdins*, *Mmp7*, *Ang4*, *Kallikreins*, and *Muc2*. **CONCLUSIONS: Spdef acts downstream of Math1 to promote terminal differentiation of a secretory progenitor pool into Paneth and goblet cells.**

Within the intestinal epithelium, the Wnt and Notch cascades cooperate to maintain the self-renewing capacity of crypt stem cells and concomitantly promote the specification and/or differentiation of progenitor cells.[1] More specifically, Wnt signaling is required for the formation of the secretory lineages (ie, Paneth, goblet, and enteroendocrine cells), whereas the Notch pathway is necessary for enterocyte development. Consequently, inactivation of Wnt signalling in progenitor cells leads to their conversion into fully differentiated enterocytes and a concomitant reduction in secretory cell types.[2] Blockage of the Notch pathway results in a massive conversion of progenitor cells into postmitotic goblet cells,[3] and, inversely, constitutive activation of the Notch receptor leads to perturbed production of secretory cells.[4–6] Current models suggest that Notch signalling drives enterocyte commitment by impeding the differentiation of progenitors along the secretory lineage. This is achieved by activating the transcription factor Hes1, a well-established Notch target gene.[7] In turn, Hes1 blocks the expression of the basic helix-loop-helix transcription factor Math1, an essential determinant of secretory lineages.[8,9] It is currently unknown whether Wnt signalling promotes secretory lineage differentiation by modulating Math1/Hes1 activity or whether Wnts act via an independent mechanism.

To elucidate further how proliferation and differentiation of gut progenitors are controlled by Wnt signalling, we performed expression profiling of *Tcf4*-deficient fetal small intestines. Initially, this approach led us to uncover a role for Wnt/Tcf4 signalling in driving Paneth cell maturation through direct regulation of anti-microbial genes such as cryptdins.[10] In this study, we describe the biologic function of a novel Wnt responsive gene, termed *Spdef*, which emerged from this screen. Spdef is a transcription factor of the Ets family, initially identified as a regulator of the prostate-specific antigen.[11] Functional studies have also indicated that manipulating the expression levels of Spdef affects migration and/or tumorogenicity of various cell lines and that overexpression in the airway epithelium results in goblet cell hyperplasia.[12–16] By generating *Spdef* knockout mice, we now demonstrate that, in the intestine, Spdef ensures the proper maturation of both Paneth and goblet cells.

Materials and Methods

Spdef *Targeting Construct and Generation of* Spdef$^{-/-}$ *Mice*

The *Spdef* targeting construct was generated using a recombineering strategy initially described elsewhere (http://recombineering.ncifcrf.gov). Briefly, starting from BAC clone, a 12-kilobase (kb) fragment of genomic DNA comprising exon 6 of the *Spdef* gene was retrieved in the pl253 plasmid using the SW102 recombinogenic strain. Similarly, loxP sites flanking exon 6 and a neo-cassette

Abbreviations used in this paper: E, embryonic; GEO, Gene Expression Omnibus; kb, kilobase; MGC, Mammalian Gene Collection; PAS, periodic acid-Schiff; RT-PCR, reverse-transcription polymerase chain reaction; SAM, significant analysis of microarrays.

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were recombined into the retrieved 12-kb genomic fragment using the pl451 plasmid as a template. The targeting construct was linearized and electroporated in 129/Ola-derived IB10 embryonic (E) stem cells. Positive clones were selected by Southern blot analysis for the presence of a recombinant 4.3-kb *Xba*I/*Cla*I band, in addition to the endogenous 5.8-kb fragment (see Supplementary Figure 1*B*). Two correctly targeted clones at both ends were injected in C57BL/6 blastocysts, and 1 of these clones produced chimeras with germ-line transmission. The floxed exon 6 was deleted using EIIaCre mice,[17] and the frt flanked neomycin cassette was released by breeding to the Flpe mice (Jackson Laboratory, Bar Harbor, ME).

Histology and Immunohistochemistry

Intestinal samples for in situ hybridization and immunohistochemistry were fixed overnight in formalin at room temperature. Samples were then dehydrated, embedded in paraffin, and sectioned at 4 μM. After antigen retrieval, the primary antibodies used in this study were rabbit anti-lysozyme (DAKO, Heverlee, Belgium), rabbit anti-synaptophysin (DAKO), and guinea pig anti-Spdef (kindly provided by Dr Jeffrey Whitsett). The EnVision+ system (DAKO) was used as a secondary antibody. Periodic acid-Schiff (PAS) for in situ hybridizations were performed according to standard protocols. The numbers of goblet cells and enteroendocrine cells within the villus epithelium were counted as a percentage of total cells. Three mice per genotype were analyzed by selecting 3 independent fields in the proximal and distal portions of the small intestine. In a similar fashion, the number of Paneth cells or Delta-like 1 (Dll1)-positive cells were counted per crypt. A Mann–Whitney *U* test was performed to verify statistical significance.

In Situ Hybridization

In situ hybridization experiments were performed as in Gregorieff et al.[18] The in situ hybridization probes utilized in this study correspond to Mammalian Gene Collection (MGC) clones obtained through the IMAGE consortium (http://hudsonalpha.org).

Transmission Electron Microscopy

Intestine samples were fixed for at least 12 hours in a mixture of paraformaldehyde (2%, wt/vol) and glutaraldehyde (2.5%, vol/vol) in sodium cacodylate buffer (0.1 mol/L, pH 7.4). The tissues were then cut into 2-mm^2 fragments, postfixed for 2 hours in osmium tetroxide (1%), dehydrated in graded alcohols, and embedded in Epon resin. Sections (60 nm) were contrasted with uranyl acetate and lead citrate staining and viewed with a Phillips (Eindhoven, The Netherlands) CM10 electron microscope.

DNA Microarrays

Total RNA was isolated from dissected whole small intestines or colons using TRIzol Reagent (Invitrogen, Breda, The Netherlands). To compare wild-type vs *Spdef*$^{-/-}$ intestinal or colonic tissue samples, pooled total RNA from 2 or 3 littermates were utilized for each experiment. In addition, tissue samples from both adult (P30) or E18.5 fetal mice were compared. Sample pairs were also dye swapped to allow for the correction of dye effects. Labeling, hybridization, and washing procedures were performed according to Agilent guidelines on 4X44K Agilent Whole Mouse Genome Microarrays (G4122F). Overall, we carried out 3 dye swap experiments for E18.5 small intestine and P30 colon resulting in 6 arrays each, and 2 dye swap experiments for E18.5 colon and P30 small intestine resulting in 4 arrays each. Raw signal intensities were corrected by subtracting local background. Negative values were changed into a positive value close to zero (standard deviation of the local background) to allow calculation of ratios between intensities for features only present in 1 sample (wild type or *Spdef*$^{-/-}$). Data were filtered if both (wild type or *Spdef*$^{-/-}$) intensities were changed or if both intensities were less than 2 times the background signal and normalized with a LOESS algorithm. Raw microarray data can be viewed at Gene Expression Omnibus (GEO) (accession number GSE14892; http://www.ncbi.nlm.nih.gov/geo).

Statistical Analysis

Statistical analysis was performed with significant analysis of microarrays (SAM) and "one class" as the response value for the small intestine and colon arrays (n = 10 each).[19] Genes were considered to be significantly up- or down-regulated in small intestine or colon if they had a q-value and a local false discovery rate (localfdr) according to SAM of <0.05 and were enriched >1.5-fold in at least 3 out of 4 arrays (E18.5 colon and P30 small intestine) or 4 out of 6 arrays (E18.5 small intestine and P30 colon).

Results

Identification of Spdef as a Marker of Gut Progenitors and Mature Goblet and Paneth Cells

To shed light on the downstream gene targets regulated by Wnt signals in the gut intestine, we previously performed microarray profiling on fetal small intestines derived from wild-type vs *Tcf4*$^{-/-}$ embryos.[10] One of the target genes that attracted our attention was the transcription factor *Spdef* (also termed *PDEF*). By in situ hybridization, we found that, at late fetal stages when the gut epithelium begins to differentiate, *Spdef* messenger RNA (mRNA) was localized in a limited number of cells scattered throughout the epithelial layer. In *Tcf4* mutants, this dotted staining pattern was abolished, thus confirming our microarray data (Figure 1*A* and *B*). In the adult small intestine, *Spdef* mRNA (data not shown) and protein expression were detected in Paneth cells, goblet cells, and a subpopulation of transit-amplifying cells