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Heparin-binding EGF-like Growth Factor Restores Wnt/ β -catenin Signaling in Intestinal Stem Cells Exposed to Ischemia/Reperfusion Injury

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Abstract

Background—We have previously demonstrated that heparin-binding EGF-like growth factor (HB-EGF) protects the intestines from injury in several different experimental animal models. In the current study, we investigated whether the ability of HB-EGF to protect the intestines from ischemia/reperfusion (I/R) injury was related to its effects on Wnt/ β -catenin signaling in intestinal stem cells (ISC).

Methods—LGR5-EGFP transgenic (TG) mice with fluorescently labeled ISC, as well as the same mice treated with intraluminal HB-EGF or genetically engineered to overexpress HB-EGF, were exposed to segmental mesenteric artery occlusion (sMAO) to the terminal ileum. Wnt/ β -catenin signaling was evaluated using immunofluorescent staining and Western blotting.

Results—LGR5 expression and Wnt/ β -catenin signaling in the ISC of the terminal ileum of LGR5-EGFP TG mice was significantly reduced 24 h post sMAO. Intraluminal administration of HB-EGF or HB-EGF overexpression in these mice led to preservation of LGR5 expression and Wnt/ β -catenin signaling.

Conclusion—These data show that HB-EGF preserves Wnt/ β -catenin signaling in ISC after I/R injury.

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DISCLOSURES/DUALITY OF INTEREST

The authors have nothing to disclose.

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INTRODUCTION

Intestinal ischemia/reperfusion (I/R) injury after surgery or trauma is commonly associated with high morbidity and mortality.¹ The intestine is one of the organs that is most sensitive to I/R injury, which typically leads to altered absorption function, gut barrier failure and bacterial translocation, followed by remote organ injury.²⁻⁴

Heparin-binding EGF-like growth factor (HB-EGF) was first identified in the conditioned medium of cultured human macrophages,⁵ and was subsequently found to be a member of the EGF family.⁶ It acts as a protective agent in response to many types of injury in different organs, and its expression is induced after I/R injury to the intestines.⁷⁻¹² Several lines of evidence from our laboratory demonstrate that HB-EGF protects the intestines from various forms of injury. We have shown that HB-EGF promotes enterocyte migration,¹³ prevents intestinal epithelial cell (IEC) apoptosis,¹⁴ decreases bacterial translocation,¹¹ and preserves gut barrier function¹⁵ after intestinal injury. We have shown that HB-EGF prevents intestinal histologic injury and reduces mortality after segmental intestinal I/R injury in rats.² *In vitro*, we showed that exposure of IEC to exogenous HB-EGF protects the cells from hypoxic necrosis and from pro-inflammatory cytokine-induced apoptosis.^{16, 17}

The intestinal mucosa is regenerated and replaced at a very fast pace by pluripotent intestinal stem cells (ISC) that reside in the base of crypts.^{18, 19} ISC have the capacity of self-renewal as well as proliferation into epithelial progenitor cells in the transit-amplifying (TA) zone, which subsequently differentiate into all of the different IEC cell lineages in order to maintain intestinal homeostasis. ISC, also known as crypt base columnar (CBC) cells due to their location at the base of the crypts, are rich in Leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5) expression.^{20, 21} Stem cells in some organs, including the intestines, have been shown to respond to stress and to promote recovery from injury.²²

Wnt/ β -catenin signaling in ISC drives intestinal regeneration through proliferation in response to the injury. Proliferation and differentiation of ISC are under the control of the Wnt/ β -catenin signaling pathway under normal conditions.^{23, 24} When secreted glycoprotein WNT binds to the frizzled (fz) receptor (the mediator of Wnt signaling), β -catenin accumulates in the cytoplasm and then translocates into the nucleus. This triggers the activation of downstream target genes such as c-Myc, Cyclin D1 and Lgr5, resulting in cell proliferation.^{25, 26} However, without Wnt, β -catenin is downregulated by axin, adenomatous polyposis coli (APC), casein kinase 1a (CK1a), protein phosphatase 2A (PP2A) and the glycogen synthesis kinase 3 (GSK3) destruction complex through ubiquitination and proteasome degradation. Moreover, Hippo/Yan signaling also counteracts Wnt/ β -catenin signaling to maintain intestinal epithelial homeostasis.

We have shown that HB-EGF promotes progenitor cell proliferation when the growth factor is overexpressed in transgenic mice.²⁷ We also showed that HB-EGF promotes crypt cell proliferation after I/R injury.¹¹ We have demonstrated that ISC expression of LGR5, a Wnt/ β -catenin target gene, is down-regulated in an animal model of necrotizing enterocolitis.²⁸ In the current study we examined the effects of HB-EGF administration, as well as intestinal HB-EGF overexpression, on ISC in Lgr5-EGFP TG mice exposed to I/R

injury. We further examined the protective effect of HB-EGF on Wnt/ β -catenin signaling and its target genes after I/R injury.

MATERIALS AND METHODS

Animal strains

C57BL/6J wild type (WT) mice and B6129P2-Lgr5^{tm1(cre/ERT)Cle/J} mice with Lgr5-EGFP expression confined to ISC (hereafter referred to as Lgr5 TG mice) were purchased from the Jackson Laboratory (Bar Harbor, ME). To generate B6129P2-Lgr5^{tm1(cre/ERT)Cle/J};Villi-HB-EGF/+ mice (Lgr5 TG mice that overexpress HB-EGF in the intestines; hereafter referred to as Lgr5-HB-EGF TG mice), B6129P2-Lgr5^{tm1(cre/ERT)Cle/J} mice were crossed with homogeneous transgenic Villi-HB-EGF/Villi-HB-EGF mice that we produced in our laboratory.²⁷ In addition, Lgr5-lacZ mice were generated by crossing Lgr5-EGFP-IREScreERT2 mice to Rosa26-lacZ Cre-reporter mice, both obtained from Jackson Laboratory (Bar Harbor, ME).²⁰ One to two month old mice were injected intraperitoneally (IP) with tamoxifen (200 μ l; 10 mg/ml) in sunflower oil to induce LacZ expression in Lgr5⁺ ISC and their progeny cells.

Segmental mesenteric artery occlusion (sMAO)

All animal procedures were approved by the Institutional Animal Care and Use Committee of the Research Institute at Nationwide Children's Hospital (Protocol #00903AR). Four to eight week old male mice weighing 20–25g were randomized to four groups ($n=4-5$ in each group): (1) Lgr5 TG mice exposed to sham surgery (Lgr5 TG-sham group); (2) Lgr5 TG mice exposed to segmental mesenteric artery occlusion (sMAO) for 45 min followed by reperfusion for 24 h (Lgr5 TG-sMAO group); (3) Lgr5 TG mice exposed to sMAO that received intraluminal HB-EGF (1200 μ g/Kg) (Lgr5 TG-sMAO+HB-EGF group), and (4) Lgr5 TG mice that overexpress HB-EGF in the intestine and that were exposed to sMAO (Lgr5-HB-EGF TG-sMAO group). In addition, three groups of WT (C57BL/6J) mice were subjected to: (1) sham surgery, (2) sMAO or (3) sMAO+ intraluminal HB-EGF (1200 μ g/Kg) for intestinal morphological injury evaluation.

For exposure to sMAO, mice were anesthetized with 3% isoflurane and the abdomen was shaved and rinsed with 70% isopropanol solution. Anesthesia was maintained using 1% isoflurane and body temperature was maintained at 37°C using a heating pad. A midline laparotomy was performed, and under a dissecting microscope a single first-order branch of the superior mesenteric artery (SMA) feeding a 5-cm length of terminal ileum was identified and occluded with a non-traumatic microvascular clamp. Arterial branches that provided collateral flow to the clamped segment were occluded as well. Paleness of the terminal ileum and lack of pulsations in the mesentery confirmed the ischemic insult. After 45 min of ischemia, mice to be treated with HB-EGF received a 100 μ l intraluminal injection of HB-EGF (1200 μ g/kg in PBS; Trillium Therapeutics Inc., Toronto, CA) into the terminal ileum, ensuring that the entire segment was filled with the HB-EGF solution. Control animals that were exposed to sMAO but not treated with HB-EGF received 100 μ l of 1X PBS solution intraluminally. Vascular clamps were removed after 45 min of total ischemia and the intestine was allowed to reperfuse for 24 h. In the sham-operated group, the ileal segmental

mesenteric artery and collateral branches were identified by dissection but were not occluded.^{2, 3}

Histologic and immunofluorescent staining

The terminal ileum was excised and processed for histologic staining and protein analysis. Specimens for histologic analyses were fixed in 10% formalin/PBS for two days and embedded in paraffin. Cross-sections (5 μ m thickness) were subjected to histochemical and immunofluorescent staining for morphological evaluation and detection of β -catenin (BD Bioscience, Franklin Lakes, NJ), proliferative cell nuclear antigen (PCNA) (Sigma-Aldrich, Saint Louis, MO) and cyclin D1 (Cell Signaling Technology, Danvers, MA) production. Intestinal morphology was assessed by H&E staining of tissue sections. H&E stained sections were examined using an Axio Scope microscope (HBO 100/W2, Zeiss, Thornwood, NY) with AxioVision software (version, 02.2002).

Fluorescent immunohistochemistry—For immunofluorescent staining, paraffin-embedded tissue sections were deparaffined, rehydrated and then blocked with 10% donkey serum/PBS for 1h at RT. Either mouse anti-PCNA (Sigma-Aldrich, Saint Louis, MO), mouse anti-cyclin D1 (Cell Signaling Technology, Danvers, MA) or mouse monoclonal anti- β -catenin (BD Bioscience, San Jose, CA) primary antibodies were applied to tissue sections according to the manufacturer's recommended dilutions in 10% donkey serum/PBS for 1h at RT or overnight at 4°C. For double staining, two antibodies were added for incubation simultaneously. After three 10 min washes with PBS/0.1% Tween 20, tissue sections were incubated with the appropriate secondary antibodies of anti-IgG (4 μ g/ml) conjugated with FITC or Cy3 (Jackson ImmunoResearch Laboratories, West Grove, PA) in 10% donkey serum/PBS for 1h at RT. Tissue sections were mounted with ProLong Gold antifade reagent with DAPI (Invitrogen, Carlsbad, CA) after three 10 min washes with PBS +0.1% Tween 20. Immunostaining in the absence of primary antibodies served as negative controls. Fluorescence microscopy was performed using an Axio Scope fluorescent microscope (HBO 100/W 2, Zeiss, Thornwood, NY). For β -catenin immunostaining, crypts from the terminal ileum were examined using a LSM 510 confocal microscope (Zeiss, Thornwood, NY).

LacZ staining was carried out according to the procedures described previously with modifications.²⁰ Intestines were isolated and immediately fixed for 2 h in 20 ml of ice-cold fixative (1% formaldehyde, 0.2% glutaraldehyde and 0.02% NP40 in PBS) at 4°C with constant agitation. The contents of the intestines were flushed out with the fixative. Tissues were washed twice in PBS for 20 min at room temperature with constant agitation. LacZ was visualized by adding β -galactosidase substrate [5mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6 \cdot 3H_2O$, 2mM $MgCl_2$, 0.02% NP40, 0.1% sodium deoxycholate and 1mg/ml X-gal in PBS] overnight at RT with constant shaking. The substrate was removed and the tissues washed twice in PBS for 20 min at RT on a rolling platform. Tissues were then further fixed overnight in 20 ml of 4% paraformaldehyde (PFA) in PBS at 4°C in the dark. The PFA was then removed and the tissues were washed twice in PBS for 20 min at RT on a rolling platform. The stained tissues were transferred to tissue cassettes and paraffin blocks

prepared using standard methods. Tissue sections (5 μ M) were prepared and counterstained with neutral red.

Whole Mount Immunohistochemistry—Intestinal whole mounts were prepared on wax plates as previously described and were fixed in ice-cold 2% formaldehyde/0.2% glutaraldehyde in PBS (pH 7.4) for 1 h.²⁹ For the generation of sections, 1–2 cm lengths of intestine were fixed on ice in 4% paraformaldehyde (PFA) for 4–6 h, immersed in 20% sucrose/PBS for 48–72 h at 4°C, snap-frozen and mounted in cryomountant (OCT) prior to cryosectioning. Air-dried sections and intestinal whole mounts were stained for β -galactosidase by incubation in 0.08% 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) substrate [2 mM MgCl₂, 5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆ · 6H₂O, 0.1 M PBS, pH 7.4, prepared from a 2% stock of X-Gal in dimethylformamide] overnight at 37°C and RT, respectively.

Isolation of LGR5-EGFP-labeled ISC and *ex vivo* crypt-villous organoid culture

Isolation of LGR5-EGFP⁺ ISC was performed as previously described with modifications.^{20, 30} WT and Lgr5 TG mice were sacrificed and the terminal ileum removed and flushed with ice-cold Ca²⁺- and Mg²⁺-free PBS. The intestine was everted on a 4 mm glass rod and assembled onto a Bulcher gradient maker and incubated in PBS/EDTA (30 mM) (pH 7.4), at 37°C for 5 min. Villi were released into ice-cold PBS after 4–5 pulses of vibration. Crypt-villus sheets were obtained after 15 min of further incubation at 37°C and several longer vibrations. Crypts were then separated from villi by pipetting the solution vigorously 10 times using 10 ml pipettes and running the solution through a 40 μ m cell strainer. Villi were retained on the cell strainer and discarded. Retained crypts were subjected to trituration to obtain single cells using a glass Pasteur pipette. High and medium expressing LGR5-EGFP⁺ cells (~1000 cells) were isolated using a FACSVantage cell sorter (Becton Dickinson, Franklin Lakes, NJ) and were then cultured in 50 μ l Matrigel supplemented with HB-EGF (50 ng/ml) at 37°C with 5% CO₂ for *ex vivo* crypt-villous organoid growth as previously described.²⁸ Crypt-villous organoid growth was documented using a LEICA DM-4000B microscope (Leica Microsystems, Buffalo Grove, IL) for fluorescent and phase-contrast images.

Quantification of Lgr5-EGFP⁺ ISC

Ileal specimens were fixed in 1% paraformaldehyde, 15% picric acid, 0.1 M sodium phosphate buffer (pH 7.0) and shaken gently at 4°C overnight. Tissues were snap-frozen and mounted in Cryomount Optimal Cutting Temperature (OCT) prior to obtaining 20 μ m cryosections.³¹ ~150–200 well aligned ileal crypts under 400x magnification were evaluated from each animal were examined using an Axioscope microscope by two independent observers (CLC and JY). A crypt with 1 distinct LGR5-EGFP⁺ ISC or more was scored as positive, whereas crypts with vague or no expression of EGFP were scored as negative. Positive crypts were expressed as the percent of total scored crypts.

Western blotting

Ileal lysates were homogenized in 100 ml/mg RIPA buffer with EDTA (50mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and 5 mM

EDTA) (Boston BioProducts, Worcester, MA) supplemented with 1X protease inhibitor cocktails (Pierce, Rockford, IL). Protein concentrations were quantified using a BCA protein assay kit (Thermo Scientific, Rockford, IL). Protein lysates were boiled with 10x sample buffer (30 μ l) at 95°C for 5 min. Proteins (100 μ g) were fractionated by SDS-PAGE using 10–20% gradient acrylamide gels (BioRad Laboratory, Hercules, CA). Target proteins were detected by probing blots with the following primary antibodies: β -catenin (BD Bioscience, San Jose, CA), phospho-AKT(S473), phospho-ERK1/2, cyclin D1, C-MYC (Cell Signaling Technology, Danvers, MA), or β -ACTIN (Sigma-Aldrich, Saint Louis, MO). Membranes were washed with PBS + 0.1% Tween 20 three times and incubated with anti-mouse or anti-rabbit (1:500 dilution, Amersham, Pittsburgh, PA) secondary antibodies conjugated with alkaline phosphatase. After three washes in PBS + 0.1% Tween 20, colorimetric visualization was developed using an alkaline phosphatase substrate mixture of lumigen TMA-6 solution A and B in the ECL advance kit (Amersham, Piscataway, NJ). Densitometric intensity of each protein band was obtained using imageJ software and normalized to that of the internal control β -actin. The average expression of each specific protein from two independent experiments was shown as fold-increase compared to the corresponding sham group which was designated as 1.

Statistical analyses

Data are presented as mean $\pm$ SD from at least three independent experiments. Statistical analyses were performed using one-way ANOVA, with Tukey-Kramer pairwise comparison tests, using Prime software. $p < 0.05$ was considered statistically significant.

RESULTS

HB-EGF protects the intestines from sMAO

WT mice subjected to sMAO for 45 min followed by 24 h of reperfusion have significant damage to the affected terminal ileum (Figure 1). This degree of sMAO leads to loss of the villi with preservation of the majority of the crypts in which the ISC reside. Administration of intraluminal HB-EGF during the ischemic interval protects the intestines from injury (Figure 1).

Lgr5-EGFP TG mice have fluorescently-labeled ISC

To examine the effects of I/R injury and the protective effects of HB-EGF on ISC, we utilized an Lgr5 promoter-driven EGFP expression mouse model that allowed us to easily identify ISC. Lgr5-EGFP TG mice have a knocked-in Lgr5-EGFP^{ires}-creERT2 in the first exon of the Lgr5 gene near the promoter, which targets EGFP expression specifically in the crypt base columnar (CBC) cells located at the base of the crypts.^{32, 33} In ileal cross-sections, well aligned crypts typically contained 3–8 Lgr5-EGFP⁺ CBC cells (Figure 2A). FACS analysis demonstrated that 6.5% of crypt cells in Lgr5-EGFP TG mice were Lgr5-EGFP⁺, with medium or high levels of green fluorescence (Figure 2B) compared to WT mice which had 0.9% auto-fluorescent cells.

LGR5-EGFP⁺ ISC have self-renewal and differentiation capacity

Lgr5-EGFP⁺ single ISC derived from the crypts of Lgr5 TG mice were isolated by FACS and cultured in matrigel. After three days in culture, single ISC grew into crypt-villous organoids that had a comparable size to organoids derived from isolated crypts after 1 day in culture (Figure 3A). In addition, Lgr5-EGFP-IREScreERT2;Rosa26-lacZ Cre mice were used for ISC lineage analysis. After 21 days of tamoxifen-induction, the Lgr5-EGFP⁺ cells and their progeny expressed β -galactosidase and were visualized as LacZ-positive collections of blue-staining cells in whole mount ileal specimens (Figure 3B). Similar findings were observed in the duodenum, jejunum and colon (data not shown). In cross sections, single ISC, their derived progenitor cells in the transit-amplifying zone, and differentiated cells (enterocytes, goblet cells) formed a continuous blue LacZ staining strip (Figure 3C–E).

HB-EGF reverses I/R-mediated reduction of Lgr5-EGFP⁺ ISC

Lgr5 TG mice exposed to sMAO had significant reductions in LGR5-EGFP⁺ ISC compared to Lgr5 TG mice exposed to sham surgery (Figure 4A, B). In Lgr5-EGFP TG mice exposed to sMAO but treated with administration of exogenous HB-EGF, or in Lgr5-EGFP TG mice that overexpressed endogenous HB-EGF in the intestine, the numbers of Lgr5-EGFP⁺ ISC were significantly increased. The numbers of Lgr5-EGFP⁺ ISC in uninjured jejunum were not affected by exposure of the ileum to sMAO (Figure 4B).

HB-EGF increases β -catenin and its downstream gene products after sMAO

Since Lgr5 is a downstream target gene of the Wnt/ β -catenin signaling pathway, we next investigated whether HB-EGF affects Wnt/ β -catenin signaling. Western blot analysis of terminal ileal specimens from Lgr5-EGFP TG mice subjected to sMAO had decreased production of the major Wnt signaling component β -catenin, as well as the downstream gene products c-MYC and cyclin D1 (Figure 5). Administration of HB-EGF reversed the sMAO-mediated reduction in these signaling molecules. In fact, administration of HB-EGF enhanced the production of c-MYC and cyclin D1 to levels that exceeded those of sham operated animals. Phosphorylation of AKT at serine 437 was also suppressed by sMAO, and was improved by HB-EGF administration. Consistent with previous studies, the ERK1/2 was slightly activated by I/R, and was suppressed by the administration of HB-EGF.

HB-EGF preserves nuclear localization of β -catenin in ISC after sMAO

Immunohistochemistry demonstrated β -catenin localization in the cell membranes and nuclei of ISC in Lgr5 TG mice exposed to sham surgery, consistent with the hallmark of β -catenin activation (Figure 6A).³⁴ β -catenin immunoreactivity of ISC cell membranes and nuclei was significantly reduced in the crypts of animals exposed to sMAO. Administration of HB-EGF in animals exposed to sMAO led to increased β -catenin immunoreactivity in ISC cell membranes and nuclei. Thus, HB-EGF activates Wnt/ β -catenin signaling in ISC by increasing the protein levels of β -catenin and preserving its translocation into the nucleus.

HB-EGF increases cyclin D1 levels and cell proliferation after sMAO

We next used anti-PCNA immunostaining to demonstrate that proliferating cells were significantly reduced in Lgr5 TG mice subjected to sMAO compared to sham surgery (Figure 6B). Anti-cyclin D1 immunostaining showed the same pattern. Administration of HB-EGF to animals subjected to sMAO led to significant increases in PCNA⁺ proliferative cells and cyclin D1⁺ cells. Many cells in the crypt bases had co-localization of PCNA and cyclin D1 expression, indicating that HB-EGF preserved cyclin D1 protein levels and proliferative activity in ISC. These results suggest that HB-EGF promotes cell proliferation by restoring the protein levels of the β -catenin target gene product cyclin D1 in progenitor cells of the transit amplifying zone and in ISC.

DISCUSSION

We have previously shown that HB-EGF protects the intestines from various forms of intestinal injury including I/R injury,^{35, 36} hemorrhagic shock and resuscitation,^{37, 38} necrotizing enterocolitis,²⁸ cecal ligation and puncture,³⁹ and radiation therapy-induced injury.⁴⁰ In the current study, we have used an animal model of intestinal I/R injury based on segmental mesenteric artery occlusion to demonstrate that administration of exogenous HB-EGF, or overexpression of endogenous HB-EGF driven by the villin promoter, preserves Wnt/ β -catenin signaling in ISC and minimizes damage to intestinal integrity. In the current study, Lgr5-EGFP TG mice proved to represent a good model in which to examine the protective effects of HB-EGF on ISC. The ISC of Lgr5-EGFP TG mice are labeled by specific EGFP expression driven by the LGR5 promoter, and are located at the base of the crypts between antibacterial protein producing Paneth cells.³² We observed that EGFP-labeled ISC proliferated and differentiated into other intestinal epithelial cell lineages in *in vivo* induction and *ex vivo* culture experiments as described previously.⁴¹ The Lgr5 gene is a downstream gene that is positively regulated by β -catenin signaling, and can serve as a direct, sensitive and quantitative indicator of Wnt/ β -catenin signaling after injury.

The results of the current studies show that HB-EGF preserves Wnt/ β -catenin signaling in ISC subjected to I/R injury. ISC differentiate into intestinal epithelial cells of all lineages during homeostasis and during regeneration after injury.^{22, 42} Decreased Lgr5-EGFP expression in ISC after I/R injury is likely due to impaired Wnt/ β -catenin signaling. This is supported by loss of expression of β -catenin on cell membranes, and decreased β -catenin nuclear accumulation, in ISC after I/R injury, as shown by both immunostaining and Western blotting in our studies. It is possible that the expression of β -catenin is decreased by the acute inflammation that is common following I/R injury.⁴³ Decreased nuclear accumulation of β -catenin may occur as a consequence of inflammatory cytokine-induced injury to ISC or to other cells in the ISC niche. Others have shown that loss of Wnt/ β -catenin makes tissues more susceptible to I/R injury.⁴⁴ A similar phenomenon has been observed in chronic intestinal colitis.⁴⁵ Thus, the ability of HB-EGF to restore β -catenin signaling in ISC likely plays an important role in the intestinal cytoprotective effects of the growth factor.

Our data also suggest that increased expression of Lgr5, c-Myc and cyclin D1 in ISC is associated with the HB-EGF-mediated Wnt/ β -catenin expression after intestinal injury. Loss

of nuclear accumulation of β -catenin leads to the decreased expression of Lgr5, c-Myc and cyclin D1 in ISC and in cells of the transit amplifying zone.⁴⁶ Diminished expression of the proliferation positive regulators c-Myc and cyclin D1 leads to defective proliferation of ISC and transit amplifying zone cells.^{46, 47} This is supported in our study by decreased expression of both the cell proliferation marker PCNA and of cyclin D1 in both ISC and in transit amplifying zone cells. Several previous studies support these findings. Expression of c-Myc in the proliferating transit amplifying zone is required for the formation of crypts.²⁶ Double mutant Lgr5/6 mice have significantly reduced nuclear accumulation of β -catenin and cyclin D1 expression in the intervillous regions (early crypts), and decreased differentiated neuroendocrine cells in the small intestine during development.⁴⁸ In our studies, partial restoration of Wnt/ β -catenin signaling by HB-EGF would be expected to result in partial restoration of Lgr5, c-Myc and cyclin D1 expression. However, expression of these three Wnt/ β -catenin-regulated genes after HB-EGF treatment was disproportionately high, and was either nearly equivalent to or higher than in sham controls. This suggests that the protective effects of HB-EGF on ISC and transit amplifying progenitor cells may be exerted through other signaling pathways in addition to Wnt/ β -catenin.

The activation of PI3K/Akt has been shown to promote Wnt/ β -catenin signaling via accumulation and nuclear translocation of β -catenin, and to protect tissues from injury.^{45, 49–51} P-Akt is known to inhibit inflammatory responses induced by stress and by radiation-induced apoptosis through suppression of PUMA signaling.^{50, 52} We observed here that activation of Akt was suppressed by sMAO and ~50% of P-Akt was restored by treatment with HB-EGF. This suggests that P-Akt may play a major role in restoring Wnt/ β -catenin signaling and its downstream gene expression in ISC in our sMAO model. Finally, ERK1/2 is activated during I/R injury, and its inhibition showed beneficial results after I/R injury in the heart and brain.^{53, 54} Here we showed that increased I/R-induced ERK 1/2 phosphorylation was attenuated by HB-EGF 24 h after I/R. This suggests that HB-EGF protection may result, in part, from ERK 1/2 suppression. We have observed activation of ERK 1/2 in the first few hours after I/R, simultaneous with the promotion of epithelial restitution.⁵⁵ This suggests a bi-modal activation response of ERK 1/2 to HB-EGF treatment after intestinal injury. The mechanism behind these interesting responses, and any existent signaling cross talk between P-Akt and ERK1/2, remain unclear at present and demand further investigation.

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Figure 1.

HB-EGF protects the intestines from sMAO injury. Shown are representative H&E stained histologic images of the terminal ileum from C57BL/6J wild type (WT) mice exposed to: A) sham surgery, B) sMAO x 45 min followed by 24 h of reperfusion, or C) sMAO x 45 min with intraluminal HB-EGF administration after 45 min of ischemia, followed by 24 h of reperfusion.

Figure 2.

Identification of EGFP⁺ ISC in Lgr5 TG mice. (A) Cross sections of terminal ileum from Lgr5 TG mice demonstrate ISC expressing EGFP. Several crypts are outlined by white dashed lines to show their location in panels *a–c*, with three to eight labeled ISC present in each crypt. *a,c*) EGFP fluorescence visualized using an Axioscope microscope, *b,e*) DAPI counterstaining, *c,f*) merged images. Single ISC at higher magnification are labeled with white arrowheads in panels *d–f*. (B) Crypts were isolated from the terminal ileum of WT and Lgr5 TG mice and subjected to FACS analysis. LGR5-EGFP^{high} and LGR5-EGFP^{medium} expressing cells comprised 6.5% of the total crypt cells in Lgr5 TG mice.

Figure 3.

Lgr5-EGFP⁺ and Lgr5-LacZ expressing ISC clonally differentiate into crypt-villous organoids and different IEC lineages. (A) Single ISC proliferate and differentiate into crypt-villous organoids in *ex vivo* culture. Panels *a* and *c* represent organoids grown from single FACS-isolated ISC after 3 days in culture. Panels *b* and *d* represent organoids grown from isolated crypts after 1 day in culture. Panels *a* and *b* represent visualization by fluorescence microscopy, and panels *c* and *d* represent visualization by phase-contrast microscopy. White arrows in *a* and *b* indicate green fluorescent Lgr5-EGFP⁺ ISC. The majority of cells in the crypt-villous organoids have lost the expression of Lgr5-EGFP, indicating a differentiated status.

(B–E) Lgr5-lacZ mice were generated by crossing Lgr5-EGFP-IREScreERT2 mice to Rosa26-lacZ Cre-reporter mice. Upon induction with tamoxifen, the stop codon in the LacZ gene was floxed out and LacZ expression occurs in ISC and their clonally derived progenies. (B) Dorsal view of a whole mount specimen of terminal ileum. White arrows indicate representative clones of blue stained cells derived from single ISC. (C) Cross-section of a whole mount specimen of terminal ileum. Each continuous blue ribbon expanding from the crypt base (white dotted line) to the tip of the villus is formed by clonal cells derived from a single ISC. The entire crypt-villus axis is outlined by black dots. M, muscular layer; L, lumen. (D–E) Histologic sections of ileum (D) and colon (E) show LacZ⁺ enterocytes as

well as goblet cells (white arrows) containing large vacuoles in the cytoplasm, which are derived from the progeny of a single ISC.

Figure 4.

The ability of HB-EGF to protect the terminal ileum from injury is closely associated with preservation of Lgr5 expression in ISC. (A) Shown are cross-sections (20 μ m) of terminal ileum examined by fluorescence microscopy from: *a–c*) Lgr5 TG mice exposed to sham surgery, *d–f*) Lgr5 TG mice exposed to sMAO, *g–i*) Lgr5 TG mice exposed to sMAO and treated with intraluminal HB-EGF, and *j–l*) Lgr5-HB-EGF TG mice exposed to sMAO. ISC are indicated by their green fluorescence. (B) Lgr5-EGFP expression levels in uninjured jejunal and I/R-injured terminal ileal crypts. The jejunum was not subject to I/R injury and serves as an internal control. Lgr5-EGFP expression is represented as the percent of crypts that contain at least one Lgr5-EGFP⁺ ISC. Values represent mean $\pm$ SD. *, $p < 0.05$ compared to the other 3 ileal groups, one-way ANOVA with Tukey-Kramer pair-wise comparison test.

Figure 5.

Administration of HB-EGF restores β -catenin signaling after sMAO-induced injury. Lysates (100 μ g) from the terminal ileum of Lgr5 TG mice subjected to sham surgery, sMAO, or sMAO with administration of intraluminal HB-EGF (n=2 in each group) were subjected to Western blot analysis of β -catenin, p-AKT(S473), p-ERK1/2, cyclin D1, and c-MYC. Densitometric intensity of each protein band was obtained using imageJ software and normalized to that of the corresponding internal control β -actin. The average level of each specific protein from two independent experiments was shown as fold-increase compared to the corresponding sham surgery group which was designated as 1.

Figure 6.

HB-EGF restores β -catenin levels in ISC after sMAO-induced injury. (A) Shown are representative photomicrographs of terminal ileum from Lgr5 TG mice subjected to: a) sham surgery, b) sMAO, and c) sMAO with intraluminal administration of HB-EGF. Tissue sections were subjected to β -catenin immunostaining. White arrows indicate representative nuclei with positive staining. (B) Double immunofluorescent labeling of PCNA and cyclin D1 in the terminal ileum of Lgr5 TG mice subjected to: a–c) sham surgery, d–f) sMAO, and g–i) sMAO with intraluminal administration of HB-EGF. Panels a, d and g represent PCNA immunostaining, panels b, e and h represent cyclin D1 immunostaining, and panels c, f and i represent merged images. Yellow arrows represent positively staining cells of the transit amplifying zone and white arrows represent positively staining ISC.