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R-Spondin1 enhances wnt signaling and decreases weight loss in short bowel syndrome zebrafish

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ABSTRACT

Background: R-spondins, including R-spondin 1 (RSPO1), are a family of Wnt ligands that help to activate the canonical Wnt/β-catenin pathway, which is critical for intestinal epithelial cell proliferation and maintenance of intestinal stem cells. This proliferation underpins the epithelial expansion, or intestinal adaptation (IA), that occurs following massive bowel resection and short bowel syndrome (SBS). The purpose of this study was to identify if recombinant human RSPO1 (rhRSPO1) could be serially administered to SBS zebrafish to enhance cellular proliferation and IA.

Methods: Adult male zebrafish were assigned to four groups: sham + PBS, SBS + PBS, sham + rhRSPO1, and SBS + rhRSPO1. Sham fish had a laparotomy alone. SBS fish had a laparotomy with distal intestinal ligation and creation of a proximal stoma. Fish were weighed at initial surgery and then weekly. rhRSPO1 was administered post-operatively following either a one- or two-week dosing schedule with either 3 or 5 intraperitoneal injections, respectively. Fish were harvested at 7 or 14 days with intestinal segments collected for analysis.

Results: Repeated intraperitoneal injection of rhRSPO1 was feasible and well tolerated. At 7 days, intestinal epithelial proliferation was increased by rhRSPO1. At 14 days, SBS + rhRSPO1 fish lost significantly less weight than SBS + PBS fish. Measurements of intestinal surface area were not increased by rhRSPO1 administration but immunofluorescent staining for β-catenin and gene expression for *cyclin D1* was increased.

Conclusions: Intraperitoneal injection of rhRSPO1 decreased weight loss in SBS zebrafish with increased β-catenin + cells and *cyclin D1* expression at 14 days, indicating improved weight maintenance might result from increased activation of the canonical Wnt pathway.

1. Introduction

Following massive small bowel resection, a marked increase in intestinal absorptive surface area allows some patients to maintain nutritional homeostasis, but this process sometimes fails. Although the cellular and molecular mechanisms resulting in successful intestinal adaptation (IA) are not well defined, it is likely that Wnt activation is involved. Multiple models have shown the importance of Wnt signaling in intestinal recovery after injury, including surgical resection [1–3].

R-spondins (RSPO) constitute a family of secreted proteins that, as agonists of the Wnt/β-catenin signaling pathway, promote cell proliferation and differentiation during development and homeostasis. The RSPO family is highly conserved among vertebrates and interacts with leucine-rich repeat-containing G protein-coupled receptors (Lgr). Lgr4 and 5 have been identified as markers of the intestinal stem cell (ISC) [4,5]. R-spondins strongly enhance but cannot initiate Wnt signaling, primarily through the canonical Wnt/β-catenin pathway [6]. This occurs as the RSPO binds to Lgr, stabilizing Frizzled (FZD), a cell membrane Wnt receptor, and low-density lipoprotein receptor related protein 5 or 6 (LRP5/6). This allows increased binding of Wnt ligands, and in turn,

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increased intracellular accumulation of β-catenin that then localizes to the nucleus and acts as a transcriptional co-factor, activating Wnt target genes and promoting cellular proliferation [7].

R-spondin markedly enhances Wnt signaling and aids in recovery of the intestinal epithelium after an insult [8]. R-spondin1 (RSPO1) appears to be of particular importance in intestinal regeneration after injury. Administration of RSPO1 in animal models of radiation induced mucositis and inflammatory bowel disease improved mucosal regeneration and recovery [9,10]. Administration of a fully characterized recombinant human RSPO1 (rhRSPO1), generated by our group in human HEK293 cells, to mice without intestinal injury resulted in increased villus height compared to vehicle control [11]. We therefore hypothesized that this rhRSPO1 might improve IA in SBS.

We have previously developed a model of short bowel syndrome in zebrafish that recapitulates the molecular and physiologic changes of successful IA. RNA sequencing analysis of the intestine of SBS zebrafish identified increased expression of genes involved in Wnt signaling, including β-catenin (*ctnnb1*), *wnt5a,* and *cyclin D1* (*ccnd1*) [12]. Administration of monensin, an inhibitor of the canonical Wnt pathway, increased weight loss in SBS fish and decreased intestinal adaptation, indicating that activation of the canonical Wnt pathway occurs in successful intestinal adaptation after massive intestinal resection [13].

Although the RSPO family of proteins is highly conserved across vertebrates, we did not know whether there was sufficient homology between human and zebrafish RSPO1 to expect a molecular response to administration of rhRSPO1. Zebrafish rhRSPO1 has only a 51% homology with human RSPO1 but the two furin-like domains have a 65% and 73% homology with human RSPO1, respectively [14,15]. The thrombospondin-like domain and the C-terminus of zebrafish RSPO1 share the least homology with human RSPO1; however, studies in Xenopus indicate these regions are dispensable and only the furin-like domains are required for enhancing Wnt signaling [16,17].

We therefore sought to identify whether, in addition to intestinal resection, zebrafish could be multiply dosed with rhRSPO1, and if this would enhance IA in SBS.

2. Materials and methods

2.1. Short bowel surgical procedure

After IACUC approval, short bowel surgeries were performed as previously described [18]. The distal intestine was ligated, and a proximal stoma created with resection of the intervening segments. Sham surgery consisted of a laparotomy alone. Only male zebrafish were included because egg production in female fish increases variability in surgery and weight measurements [18].

2.2. Production and purification of recombinant human R-spondin1

Recombinant human R-spondin1 (rhRSPO1) was expressed in HEK293 cells and purified as previously described [12]. The protein was lyophilized and then reconstituted in sterile PBS prior to injection.

2.3. One-week protocol for R-spondin1 administration

Adult male zebrafish were allocated to four groups: sham + PBS (n = 12), SBS + PBS (n = 12), sham + rhRSPO1 (n = 12), and SBS + rhRSPO1 (n = 12). Fish were anesthetized in 0.02% tricaine (Sigma-Aldrich, Cat# E10521) and injected intraperitoneally with either 0.8 μg of rhRSPO1 reconstituted in 30 μL of sterile PBS or 30 μL of PBS alone. The dose of rhRSPO1 was extrapolated from previous studies in mice and adjusted for the mean weight of an adult zebrafish [12,19]. Each zebrafish received injections starting on post-operative day (POD) four and continuing daily until harvest, totaling three injections of rhRSPO1 or PBS in this period. Zebrafish were maintained on a standard diet and then harvested on POD7 with collection of the most proximal intestinal segment, S1. Four hours prior, fish were injected intraperitoneally with bromodeoxyuridine (BrdU) (Sigma Life Sciences, B5002-1G).

2.4. Two-week protocol for R-spondin1 administration

Adult male zebrafish were allocated to four groups: sham + PBS (n = 12), SBS + PBS (n = 12), sham + rhRSPO1 (n = 12), and SBS + rhRSPO1 (n = 12). Starting on POD5, zebrafish were injected with rhRSPO1 or sterile PBS alone, as described above. Each zebrafish received injections every other day until harvest on POD14 for a total of five doses. Injections were started on POD5 to allow longer recovery time after the surgical procedure and spaced out to every other day to decrease the morbidity of repeated anesthesia. Zebrafish were maintained on a standard diet and then harvested on POD14 with collection of the most proximal intestinal segment, S1. Four hours prior, fish were injected intraperitoneally with BrdU.

2.5. Measurement of post-operative weights

Zebrafish were weighed after the initial surgery, on POD7, and, for the two-week protocol, on POD14. Zebrafish were anesthetized, patted dry, and weighed on a precision scale. All weights were reported as percentage of initial weight ± standard error of the mean (SEM).

2.6. Measurement of intestinal surface area

Sections from formalin-fixed, paraffin-embedded proximal intestinal segments (S1) were deparaffinized, rehydrated, and stained with hematoxylin (Sigma-Aldrich, Cat #MHS16-500 ML) and eosin (Sigma-Aldrich, Cat #HT110332-1L) then imaged on a brightfield microscope (Leica DM5500, Buffalo Grove, IL). Histological markers of intestinal adaptation were measured from representative images of a single intestinal section for each fish, including villus height (VH) and villus epithelial perimeter (VEP), as validated in our prior work [18]. Only complete villus folds were included in analysis. All measurements were performed with ImageJ software (NIH.gov) and are reported in μm±SEM.

Quantification of epithelial cell proliferation and epithelial cell death with immunofluorescent staining for BrdU and cleaved caspase-3 (CC3).

Proximal intestinal sections were stained for BrdU and CC3 as described [14,18]. Images were taken with a Leica immunofluorescent microscope (Leica DM1600B, Buffalo Grove, IL). Staining was quantified by counting the number of positively staining epithelial cells per villus fold and reported as BrdU + cells/villus fold ±SEM or CC3+ cells/villus fold±SEM.

2.7. Quantification of β-Catenin

Proximal intestinal sections were stained for β-catenin using our previously reported protocol [12]. Images were obtained with a Leica immunofluorescent microscope and staining was quantified as the number of positively staining epithelial cells per villus fold. This was reported as β-catenin + cells/villus fold±SEM.

Reverse transcriptase quantitative polymerase chain reaction for ctnnb1, ccnd1, c-myc, and yap1.

RNA was extracted from proximal intestinal segments (S1) of sham + PBS, SBS + PBS, sham + rhRSPO1 and SBS + rhRSPO1 fish with a RNeasy Micro Kit (Qiagen, Hilden, Germany, Cat. no. 74034). The RNA concentration of each sample was determined by spectroscopy with a Nanodrop2000 (Thermo Scientific, Waltham, MA). Five hundred nanograms of RNA were reverse transcribed to complementary DNA with iScript Reverse Transcription Supermix (Bio-Rad, Hercules, CA, no. 170–8841). Reverse transcriptase quantitative polymerase chain reaction was performed with SYBRgreen (Roche, Basel, Switzerland, Cat. no. 44172908) on a LightCycler 480 (Roche). Primers for *beta 1 catenin* (*ctnnb1*), *cyclin D1* (*ccnd1*), *MYC proto-oncogene* (*c-myc*) and *Yes1*

associated transcriptional regulator (*yap1*) are specified in Supplementary Table 1. Data were reported as messenger RNA fold change normalized to the housekeeping gene *glyceraldehyde 3-phosphate dehydrogenase* (*gapdh*).

2.8. Statistical analysis

All statistical analysis was performed with Graphpad Prism software (Graphpad, San Diego, CA) and included ROUT, two-way ANOVA, and one-way ANOVA.

3. Results

3.1. Repeated intraperitoneal injections are a feasible dosing strategy in zebrafish

While intraperitoneal injection has been described in zebrafish, repeated intraperitoneal injections following intra-abdominal surgery have not. In order to determine whether repeated intraperitoneal injections were feasible, we initially designed a pilot experiment in which rhRSPO1 was administered to SBS zebrafish over one week with 12 fish in each group (Fig. 1A). Zebrafish received injections starting on POD4 and received a total of three injections during the experiment (Fig. 1A). Day four was chosen because the peritoneum has healed closed by this time, so that intraperitoneal medication would not leak out of the wound. Repeated injection was well tolerated with an overall survival of 85%, and with a survival of 75% in the SBS + rhRSPO1 group, 91% in the SBS + PBS group, 83% in the sham + rhRSPO1, and 91% in the sham + PBS. This is consistent with our previously reported survival rate (67%–90%) [14,18]. At seven days, there was no difference in weight loss between any of the groups despite the additional stress of repeated injections (Fig. 1B). This is consistent with our previous studies where a difference in post-operative weight was not noted until POD14 [19,18].

3.2. SBS increases measurements of intestinal surface area at seven days

At seven days, the villus folds of the most proximal intestinal segment (S1) of both SBS + PBS and SBS + rhRSPO1 fish were larger and more complex than in the respective sham fish (Fig. 1C–F). Validated markers of intestinal adaptation, VH and VEP, were measured from histologic sections. VH was increased in SBS + PBS fish compared to sham (222.2 μm ± 9.6 vs 170.8 μm ± 10.3, $p = 0.006$), consistent with our prior work (Fig. 1G) [18]. There was no difference in VH between SBS + rhRSPO1 fish and sham + rhRSPO1 (229.8 μm ± 12.8 vs 200.3 μm ± 7.4, $p = 0.2$) (Fig. 1G). There was no difference in VH between SBS + PBS and SBS + rhRSPO1 (222.2 μm ± 9.6 vs 229.8 μm ± 12.8, $p = 0.9$) (Fig. 1G). Similarly, VEP was increased in both SBS + PBS and SBS + rhRSPO compared to respective sham groups (512.4 μm ± 19.3 vs 374

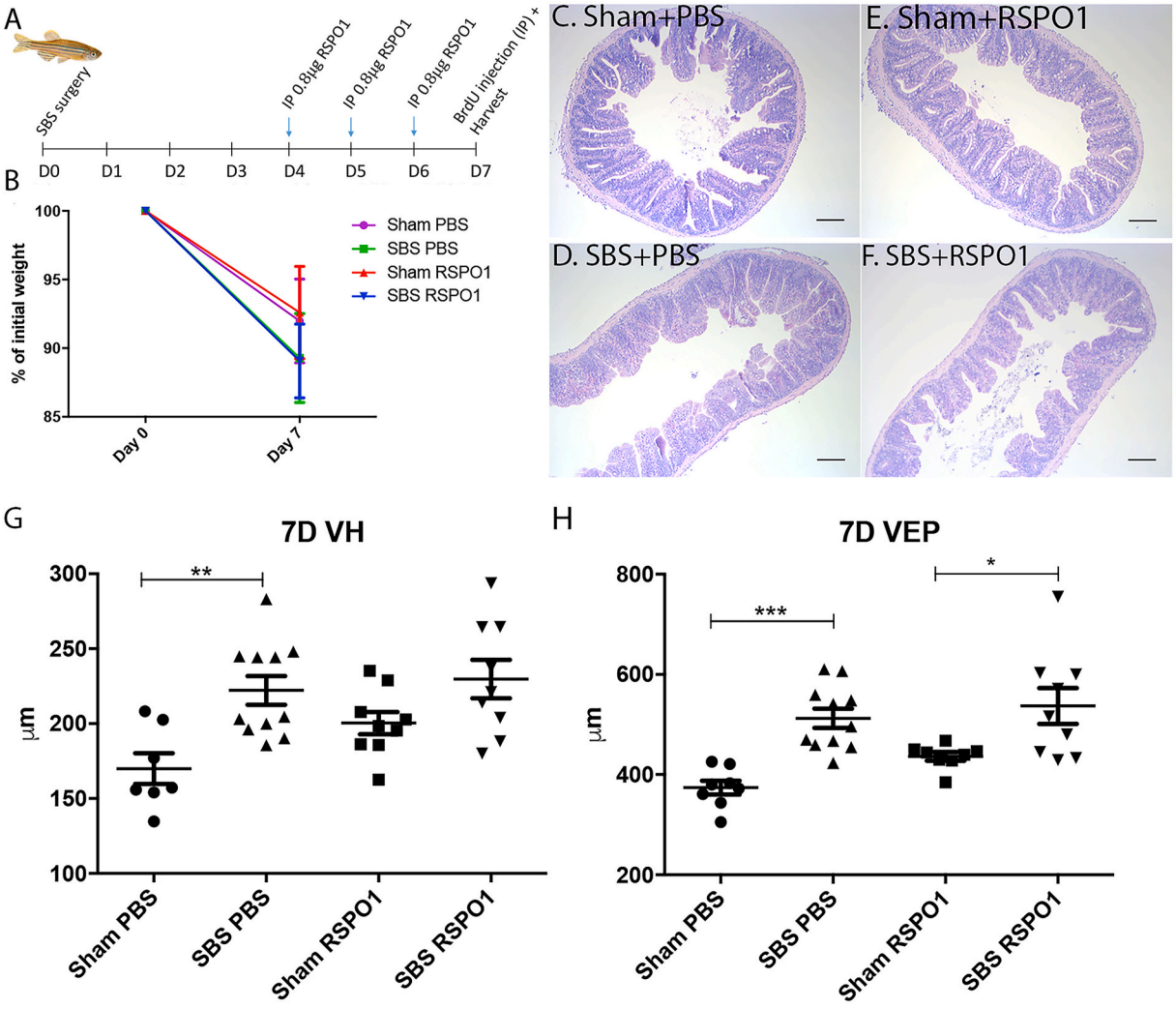

Fig. 1. Repeated intraperitoneal injections did not affect weight loss or intestinal adaptation. One-week dosing schedule for rhRSPO1 or vehicle control(A). Post-operative weights(B). Hematoxylin and eosin staining of proximal intestinal segments at 7 days from Sham + PBS(C), SBS + PBS(D), Sham + RSPO1(E), and SBS + RSPO1(F). Measurements of VH(G) and VEP(H). Scale bars 100 μm *p < 0.05, **p < 0.01, ***p < 0.001.

μm ± 13.9, $p = 0.0007$, 537.1 μm ± 35.7 vs 436.4 μm ± 8.6, $p = 0.02$, respectively) but there was no difference between the two SBS groups (512.4 μm ± 19.3 vs 537.1 μm ± 35.7, p = 0.8) (Fig. 1H).

3.3. rhRSPO1 increases intestinal epithelial cell proliferation at seven days

In the established model, SBS induces increased epithelial cell proliferation starting as early as three days post-operatively, so we next sought to evaluate how administration of rhRSPO1 would affect epithelial cell proliferation at seven days [14]. BrdU is a synthetic analog of thymidine, taken up by actively dividing cells and then incorporated into DNA. BrdU was administered to zebrafish prior to harvest. The number of positive cells was quantified by immunofluorescent staining (Fig. 2A–D). SBS + PBS fish had increased BrdU + cells/villus folds compared to sham + PBS fish (3.8 BrdU + cells/villus fold ±0.5 vs 0.66 BrdU + cells/villus fold ±0.3, $p = 0.002$), consistent with our previous findings (Fig. 2E) [18]. Similarly, SBS + rhRSPO1 fish had increased BrdU staining compared to sham + rhRSPO1 (5.8 BrdU + cells/villus fold±0.5 vs 1.4 BrdU + cells/villus fold±0.3, p = 0.0003) (Fig. 2E). Interestingly, SBS + rhRSPO1 fish had increased BrdU staining when compared to SBS + PBS fish (5.8 BrdU + cells/villus fold±0.5 vs 3.8 BrdU + cells/villus fold±0.5, p = 0.04) (Fig. 2E).

3.4. rhRSPO1 does not affect cell death at seven days

We also evaluated whether rhRSPO1 improved cell survival at seven days by immunofluorescent staining for cleaved caspase-3 (CC3), a marker of cell death (Supplementary Figs. 1A–D). There was no statistically significant difference in CC3 staining between any of the groups (Supplementary Fig. 1E).

3.5. SBS increases the number of β-catenin + cells at seven days

As rhRSPO1 potentiates Wnt signaling by activating the canonical Wnt/β-catenin pathway, we next sought to evaluate if increased β-catenin and Wnt activation accompanied increased proliferation (Fig. 2F–I). In our previous studies, we have demonstrated an increase in β-catenin staining in SBS fish at 14 days, although we had not evaluated earlier time points [12]. Intestinal segments from SBS + PBS and SBS + rhRSPO1 had both increased β-catenin + cells when compared to respective sham groups (3.26+cells/villus fold ±0.21 vs 1.01+cells/villus fold ±0.12, $p < 0.0001$), (3.25+cells/villus fold ±0.28 vs 1.39+cells/villus fold ±0.15, $p < 0.0001$) (Fig. 2J). However, there was no significant difference between the two treatments in the SBS groups (3.07+cells/villus fold ±0.23 vs 3.35+cells/villus fold ±0.28, $p = 0.8$) (Fig. 2J).

3.6. At 14 days, rhRSPO1 decreases weight loss in SBS zebrafish

Because we had established that repeated injections were well tolerated and increased epithelial cell proliferation, we next evaluated the effect of an increased number of rhRSPO1 injections over a longer period. We designed a second protocol in which zebrafish received

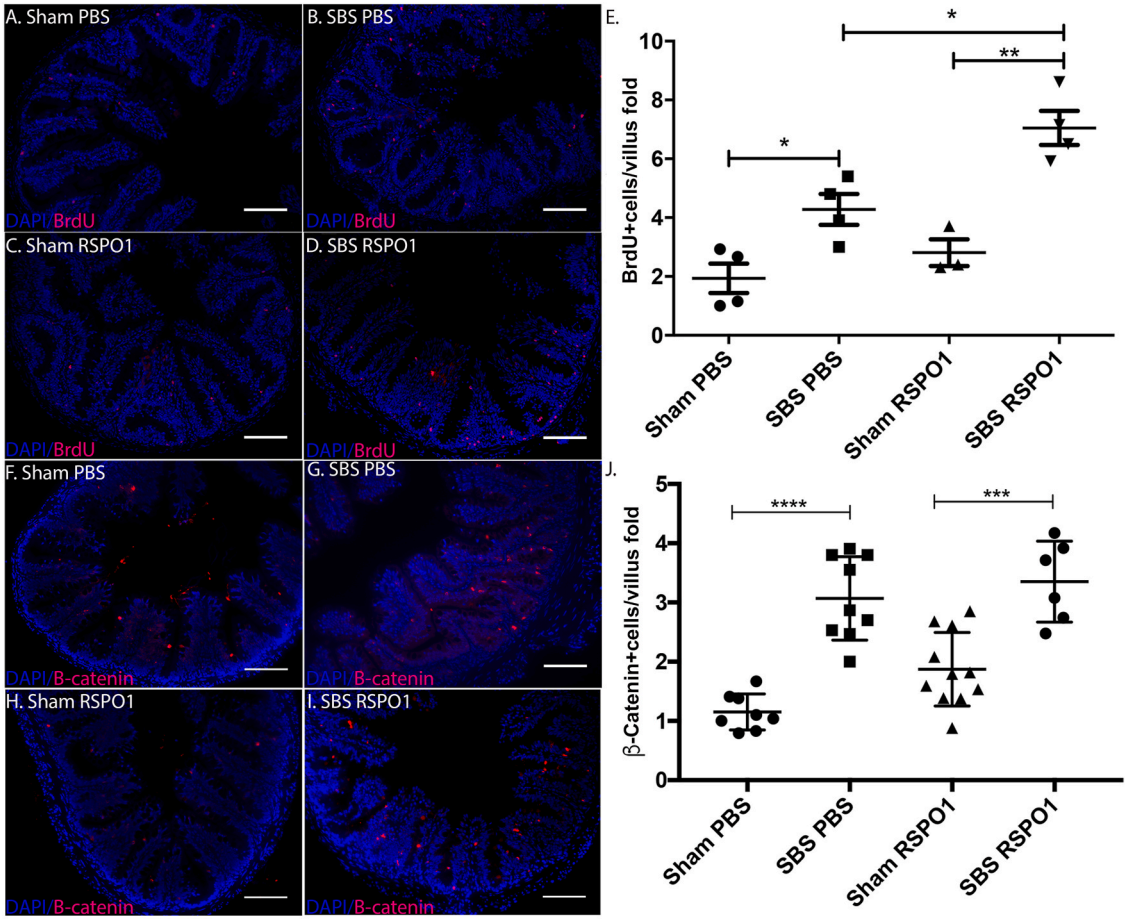

Fig. 2. Intestinal epithelial cell proliferation is increased with rhRSPO1 at 7 days. Immunofluorescent staining of proximal intestinal segments for bromodeoxyuridine (BrdU) for Sham + PBS(A), SBS + PBS(B), Sham + RSPO1(C), and SBS + RSPO1 fish(D) at POD7. Quantification of BrdU + cells/villus fold(E). Immunofluorescent staining of proximal intestinal segments for β-catenin for Sham + PBS(F), SBS + PBS(G), Sham + RSPO1(H), and SBS + RSPO1 fish(I) at POD7. Quantification of β-catenin + cells/villus fold(J). Scale bars 100 μm ***p < 0.001.

intraperitoneal injections starting on POD5 and then every other day until harvest on POD14, for a total of five injections as opposed to three in the one-week protocol (Fig. 3A). Injections were administered every other day instead of daily to allow for increased recovery time and to reduce the morbidity of repeated anesthesia. After 14 days and five injections, SBS fish that received rhRSPO1 lost 16.7% less weight than SBS fish that received PBS (89.6% ± 5.8 vs 72.9% ± 3.3, $p = 0.017$) (Fig. 3B). Consistent with previous work, SBS + PBS fish lost a significant amount of weight when compared to sham + PBS fish (72.9% ± 3.3 vs 95.4 ± 4.0, $p = 0.0002$) (Fig. 3B) [18]. There was no difference in weight loss between SBS + rhRSPO1 and either sham group (Fig. 3B).

3.7. Despite a physiologic effect, at 14 days, rhRSPO1 does not increase measurements of intestinal surface area in SBS fish

As rhRSPO1 administration decreased weight loss in SBS fish, we evaluated measurements of intestinal surface area as a marker for possible increased intestinal adaptation. Histologic sections of the most proximal intestinal segment from SBS + PBS, sham + rhRSPO1 and SBS + rhRSPO1 fish appeared to have increased villus fold complexity (Fig. 3C–H). As expected from our previous studies, VH was increased in SBS + PBS compared to sham + PBS fish (203 μm ± 6.2 vs 138.8 μm ± 3.9, $p = 0.03$) (Fig. 3G). There was no difference in VH between SBS + PBS and SBS + rhRSPO1 fish (203 μm ± 6.2 vs 196.8 μm ± 21.1, $p = 0.99$) (Fig. 3G). Interestingly, there was no difference in VH between SBS + rhRSPO1 and sham + rhRSPO1, or between sham + rhRSPO1 and SBS + PBS groups (196.8 μm ± 21.1 vs 196.4 μm ± 21.1, $p > 0.99$), (196.4 μm ± 21.1 vs 203 μm ± 6.2, $p = 0.99$) (Fig. 3G), and VH was increased in sham + rhRSPO1 compared to sham + PBS fish (196.4 μm ± 21.1 vs 138.8 μm ± 3.9, p = 0.04) (Fig. 3G). There was a similar pattern for VEP, with increased VEP in SBS + PBS compared to sham + PBS fish (458.2 μm ± 17.9 vs 325.3 μm ± 15.9, $p = 0.04$) (Fig. 3H). There was no difference between either SBS + rhRSPO1 or sham + rhRSPO1 and SBS + PBS fish (464.9 μm ± 42.7 vs 458.2 μm ± 17.9, $p = 0.99$), (440.4 μm ± 39.3 vs 458.2 μm ± 17.9, $p = 0.97$) (Fig. 3H).

3.8. Increased epithelial cell proliferation with rhRSPO1 administration does not persist at 14 days

As we did not observe increases in intestinal surface area, we then tested if the increased intestinal epithelial cell proliferation we observed at seven days persisted at 14 days (Fig. 4A–D). As in previous SBS experiments, at 14 days post-surgery, there was increased epithelial BrdU staining in SBS + PBS compared to sham + PBS fish (5.6 BrdU + cells/villus fold±1.0 vs 1.6 BrdU + cells/villus fold±0.5, $p = 0.02$) (Fig. 4E). However, there was no difference in BrdU staining between SBS + rhRSPO1 and sham + rhRSPO1 fish (4.0 BrdU + cells/villus fold±1.1 vs 2.2 BrdU + cells/villus fold±0.2, $p = 0.66$) (Fig. 4E). There was no difference between either SBS + rhRSPO1 or sham + rhRSPO1 and SBS + PBS fish (4.0 BrdU + cells/villus fold±1.1vs 5.6 BrdU + cells/villus fold±1.0, $p = 0.56$), (2.2 BrdU + cells/villus fold±0.2 vs 8.56 BrdU + cells/villus fold±1.0, $p = 0.25$) (Fig. 4E).

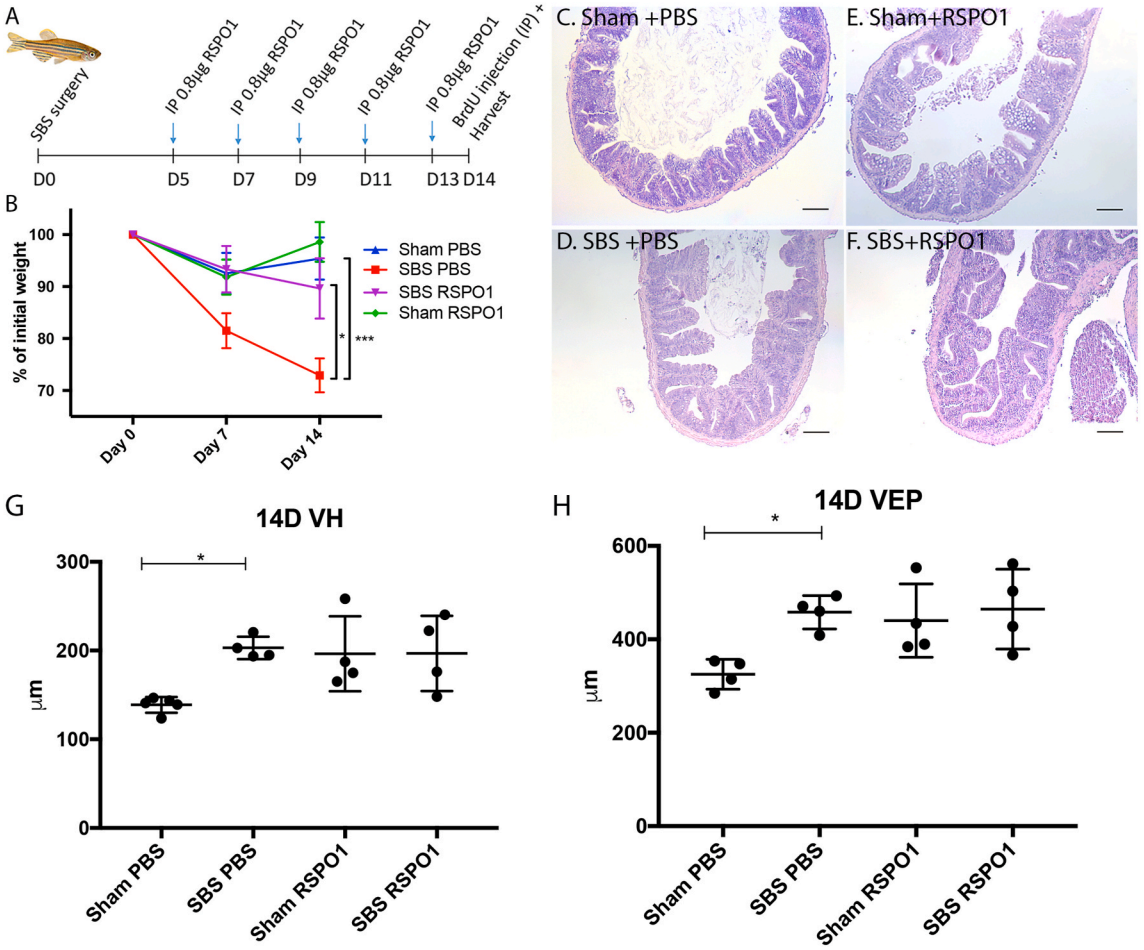

Fig. 3. Two-week dosing schedule for rhRSPO1 or vehicle control(A). Post-operative weights(B). Hematoxylin and eosin staining of proximal intestinal segments at 14 days from Sham + PBS(C), SBS + PBS(D), Sham + RSPO1(E), and SBS + RSPO1(F). Measurements of VH(G) and VEP(H). Scale bars 100 μm *p < 0.05, ***p < 0.001.

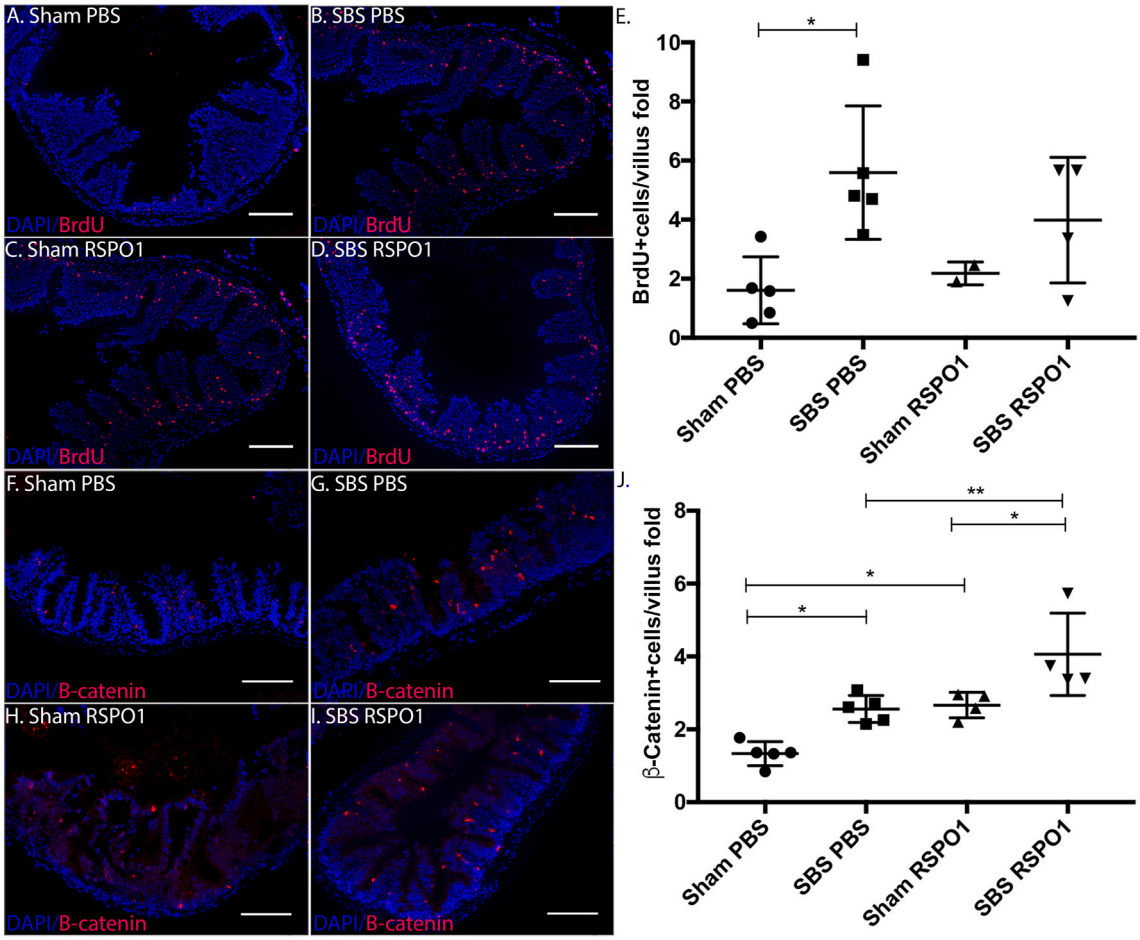

Fig. 4. Intestinal β-catenin is increased with rhRSPO1 at 14 days. Immunofluorescent staining of proximal intestinal segments for bromodeoxyuridine (BrdU) for Sham + PBS(A), SBS + PBS(B), Sham + RSPO1(C), and SBS + RSPO1(D) fish at POD14. Quantification of BrdU + cells/villus fold(E). Immunofluorescent staining of proximal intestinal segments for β-catenin for Sham + PBS(F), SBS + PBS(G), Sham + RSPO1(H), and SBS + RSPO1 fish(I) at POD14. Quantification of β-catenin + cells/villus fold(J). Scale bars 100 μm *p < 0.05, **p < 0.01.

3.9. rhRSPO1 administration increases β-catenin staining at 14 days in SBS

In order to determine how weight maintenance was improved in SBS + rhRSPO1 fish without a measurable increase in intestinal surface area, we next looked at β-catenin staining to identify whether there was increased activation of the Wnt pathway at a longer time point and with an increased number of rhRSPO1 injections (Fig. 4F–I). Again, there was an increased number of β-catenin + cells in SBS + PBS compared to sham + PBS fish (2.66+cells/villus fold ±0.16 vs 1.33+cells/villus fold ±0.14, $p = 0.03$) (Fig. 4J), consistent with previous studies. β-catenin + cells in SBS + rhRSPO1 were increased compared to sham + rhRSPO1 (4.06+cells/villus fold ±0.56 vs 2.7+cells/villus fold±0.17, $p = 0.02$) (Fig. 4J). There was also increased β-catenin + cells in SBS + rhRSPO1 compared to SBS + PBS fish (4.06+cells/villus fold ±0.56 vs 2.66+cells/villus fold ±0.16, $p = 0.01$) (Fig. 4J), indicating increased activation of the Wnt pathway.

3.10. rhRSPO1 administration increases ccnd1 gene expression at 14 days

We further evaluated the effect of rhRSPO1 on Wnt pathway targets *ctnnb1, ccnd1, c-myc, and yap1* in the proximal intestine of fish at 14 days. There was increased mRNA expression of *ccnd1* in SBS + PBS compared to SBS + rhRSPO, and sham + rhRSPO compared to SBS + rhRSPO groups (3.1-fold increase, p = 0.0049, and 2.7-fold increase, p = 0.03, respectively) (Fig. 5B). There was no significant difference identified between the other groups for *ccnd1* (sham + rhRSPO vs. sham + PBS, p = 0.99, and SBS + PBS vs. sham + PBS, p = 0.8) or for additional genes *ctnnb1, c-myc, and yap1* (Fig. 5).

3.11. rhRSPO1 does not affect cell death at 14 days

Finally, the samples were subjected to immunofluorescent staining for CC3 at 14 days (Supplementary Figs. 2A–D) to determine relative amounts of apoptotic cell death. There was no difference in CC3 staining at 14 days between any of the groups (Supplementary Fig. 2E).

4. Discussion

Recombinant human R-spondin1 may have future applications as a short-term postoperative therapy to improve intestinal adaptation and diminish immediate weight loss after massive bowel resection as it did in these preliminary results. RSPOs have been considered as possible therapeutics for intestinal disease processes in which there is injury and regeneration [11]. Here, we report for the first time that repeated intraperitoneal injection after intestinal resection is a feasible method for medication administration in zebrafish. Furthermore, intraperitoneal administration of rhRSPO1 decreased weight loss in SBS zebrafish (Fig. 3B) with concurrent increase in β-catenin + cells (Fig. 4J) and *cyclin D1* gene expression in the intestine (Fig. 5B). Taken together, this implies that there is increased activation of the Wnt signaling pathway,

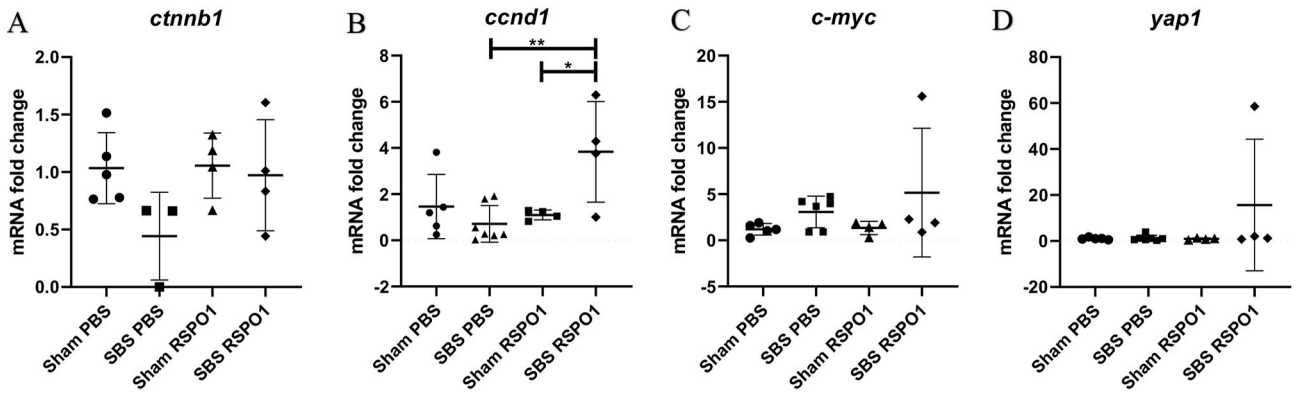

Fig. 5. Intestinal *ccnd1* gene expression is increased with rhRSPO1 at 14 days. RT-qPCR of proximal intestine messenger RNA for *ctnnb1*(A), *ccnd1*(B), *c-myc*(C) and *yap1*(D). *p < 0.05, **p < 0.005.

possibly multiplicative with the increase in β-catenin that is already induced by SBS (Fig. 4I) [20,12].

At one week, repeated intraperitoneal injections did not affect survival or weight loss, concordant with our previous studies [18,20]. However, after 14 days, rhRSPO1 ameliorates the weight loss associated with SBS in zebrafish. After 14 days with a total of five doses of rhRSPO1, SBS zebrafish lost significantly less weight than SBS zebrafish that received vehicle control alone and had equivalent weights to sham-operated fish (Fig. 3B). While there was no difference in weight loss in the shorter protocol with three doses of rhRSPO1, there was increased intestinal epithelial cell proliferation demonstrated by increased BrdU-positive cells in the intestinal epithelium in SBS + rhRSPO1 fish compared to SBS + PBS fish (Fig. 2E). However, this increase was no longer present at 14 days (Fig. 4E). Yet in our original model, BrdU-positive cells were still increased at this time point [18]. One explanation may be that rhRSPO1 administration increases proliferation initially but by 14 days after repeated rhRSPO1 doses, the proliferative response to SBS may already be maximized. Administration of rhRSPO1 did not alter the number of CC3-positive cells per villus fold, and thus does not change relative events of cell death.

Similarly, measurements of intestinal surface area, markers for intestinal adaptation, while increased following intestinal resection, were not different after administration of rhRSPO1 at either time point. However, at 14 days and five injections of rhRSPO1 there did appear to be an increase in villus fold complexity in the sham + rhRSPO1 fish as there was no difference in VH or VEP between sham + rhRSPO1 and either SBS group. VH was significantly increased in sham + rhRSPO1 fish, indicating that administration of rhRSPO1 does increase intestinal surface area to some extent (Fig. 3G).

Administration of the same recombinant human RSPO1 to adult BALB/c mice without prior resection or intestinal injury also resulted in increased crypt-villus height [12]. Yan et al. previously identified over-expression of rhRSPO1 alone in mice expands Lgr5+ ISCs within the crypt but does not induce villus hypertrophy unless there is concomitant administration of a Wnt ligand, suggesting Wnt ligand priming of ISCs is necessary for the proliferative response of ISCs to rhRSPO1 [8]. This priming may also be induced by massive intestinal resection, but because there is already a marked increase in intestinal surface area, further increases may not be possible with administration of rhRSPO1 alone.

Although there was no demonstrable statistical difference in measurements of intestinal surface area, intestinal epithelial cell proliferation was increased at seven days after surgery but not at 14 days (Figs. 2E and 4E). Conversely, β-catenin staining was not significantly increased by rhRSPO1 administration until POD14 (Fig. 4I). Previously, we have found that intestinal epithelial cell proliferation occurs as early as three days, peaks around 7–14 days, then returns to baseline by postoperative day 30 [14]. rhRSPO1 administration increases this early epithelial cell proliferation, which may help to preserve weight through early expansion of the zebrafish ISC population.

Overexpression of R-spondin1 in mice increased the number of Lgr5+ ISCs within the crypt but, as demonstrated by lineage tracing studies, did not increase proliferation of these cells [8]. In this study, there was a significant increase in *ccnd1* at 14 days in fish treated with rhRSPO1 but no change was identified in other Wnt targets, including *ctnnb1, c-myc, and yap1* (Fig. 5). In other studies, there was no significant increase of *ccnd1* reported after RSPO1 administration in mouse intestinal organoid units or in the arcuate nucleus after LGR4 knockout [12,21]. Although there was no observed difference in apoptotic cell death, expansion of the yet unidentified zebrafish intestinal stem cell may improve efficiency of replacement of cellular shedding from villus tips.

As intestinal surface area was not increased, the exact mechanism by which rhRSPO1 administration decreased weight loss in SBS remains to be elucidated. Part of this mechanism may result from increased Wnt activation with increased gene expression of the downstream target *ccnd1*. Or, the cellular composition of the intestinal epithelium may be altered to improve efficiency of nutritional uptake. In models of ovarian and colorectal cancer, Wnt signaling has been implicated in changes of cellular metabolism, particularly with respect to glucose metabolism and fatty acid oxidation [22,23]. LRP5 and LRP6, co-receptors that form a complex with Wnt and FZD, have also been postulated to interact with R-spondins and have both been implicated in lipid homeostasis and glucose metabolism [24,25].

The canonical Wnt/β-catenin pathway is crucial for maintenance of the ISC niche, intestinal epithelial homeostasis, and epithelial regeneration following injury. Here we have shown that administration of rhRSPO1 increases accumulation of β-catenin and the expression of *ccnd1* and improves weight loss after the surgical induction of short bowel syndrome, implying sufficient homology between zebrafish and rhRSPO1 for a measurable biologic effect. Improved understanding of the complex signaling pathways that drive intestinal adaptation following SBS might help to identify future human therapies.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2020.100874.

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