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Smad3 contributes to positioning of proliferating cells in colonic crypts by inducing EphB receptor protein expression

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ABSTRACT

Deficiency of Smad3, an intracellular mediator of TGF-β, was shown to significantly accelerate re-epithelialization of the colonic mucosa. This study was performed to investigate the molecular mechanisms by which Smad3 controls colonic epithelial cell proliferation and crypt formation. *Smad3*$^{ex8/ex8}$ C57BL/6 mice were used in this study and wild-type littermates served as controls. The number of proliferating cells in the isolated colonic epithelium of *Smad3*$^{-/-}$ mice was significantly increased compared to that in wild-type littermates. Protein levels of the cell cycle inhibitors p21 and p27 were significantly decreased, while that of c-Myc was increased in the isolated colonic epithelium from *Smad3*$^{-/-}$ mice. In the colonic tissue of wild-type mice, cell proliferation was restricted to the bottom of the crypts in accordance with nuclear β-catenin staining, whereas proliferating cells were located throughout the crypts in *Smad3*$^{-/-}$ mice in accordance with nuclear β-catenin staining, suggesting that Smad3 is essential for locating proliferating cells at the bottom of the colonic crypts. Notably, in *Smad3*$^{-/-}$ mice, there was loss of EphB2 and EphB3 receptor protein expression, critical regulators of proliferating cell positioning, while EphB receptor protein expression was confirmed at the bottom of the colonic crypts in wild-type mice. These observations indicated that disturbance of the EphB/ephrin B system brings about mispositioning of proliferating cells in the colonic crypts of *Smad3*$^{-/-}$ mice. In conclusion, Smad3 is essential for controlling number and positioning of proliferating cells in the colonic crypts and contributes to formation of a "proliferative zone" at the bottom of colonic crypts in the normal colon.

1. Introduction

The colonic mucosa contains a number of flask-like invaginations termed crypts. Pluripotent stem cells reside near the bottom of the crypts and generate progenitors that occupy the lower third of the crypt. Epithelial renewal occurs in these crypts through a coordinated series of events involving proliferation, differentiation and migration toward the intestinal lumen. In the midcrypt region, the cells differentiate into one of the functional cell types of the colon [1]. However, the molecular mechanisms responsible for the induction and maintenance of this process remain undetermined [2].

Transforming growth factor-β (TGF-β) has been shown to play an essential role in the process of mucosal healing [3]. The recent identification of TGF-β signal transduction pathways involving Smad proteins, intracellular mediators and latent nuclear transcriptional activators of TGF-β, has made it possible to investigate their contributions to the activities of TGF-β *in vivo*. Ashcroft et al. demonstrated that, in contrast to predictions made based on the ability of exogenous TGF-β to improve wound healing, Smad3-null (*Smad3*$^{ex8/ex8}$) mice showed accelerated cutaneous wound healing as compared to wild-type controls. This result was explained by an increased rate of re-epithelialization and significantly reduced local infiltration of monocytes in *Smad3*$^{-/-}$ mice [4]. Using a murine model of colitis induced by TNBS (2,4,6-trinitrobenzene sulfonic acid), we recently reported that Smad3 deficiency significantly accelerates re-epithelialization of the colonic mucosa without enhancing inflammation, suggesting that Smad3 is involved in not only specific pathways of tissue repair of cutaneous wounds but also those of colonic epithelial restitution [5]. These findings prompted us to examine whether Smad3 regulates proliferating cells in the colonic crypts and further influences the crypt/villus axis of the intestinal epithelium. Therefore, the present study was performed to investigate the molecular mechanisms by which Smad3 controls colonic epithelial cell proliferation and colonic crypt formation in terms of the distribution of proliferating cells.

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2. Materials and methods

2.1. Animals

Smad3$^{ex8/ex8}$ C57BL/6 mice generated by targeted disruption of the *Smad3* gene by homologous recombination were generously donated by Dr. Anita B. Roberts (NCI, National Institutes of Health, Bethesda, MD). Eight- to 12-week-old Smad3-null mice and wild type littermates were used in this study. Animals were maintained in a restricted access room with controlled temperature. The animals were housed in wire cages with a maximum of 6 mice per cage. Standard laboratory pelleted formula and tap water were provided *ad libitum*. The present study was approved by the Chiba University Ethical Committee. *Smad3*$^{-/-}$ mice at 8–12 weeks old were smaller than their wild-type littermates and had an incompletely penetrant forepaw defect with torqued-wrist defect.

2.2. Isolation of colonic epithelial cells

Viable colonic epithelial cell layers were isolated from crypt units by an EDTA-perfusion method [6]. Briefly, mice were anesthetized by intraperitoneal injection of pentobarbital sodium (70 mg/kg). The thoracic cavity was opened and perfused through the left ventricle of the heart with 30 mM EDTA in calcium–magnesium free Hank's balanced salt solution (HBSS) warmed to 37 °C. The abdomen was then opened and the lumen of the colon was washed with 10 mL of 1 mM EDTA in HBSS warmed to 37 °C. After perfusion, the entire colon excluding the cecum was removed, inverted, cut into 1-cm segments, and placed in a cold tube filled with 2 mL of cold HBSS. The tube was shaken at 2500 rpm for 60 s using a mini-beater. The tissue remnants were discarded and crypts in the supernatant were allowed to settle to the bottom of the tube and then washed three times with cold HBSS. The isolated crypts were then incubated with collagenase (1.0 mg/mL; Sigma, St. Louis, MO) for 20 min at 37 °C. Proliferation of the suspended colonic epithelial cells was quantified using a Cell Proliferation ELISA, BrdU Kit (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions.

2.3. Western blotting

Proteins from the mouse colon were lysed by boiling in PBS and 1% SDS containing 100 μg/mL of phenylmethylsulfonyl fluoride and 1 mM sodium orthovanadate at the indicated times after plating. Aliquots of 20 μg of proteins were separated by SDS–PAGE (10–20% resolving gels) and electroblotted onto nitrocellulose membranes (NEN™ Life Science Products, Inc., Boston, MA). The membranes were saturated for 30 min at room temperature with blocking buffer (0.6% Tween 20, 1% bovine serum albumin and 10% skimmed milk) and incubated with anti-p21 antibody (1:100, #sc-6246; Santa Cruz Biotechnology, Santa Cruz, CA), anti-p27 antibody (1:100, #sc-1641; Santa Cruz Biotechnology), anti-c-myc antibody (1:100, #sc-42; Santa Cruz Biotechnology), anti-EphB2 antibody (1:1000, #AF467; R&D Systems, Minneapolis, MN), anti-EphB3 antibody (1:1000, #AF432; R&D Systems), or anti-β-actin antibody (1:1000; Sigma–Aldrich, St. Louis, MO) at room temperature overnight. The membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (1:1000; Vector Laboratories, Burlingame, CA) or HRP-conjugated anti-goat IgG antibody (1:1000; #HAF109, R&D Systems) at room temperature for 45 min, and detection was carried out using an enhanced chemiluminescence (ECL) Western blotting system (Bio-Rad Laboratories, Hercules, CA).

2.4. Immunohistochemistry

Colons were removed from the mice and washed with saline to remove any fecal material, and then fixed in 10% buffered formalin. Tissues were embedded in paraffin, and sections 7 μm thick were cut. Colonic tissue sections were deparaffinized, and endogenous peroxidase activity was blocked. Sections were incubated overnight with primary antibodies: monoclonal rat anti-mouse Ki-67 antibody (1:25, #M7249; Dako, Tokyo, Japan), polyclonal goat anti-mouse EphB2 antibody (1:10, #AF467; R&D Systems), polyclonal goat anti-mouse EphB3 antibody (1:10, #AF432; R&D Systems), anti-mouse β-catenin antibody (1:20, #160153; BD Biosciences, San Jose, CA). Specimens were then incubated with biotinylated polyclonal rabbit anti-rat immunoglobulin (1:200, #E0468, Dako) for 60 min. Signals were detected using the streptavidin peroxidase complex with DAB substrate chromogen system (Dako).

2.5. Data and statistical analysis

Signals were quantified using ImageJ 1.43u (Wayne Rasband, NIH, Bethesda, MD). Quantitative values are expressed as means ± SEM. Statistical significance was assessed by the unpaired Student's *t* test. In all analyses, $P < 0.05$ was taken to indicate statistical significance.

3. Results

3.1. Proliferation of isolated colonic epithelial cells

Viable colonic epithelial cells were isolated and proliferating cells were quantified. As shown in Fig. 1A, the number of cells in the cell cycle from the colonic epithelium of *Smad3*$^{-/-}$ mice was significantly increased (205 ± 27%) compared to that of wild-type controls (100%), even when treated with 10 ng/mL TGF-β (288 ± 83%) that was shown to reduce epithelial cell proliferation [7]. We then extracted proteins from the isolated colonic epithelial cells and expression levels of the cell cycle proteins were evaluated. Protein levels of the cell cycle inhibitors p21 and p27 were significantly decreased and that of c-Myc was increased in the colonic epithelium of *Smad3*$^{-/-}$ mice (Fig. 1B). These findings demonstrated that, in the colonic crypts of *Smad3*$^{-/-}$ mice, proliferation of the colonic epithelial cells was accelerated due to enhanced cell cycle by downregulation of p21 and p27 and upregulation of c-Myc protein expression. These results indicated that Smad3 in the normal colon plays an important role in negatively regulating colonic epithelial cell proliferation by controlling expression levels of cell cycle-related proteins, such as p21, p27 and c-Myc.

3.2. Ki-67 staining of the colonic mucosa

Ki-67 staining of the colonic mucosa was performed to examine the distribution of proliferating cells in the colonic epithelium. Intriguingly, Ki-67-positive proliferating cells were dispersed throughout the colonic crypts in *Smad3*$^{-/-}$ mice, whereas they were restricted to the bottom of the crypts in *Smad3*$^{+/+}$ littermates (Fig. 2A). These findings demonstrated that Ki-67-positive proliferating cells and Ki-67-negative differentiated epithelial cells were intermingled throughout the colonic crypts of the *Smad3*$^{-/-}$ mice. As shown in Fig. 2B, counting the numbers of Ki-67-positive epithelial cells showed there were twice as many proliferating cells in the crypts of *Smad3*$^{-/-}$ mice as there were in wild-type littermates, demonstrating again that the number of proliferating cells of colonic crypts was markedly increased in the *Smad3*$^{-/-}$ mice compared to wild-type controls. These results indicated that