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Adult zebrafish intestine resection: a novel model of short bowel syndrome, adaptation, and intestinal stem cell regeneration


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Schall KA, Holoyda KA, Grant CN, Levin DE, Torres ER, Maxwell A, Pollack HA, Moats RA, Frey MR, Darehzereshki A, Al Alam D, Lien C, Grikscheit TC. Adult zebrafish intestine resection: a novel model of short bowel syndrome, adaptation, and intestinal stem cell regeneration. Am J Physiol Gastrointest Liver Physiol 309: G135–G145, 2015. First published June 18, 2015; doi:10.1152/ajpgi.00311.2014.—Loss of significant intestinal length from congenital anomaly or disease may lead to short bowel syndrome (SBS); intestinal failure may be partially offset by a gain in epithelial surface area, termed adaptation. Current in vivo models of SBS are costly and technically challenging. Operative times and survival rates have slowed extension to transgenic models. We created a new reproducible in vivo model of SBS in zebrafish, a tractable vertebrate model, to facilitate investigation of the mechanisms of intestinal adaptation. Proximal intestinal diversion at segment 1 (S1, equivalent to jejunum) was performed in adult male zebrafish. SBS fish emptied distal intestinal contents via stoma as in the human disease. After 2 wk, S1 was dilated compared with controls and villus ridges had increased complexity, contributing to greater villus epithelial perimeter. The number of inter villus pockets, the intestinal stem cell zone of the zebrafish increased and contained a higher number of bromodeoxyuridine (BrDU)-labeled cells after 2 wk of SBS. EGF receptor and a subset of its ligands, also drivers of intestinal adaptation, were upregulated in SBS fish. IGF has been reported as a driver of intestinal adaptation, a poorly understood response in which the epithelial surface area is expanded. This is a critical response for human patients to enhance the absorption of enteral nutrition and to reduce the need for TPN, but in many patients adaptation is inadequate. In vivo modeling of adaptation is described for mice and larger mammals (45). These models involve massive small bowel resection and are complicated, time consuming, and associated with low survival and high cost. Long-term end points are difficult to achieve. For these reasons, adaptation is still poorly understood. An im-

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proved model of intestinal adaptation would allow the identification of key factors for future human therapies.

Zebrafish (Danio rerio) is an ideal organism for the study of vertebrate intestine and is equivalent to the mouse with regards to precise genome modification (58). Zebrafish intestine is subdivided into seven segments of equal length that are strikingly similar to mammalian correlates. The proximal five subdivisions (S1–S5) are homologous to the regions of human small intestine and express genes related to metabolism of fatty acid, organic acid, lipid, vitamins, and carbohydrates as well as genes associated with the hydrolyase and transferase activity of absorption. The distal segments (S6, S7) demonstrate ion transport and fecal dehydrolysis as in mammalian colon and rectum (51). In contrast to mammalian intestine, zebrafish lack a stomach and the defense-secretory Paneth cell (29, 49). The zebrafish also lacks the fingerlike projections and microvilli of mammalian villi. Instead, S1–S5 contain wide folds called villus ridges that are composed of enterocytes, goblet cells, and enteroendocrine cells in similar ratios to human mucosa. The zebrafish intestinal stem cells are found in the valleys between villus ridges, known as the intervillus pockets (49).

To date, few techniques have been described for the manipulation or response to injury of the adult zebrafish. Success with tail fin amputation, retroorbital injections, pancreatectomy, and injection of cells directly into the kidney via lateral incision have been accomplished (1, 7, 28, 37). However, survival surgery following resection and diversion of the adult intestine has not been reported. Our method is novel in modeling human SBS and as a proof of the feasibility of adult zebrafish intraperitoneal surgery for investigation of a human disease that demands improved therapies.

MATERIALS AND METHODS

The Children’s Hospital Los Angeles animal care facility and IACUC approved all protocols.

Generation of short bowel syndrome. One-year-old, male zebrafish (wild type, Ekk) were obtained for surgery. Each fish was anesthetized with 0.02% tricaine and placed in a solution consisting of 10 mg strychnine (Sigma) and 1 mg dantrolene (Sigma) in 100 ml system water. After 15 min in solution, the fish were immediately imaged by using a Skyscan 1,172 micro-CT (Bruker). The optimal settings were determined to be 36 kVP, 161 μAmp, 0.5° steps for 360°, and two averages with an aluminum filter at a resolution of 2.07 μm. The fish were scanned for 90 min. The Bruker Skycan 1172 software was used for reconstruction, by a Feldkamp-type algorithm running on an array of processors supplied by Bruker. After assessment of images, 3D reconstruction was performed with Mimris (Mimris), Amira (FEI) and Lightwave (Newtek) software running on dedicated Windows-based workstations.

Determination of BrdU incorporation. At 2 wk, sham (n = 9) and SBS (n = 14) fish underwent intraperitoneal injection with 30 μl of 2.5 mg/ml bromodeoxyuridine (BrdU) (Sigma Life Sciences B5022-1G) and were euthanized after 4 h. At 4 wk, sham (n = 13) and SBS (n = 13) were similarly injected. S1 was harvested, fixed in formalin, and paraffin embedded. Antigen retrieval was performed by boiling the slides for 12 min in 10 mM N-citrate (pH 6.0). Samples were incubated overnight at 4°C with primary antibody anti-BrdU (1/100, BD Bioscience). Slides were washed and incubated with secondary antibody Cy3 goat anti-mouse IgG (1/200, Life Technologies), then counterstained with 4,6-diamidino-2-phenylindole (DAPI; Vector), mounted with ProLong Gold (Life Technologies), and imaged under an upright immunofluorescent microscope. All nuclei positive for BrdU incorporation were counted per semi-intervillus pocket to the tip of the villus ridge and represented as a percentage of all DAPI-stained epithelial cell nuclei. All areas with a complete intervillus pocket and villus ridge were included for analysis. Counts were performed on sham and SBS fish by a single trained, blinded observer to prevent interobserver variations. Data were expressed as a percentage of BrdU-positive cells per total epithelial cells ± SE (significance, P < 0.05).

Igf1r inhibition experiment. To determine whether the inhibition of Igf1r reduced intestinal adaptation, sham and SBS fish were exposed to 5 mM concentration of the Igf1r inhibitor NVP-AEW541 (sham n = 12, SBS n = 13) (Cayman Chemical 13541) or vehicle control DMSO (sham n = 18, SBS n = 14) (Sigma Life Sciences D2650) administered in system water and refreshed every 48 h for 2 wk following surgery. Intraperitoneal BrU (Sigma Life Sciences B5002-1G) was injected 4 h prior to euthanasia. S1 was collected for histology and quantitative PCR to determine the relative expression of the ligands igf1a, igf1b, igf2a, and igf2b, as well as the receptor igf1r.

Based on hematoxylin and eosin staining on complete cross-sectional samples, the inner epithelial perimeter and circumference were measured manually by outlining the luminal surface of the epithelium and the outer serosal portion of the sample, respectively, with ImageJ by a trained, blinded observer.

Determination of Alcian blue quantification. Slides were stained with Alcian blue and counterstained with Nuclear fast red [0.2 g Nuclear fast red (Kernechtrot), 10 g of aluminum sulfate (Sigma-Aldrich), 100 ml deionized water]. A single trained, blinded observer counted the total number of nuclei and the number of Alcian blue-positive cells per semi-intervillus pocket to the tip of the villus ridge within a complete cross section of S1 for each fish. Data were expressed as means ± SE (significance, P < 0.05).

Quantitative real-time PCR. In the Egf PCR, without Igf1r inhibitor, S1 segments were resected from sham (n = 6) and SBS (n = 7) fish at 2 wk postsurgery. In the Igf experiment, S1 segments were resected from sham (n = 9) and SBS (n = 19) fish at 2 wk postsurgery. The RNA was extracted by using Qiagen RNeasy minin Kit. RNA concentration was determined by use of NanoDrop (Thermo Scientific). RNA integrity was determined on a bioanalyzer (Agilent technologies). All samples had a RNA integrity number of at least 8.1.
One microgram of RNA was reverse-transcribed to cDNA and quantitative PCR (qPCR) was carried out on Roche LightCycler 480 reagents by using SYBR Green and the following primers: egf F: 5’GAACAAGGGCTAAAGGATTGC, R: 5’GATGCAAGCAGTTAGGCT; efg F: 5’TCTTACTTCTGACCTGTTCTG, R: 5’ATGACATATCCACACGTTTC; bte F: 5’CCCCAGCAGTAGATGTTGTTT, R: 5’ACTTGGGTCTTGGTGTGACT; bbe F: 5’ATTTCTGACATTGAGGTTGTTGACT; bbt F: 5’GGGCTAGGGGCTGAATGTTGTTT, R: 5’CCAGTGAAGGGTGTGGTGA; igtla: F: 5’CCTTTGCTTATTTGATGAATC, R: 5’AGACATTTTTCACAGGAAACGC; igtla2: F: 5’TGAAGTGGGGAGCAAGGTTGGAGT, R: 5’GGGAGAAGCTTGGTGGACGCTCTACA, R: 5’AGAACGTGGCAATCACCACATCG; igtlb: F: 5’AGCTGTTGAGCCTGCTACAG, R: 5’GAAGAAGCTGGACTTGGACCT; igtlr: F: 5’AGACATTTTTCACAGGAAACGC; igtlb2: F: 5’TGAAGTGGGGAGCAAGGTTGGAGT, R: 5’GGGAGAAGCTTGGTGGACGCTCTACA, R: 5’AGAACGTGGCAATCACCACATCG.

For all samples, the expression level was determined by using Roche LightCycler 480 software release 1.5.0. Each PCR reaction was run in triplicate, and single outliers that occurred in the technical replicates were removed for quantification. Relative expression of the genes investigated compared with the housekeeping gene ef1a was determined by 2\(^{-\Delta\Delta CT}\) algorithm. All the samples were normalized to the resected S2 segment.

Statistical analyses. Quantifications were performed on sham and SBS fish by a single trained, blinded observer to prevent interobserver variations. Cell proliferation was expressed as a percentage of BrdU-positive cells per total epithelial cells per hemivillus ± SE. Goblet cell quantification was expressed as a percentage of Alcian blue-positive cells per total nuclei per hemivillus ± SE. Outliers were determined by the ROUT method with the value of Q set to 0.5%. Statistical significance was determined by one-way ANOVA or Student’s t-test in Prism software.

RESULTS

Intestinal resection in the zebrafish model is fast and technically straightforward. On average, the SBS procedure took 329.6 s (5.5 min) vs. 17.6 s for sham. The procedure is presented step by step in Supplementary Video S1 (Supplemental Material for this article is available online at the Journal website). Each fish was anesthetized in 0.02% tricaine diluted in system water for ~3 min until fin motions ceased. The fish was then placed supine (dorsal fin down) on a moist operating sponge beneath a stereomicroscope. The peritoneal cavity was entered with microscissors through a 3-mm midline incision (laparotomy) started immediately cephalad to the origin of the anal fins (Fig. 1Ai; Supplemental Material for this article is available online at the Journal website). The liver overlying the intestine was gently swept cephalad and the segment 2 (S2) loop of intestine was grasped with microforceps (Fig. 1Aii). A 22-gauge bare copper wire was inserted underneath S2 to prevent reduction of intestine back into the abdomen (Fig. 1Aiii). The proximal intestine was sutured to the body wall with a single 0-0 monofilament polypropylene suture (Fig. 1Aiv) and the distal intestine was suture ligated (Fig. 1Av). The close anatomic proximity of the swim bladder to the distal intestine precludes complete resection of the distal intestine. S2 and S3 were excised, leaving behind the shortened proximal segment 1 (S1) as a functional proximal ostomy. The distal segment was reduced back into the abdomen (Fig. 1Avi). Sham operation consisted of laparotomy alone with no intestinal manipulation (Fig. 1Ai). The ventral laparotomy incision did not require surgical closure and healed around the open stoma (Fig. 2, A–C). Of note, in the subsequent inhibitor experiments, the incision did not heal in the sham or SBS groups by 2 wk.

Fig. 1. Zebrafish with short bowel syndrome (SBS) lose significantly more weight compared with sham-operated fish. A: SBS operation. B: Ventral laparotomy incision (solid black arrowhead). The head of the fish is located at the top of the image, while the tail is oriented at the bottom. C: micro-CT of intestine following sham operation with proximal intestinal incision at the bottom of the image. D: micro-CT intestine following sham operation with proximal intestinal incision at the top and distal intestinal incision at the bottom of the image. Sham intestine is intact with normal caliber. C: micro-CT of intestine 2 wk after SBS surgery. The ostomy is present at the bottom of the image with the proximal intestine profoundly dilated. Scale bar = 500 µm. D: Percentages of preoperative weight were plotted at each weekly time point with the surviving fish euthanized at 2 and 4 wk. Zebrafish with SBS lose a significant amount of weight compared with sham-operated fish. Error bars indicate SE.
2–4 wk following surgery, the fish were weighed each week and monitored for loss of body weight, a common indication of malabsorption and malnutrition related to SBS. The change in body weight was expressed as percentage of the preoperative body weight ± SE. At 2 wk, SBS fish weighed 87.77 ± 1.33% of preoperative body weight compared with sham fish, which weighed 99.76 ± 0.94% of preoperative body wt \( P < 0.01 \) (Fig. 1D). Following a period of 2 wk, the majority of fish were euthanized and dissected for morphological evaluation of the proximal intestine. Another large cohort of zebrafish were followed and euthanized at 4 wk. Whereas the sham fish gained weight over the 4-wk period, SBS fish continued to lose weight, reaching a nadir of 85.91 ± 1.28% preoperative body weight compared with sham fish that weighed an average of 103.14 ± 1.38% body wt \( P < 0.01 \) (Fig. 1D). The remnant proximal intestine, or ostomy, at the ventral surface of the skin of the experimental group of fish was visible and patent as seen grossly and on histological cross section (Fig. 2, A–C).

Micro-CT was performed to allow for better visualization of the intestinal lumen with the sham fish having completely intact native intestine with normal caliber (Fig. 1B), whereas the SBS fish demonstrated profoundly dilated proximal intestine with an end stoma (Fig. 1C). Further evaluation of the SBS surgery model included three-dimensional reconstructions of the sham and SBS Micro-CT with renderings at various angles included in Supplemental Videos S2 and S3, respectively.

Following dissection of the abdominal cavity at 2 wk, S1 of the SBS fish was considerably hypertrophied and dilated compared with the controls. In contrast, the distal bowel in SBS fish was small in size and had no visible luminal content (Fig. 2D). In addition to the proximal segment dilation, lack of luminal content in the distal remnant intestine confirmed that the intestine had not reconnected, or formed a fistula, following the operation. All SBS fish demonstrated this proximal dilation and remnant decompression at the time of dissection. The intestine of sham fish remained normal in caliber with food content visible throughout (Fig. 2E).

Hematoxylin and eosin staining demonstrated increased complexity of each villus ridge in SBS compared with sham (Fig. 3, A–D), contributing to a greater villus epithelial perimeter (Fig. 3E). At 2 wk, the villus epithelial perimeter was significantly longer in the SBS group compared with sham (412.36 ± 13.76 vs. 348.85 ± 14.64 \( \mu \)m; \( P < 0.01 \), Fig. 3E). Similarly, the inner epithelial perimeter was significantly longer in the SBS group compared with sham (11,919.68 ± 1,899.65 vs. 7,146.05 ± 369.88 \( \mu \)m; \( P < 0.01 \)). At 4 wk, the villus epithelial perimeter, the inner epithelial perimeter, and the overall circumference of the proximal intestine were significantly increased in the SBS compared with sham fish. Each individual villus was also more complex in the SBS fish at 4 wk with a mean villus epithelial perimeter of 486.40 ± 12.10 \( \mu \)m compared with 352.36 ± 9.50 \( \mu \)m in sham fish \( P < 0.01 \) (Fig. 3E). Similarly, the inner epithelial perimeter was also significantly greater in SBS fish compared with sham fish, with means of 13,265.39 ± 1,429.89 vs. 8,337.63 ± 338.313 \( \mu \)m, respectively \( P < 0.01 \). The mean circumference of the proximal intestine was 4,038.98 ± 255.492 \( \mu \)m for SBS fish vs. 2,719.66 ± 118.971 \( \mu \)m in sham fish \( P < 0.01 \).

Response to intestinal truncation in zebrafish short bowel surgery is similar to that in humans. At 2 wk following SBS surgery, the S1 segment becomes significantly more dilated in SBS (Fig. 1C) compared with sham (Fig. 1B) fish. In the demonstrating the global inhibitor effect, but survival was not affected. Survival rates in these untreated groups were 90.7% for sham (N = 39/43) and 66.2% for SBS (N = 43/65), with deaths predominantly in the first week coincident with rapid weight loss. In an additional control group to verify that this is not a model of intestinal obstruction, the reversed surgery was performed in 10 fish: a proximal ligation and distal stoma. In these zebrafish, we caused complete obstruction of the intestine. We performed postmortem examinations on all zebrafish. Most zebrafish that are obstructed as a result of a proximal ligation die early in the postoperative period. These animals were all confirmed to be obstructed, with hugely dilated intestines (larger than in the SBS model at the same time point) and no fistula or connection to the distal bowel. In two animals that did survive complete obstruction through proximal ligation, a fistula that had formed to the distal bowel was identified. Obstruction and therefore elevated proximal pressure is a known risk factor for fistula formation. We do not identify fistula forming in the SBS model, which further validates that there is egress of intestinal contents via the stoma, and therefore nonpathological pressures in the proximal intestine.
Increased proliferation in the stem cell zone following intestinal resection is associated with increased villus epithelial perimeter. The intervillus pocket is the intestinal stem cell zone of the zebrafish, corresponding to the human intestinal crypt (49). Severely decreased intestinal length available to absorb nutrition through the creation of a proximal stoma led to a significant increase in the villus epithelial perimeter of the proximal intestine. To investigate whether there was an increase in the number of proliferating cells, epithelial cells positive for BrdU incorporation, injected 4 h prior to harvest, were quantified as a percentage of total epithelial cells along the hemi-intervillus pocket to tip of villus ridge axis. The percent of BrdU-positive epithelial cells was tripled in SBS (Fig. 4B) compared with sham (Fig. 4A) fish at 2 wk (6.36 ± 0.80 vs. 1.84 ± 0.19%; P < 0.01, Fig. 4E) and localized predominantly to the intervillus stem cell zone. The percent of BrdU-positive epithelial cells did not remain increased at the 4-wk time point. At 4 wk, there was no statistically significant

Fig. 3. The villus epithelial perimeter is significantly increased in SBS fish, suggesting increased adaptation. The sham fish at 2 wk (A) and 4 wk (C) postoperatively vs. SBS fish at 2 wk (B) and 4 wk (D) postoperatively. S1 segments were harvested and stained with hematoxylin and eosin, and composite ×10 images were tiled for a complete cross section. E: quantification of villus epithelial perimeter (μm) of the sham and SBS groups at 2 and 4 wk. Results are reported as means ± SE. Scale bar = 200 μm. *P < 0.01.

Fig. 4. Cell proliferation increased 2 wk after SBS. A–D: immunofluorescence (IF) staining showing bromodeoxyuridine (BrdU)-labeled cells (in red) in sham fish at 2 wk (A) and 4 wk (C) postoperative vs. SBS fish at 2 wk (B) and 4 wk (D) postoperative. E: quantification of BrdU-positive cells as a percentage of total epithelial cells per hemivillus ridge at 2 wk and 4 wk postoperative. Results are reported as means ± SE. Scale bar = 50 μm. *P < 0.01.
Intestinal adaptation following short bowel resection is associated with increased mRNA expression of epidermal growth factor receptor, as well as its ligands epidermal growth factor and betacellulin. Studies in rodent models show that epidermal growth factor (EGF) receptor (EGFR) signaling plays a critical role in intestinal adaptation following either resection or fasting/refeeding (4, 8, 16). EGFR ligand expression increases following resection in mammals, and exogenous EGFR ligand administration amplifies intestinal adaptation (6, 31). To test whether this ligand-receptor system is also regulated during adaptation in zebrafish, RNA isolated from the proximal intestine of sham and SBS zebrafish was subjected to RT-qPCR analysis for egfr and the ligands egf, bte, hb-egfa, and hb-egfb. There was a 13.5-fold increase in expression of egfr in the SBS proximal intestine compared with sham fish (P < 0.01) (Fig. 5A). We also found 71.2-fold and 9.6-fold increases in expression of egf and bte (P < 0.05 and P < 0.01), respectively (Fig. 5, A–C); in contrast, there were no differences in hb-egfa or hb-egfb expression with experimental SBS (data not shown).

Intestinal adaptation following short bowel syndrome is associated with increased mRNA expression of insulin-like growth factor receptor (Igf1r) ligands igf1a and igf2a, and Igf1r inhibitor abrogates intestinal adaptation after intestinal resection in zebrafish. Insulin-like growth factor (Igf) has been shown in previously described animal models to promote intestinal adaptation (23, 47). To investigate whether Igf was an effector of the phenotype observed in our model, we treated sham or SBS fish with the Igf1r inhibitor NVP-AEW541, with the required solvent DMSO alone applied to the control sham and SBS fish. RT-qPCR results were measured as a ratio of the housekeeping gene, ef1a, demonstrating that there were no differences in Igf receptor or ligand expression in sham fish with or without inhibitor treatment. For SBS fish, there was no significant difference between SBS/inhibitor and SBS/DMSO-treated fish in the mRNA quantification of ligands igf1b, and igf2b, or the receptor igf1r by RT-qPCR analysis (data not shown). Compared with the intestine resected during the surgery to create the SBS fish (S2 and S3), SBS/DMSO fish had a 6.6-fold change increase in igf1a expression (P < 0.05, Fig. 5D) and a 3.9-fold increase in igf2a expression (P < 0.05, Fig. 5E) compared with S2 and S3. This difference was not observed in SBS fish when treated with inhibitor. Compared with SBS/DMSO, SBS/inhibitor fish had decreased igf1a (P < 0.01, Fig. 5D) and igf2a (P < 0.05, Fig. 5E) expression; in the presence of the inhibitor, igf1a and igf2a expression were not increased. In addition, fish did not heal their laparotomy wounds in the presence of the inhibitor, whereas those only exposed to DMSO did close their wounds, indicating a definite biological effect. Consistent with the initial experiments, inhibitor-treated SBS fish demonstrated greater weight loss than inhibitor-treated sham fish (losing 23.9 vs. 5.2% initial body weight). SBS fish treated with inhibitor also lost more weight than SBS fish exposed only to the vehicle control DMSO (losing 12.9 vs. 5.2% initial body weight) (Fig. 6E).
In addition to visually dilated bowel, increased villus ridge complexity resulted in an increased inner epithelial perimeter in SBS/DMSO (Fig. 6B) compared with sham/DMSO (Fig. 6A) (15,423 ± 1,362 vs. 9,369 ± 559 μm, P < 0.01, Fig. 6F) and SBS/DMSO (Fig. 6B) compared with SBS/inhibitor (Fig. 6D) (15,423 ± 1,362 vs. 10,498 ± 777 μm, P = 0.01, Fig. 6F). This increase was not demonstrated in measurements of the inner epithelial perimeter for the SBS/inhibitor zebrafish compared with sham/inhibitor. The serosal circumference is significantly increased in SBS/DMSO compared with sham/DMSO fish (5,213 ± 465 vs. 3,303 ± 46 μm, P = 0.05, Fig. 6G). With the addition of Igf inhibitor, this effect is blunted and there is no significant increase in circumference of the intestine between SBS/inhibitor and sham/inhibitor (3,694 ± 308 vs. 3,274 ± 249 μm, P > 0.05, Fig. 6G).

As in the initial experiments conducted in water alone, the percentage of BrdU-positive cells increased significantly in SBS/DMSO (Fig. 7B) compared with sham/DMSO (Fig. 7A) fish (3.9 vs. 0.5%, P = 0.01, Fig. 7E). There was also a significant increase in sham/inhibitor (Fig. 7C) compared with sham/DMSO (Fig. 7A) fish (2.1 vs. 0.5%, P < 0.05, Fig. 7E). However, there was no significant difference in the percentage of BrdU-positive cells between the SBS/DMSO (Fig. 7B) and SBS/inhibitor (Fig. 7D). There was also no significant difference between the sham/inhibitor (Fig. 7C) and SBS/inhibitor (Fig. 7D) groups, indicating that the inhibitor blocked adaptation in the SBS group.

There was no significant change in the percentage of Alcian blue-positive epithelial cells in SBS/DMSO (Fig. 7G) compared with sham/DMSO (Fig. 7F) (6.8 ± 1.6 vs. 6.8 ± 0.8%; P = 0.99, Fig. 7J). There was a significant increase in percentage of Alcian blue-positive cells per hemivillus in sham/inhibitor (Fig. 7H) compared with sham/DMSO (Fig. 7F) (9.7 ± 0.6 vs. 6.8 ± 0.8%; P = 0.01, Fig. 7J). The SBS/inhibitor (Fig. 7I) appeared to demonstrate an increased percentage of Alcian blue-positive cells per hemivillus compared with SBS/DMSO (Fig. 7G), though this was not significant (8.6 ± 1.2 vs. 6.9 ± 1.6%; P = 0.38, Fig. 7J).

DISCUSSION

We describe a novel model of SBS and intestinal adaptation that closely recapitulates the physiological effects seen in the human correlate condition. The small size of the adult zebrafish may appear to preclude attempts to surgically modify intraperitoneal organs, but this model is straightforward and reproducible and demonstrates a number of the characteristics necessary to study intestinal adaptation. With an average operative...
time of 329.6 s (5.5 min) for SBS and 17.6 s for sham operation, these procedures are much faster than murine SBS surgery, and we were able to demonstrate long postoperative time points in high experimental numbers at low cost. The animals survive the brief operation and demonstrate weight loss in response to the severely truncated intestinal surface area available to absorb nutrition. Housed and fed together with their sham counterparts, SBS zebrafish have a lower survival rate and demonstrate significantly greater weight loss within the first 2 wk following surgery than the sham laparotomy controls. In the subsequent 2 wk, there is continued weight loss to ~85% of preoperative weight. As in humans, the remaining proximal intestine is grossly dilated with increases in length and cross section. This adaptive response has been well described in mammalian models and humans with SBS and is characterized by compensatory dilation, thickening, and lengthening of the remaining small intestine (41, 55).

There is no single efficient and well-accepted model of SBS and intestinal adaptation. The majority of investigations involve 50% or greater small bowel resection (SBR) in mouse, rat, and pig models. Piglet models benefit from the larger size of the animals, which allows a technically easier bowel resection and anastomosis, and authors also cite more anatomical, physiological, and biochemical similarities to newborn human infants than rodent models (33). However, housing, procurement, and surgical supplies are more expensive, typically thousands of dollars per piglet. A technical review of 27 articles reporting porcine SBS models demonstrated great heterogeneity in age, weight, and sex of pigs, as well as percent and location of SBR performed. Experimental numbers were low, and mortality ranged from 6 to 18% where reported (52). One report of 75% SBR in 7-day-old piglets reported 8% mortality rate at 28 days; however, the animals were fed a standard amount of calories per body weight daily and were supplemented with electrolytes, additional costly and work-intensive interventions (15).

Rodent models offer more options for transgenic investigations than pigs, but experimental approaches are also diverse and a gold standard has not been achieved. Few authors include mortality data when describing their models, but it is understood through the few centers that publish data from these models that rodent bowel resection and anastomosis is a very specialized skill. After achieving only a 16% survival rate after 75% SBR, which closely mimics human disease, Warner and colleagues (45) modified their approach and developed a robust model of intestinal adaptation with 85% survival following 50% SBR with reanastomosis. Mouse models also report a similarly modest resection, likely due to prohibitively high
mortality rates (32). It is important to note that clinical SBS does not result after 50% bowel resection in humans, and adaptation is typically seen at higher levels of intestinal loss. In addition to modeling adaptation in response to greater than 75% SBR by creation of a proximal stoma, our model anatomically recapitulates severe SBS and the early, rapid weight loss of the resultant intestinal failure, while allowing high-throughput investigations given the low cost, high survival rate, and reproducibility of the intestinal response. We are the first to describe the time per proximal diversion operation and, given our experience with intra-abdominal surgery in rodent and pig models, believe it to be a reasonable claim that it is in fact the shortest operative time of any existing SBS model despite the size of the zebrafish. Additional confounding interventions including parenteral nutritional support are not required, and not providing supplementary therapeutic agents will be less costly as well. Advances in zebrafish genetics make this a reasonable vertebrate model to develop possible future human therapies. In addition, slight variations in technique could facilitate more in-depth investigations of adaptation in other more distal regions of the intestine.

Histologically, human adaptation is associated with crypt proliferation with cells migrating at increased rates into the villus (53). There is a coincident increase in apoptosis, with the overall effect of increased intestinal mass, surface area, and hyperplasia of the muscularis propria (13, 42, 55). In the zebrafish, quantification demonstrates increased villus epithelial perimeter and greater than threefold increase in the number of cells incorporating BrdU in the fish with severely truncated intestine at 2 wk. These proliferating cells were primarily located in the intervillus pocket, the zebrafish intestinal epithelial stem cell zone. In the zebrafish, the increase in BrdU-positive cells was demonstrated only at the 2-wk time point following the SBS operation and did not persist at the 4-wk time point. In humans, intestinal adaptation is noted mainly in the first 1–2 years following massive small bowel resection, although large cohorts with replicated time points are of course not possible. Future identification of these proliferating cells may be informative about the mechanisms that underpin adaptation.

Multiple factors contribute to intestinal adaptation. The magnitude of the adaptation response in human patients is thought to correlate with extent of resection but is also altered by luminal factors in recovery after resection, including nutrition, pancreaticobiliary secretions, and the microbiome. EGF, glucagon-like peptide 2 (Glp2), growth hormone, and insulin-like growth factor (IGF) are among the nonnutritive factors that have been confirmed to stimulate adaptation in multiple rat, rabbit, and piglet studies (5, 11, 23, 26, 30, 43, 47, 50). A glucagon-like peptide 2 analog (GLP-2) was recently approved in Europe and the United States for the treatment of intestinal failure and is believed to exert its effect through IGF-1, IGF-2, and ERBB ligands (39). Experimental models in animals have focused on withdrawal or addition of these factors to investigate the mechanism underlying the adaptive response.

IGF-1 and -2 are produced in the liver, and locally in the intestine, and are important regulators of metabolism and organ regeneration. Whereas IGF-2 is primarily involved in growth during the fetal period (18), IGF-1 is thought to be one of the primary effectors of growth hormone; IGF-1 stimulates duodenal crypt cell proliferation in humans and increased intestinal adaptation when administered to rats after massive small bowel resection (18, 23, 47, 54, 56). IGF is preserved across vertebrate species. In the zebrafish, signaling occurs through four IGF genes: igf1a, -1b, -2a, and -2b. These genes encode four peptides that signal through the receptor, Igf1r (19, 57). Igfs are potent mitogens for zebrafish embryonic cells through activation of MAPK and PI3K signaling pathways and are involved in a variety of physiological processes including metabolism, growth, and reproduction (35, 38). Transcript levels are regulated tightly by hormonal and environmental factors such as feeding status (2). The role of Igf in intestinal regeneration in the zebrafish is undefined.

We investigated whether Igf contributed to intestinal adaptation in our model. By exposing sham or SBS fish to the Igf1r inhibitor NVP-AEW541 or vehicle control (DMSO), we demonstrated that inhibition reduced measurements associated with intestinal adaptation and prevented the increase in BrdU-marked intervillus pocket cells that is seen in SBS in the absence of NVP-AEW541. In the control group exposed to DMSO only, SBS fish demonstrated significantly increased BrdU incorporation compared with sham fish. This effect was not observed when inhibitor was added, suggesting that Igf signaling is important in the increase of marked progenitor cells associated with intestinal adaptation in our model. We noted an increase in goblet cells, as stained by Alcian blue, in the sham group exposed to Igf1r inhibitor compared with the sham group exposed to vehicle. There was no difference in goblet cells per hemivillus between sham and SBS groups. Previous studies have reported significant increase in goblet cell density in mouse and pig SBS models (17, 33), but increases in goblet cells are also noted in response to ATOH1 and SPDEF (12, 21).

Although NVP-AEW541 is a kinase inhibitor reported to inhibit receptor activation and downstream signaling, likely to be most effective through these downstream pathways, we evaluated igf-1a, 1b, 2a, 2b, and 1r by RT-qPCR. There was no significant difference between SBS/inhibitor and SBS/DMSO fish in the mRNA quantitation of ligands igf1b, and igf2b, or the receptor igf1r. In SBS fish, igf1a and igf2a expression was increased relative to the sample of intestine (S2 and S3) immediately adjacent to the tested intestine (S1) resected at the time of initial surgery. There was a relative decrease in igf1a and igf2a expression in fish with SBS exposed to the inhibitor compared with those exposed to DMSO alone. We chose inhibition through NVP-AEW541 as an initial proof-of-concept of this SBS model because there are good data relating Igf to SBS in other animal models. Igf1 potently stimulates mucosal growth in uninjured and irradiated mouse small bowel. It has the ability to activate quiescent and constitutively active stem cell populations by different molecular mechanisms (46). However, addition of NVP-AEW541 to the tank water is a relatively blunt force approach, expected to have systemic effects as well as downstream signaling actions, and the modest mRNA changes are unlikely to be the major effector of the marked increase in BrdU-positive cells and intestinal villus epithelial perimeter identified in the SBS fish.

In further evaluation of SBS fish in the presence of NVP-AEW541, the histological changes associated with intestinal adaptation, including increased inner epithelial perimeter, were reduced in the presence of the inhibitor. However, all three groups of SBS fish, whether exposed to tank water alone,
DMSO, or inhibitor, all demonstrated known characteristics of SBS as it is identified in human patients who have severely truncated small intestines. All of the SBS fish had an increased inner epithelial perimeter of the intestine with greater villus ridge complexity, dilated girth of the intestine, and increased serosal circumference. Linked to the increase in BrdU-positive cells identified in conjunction with these histological findings when not exposed to inhibitor, a stem/progenitor cell mechanism is a likely candidate.

Given the limitations of the Igf1r inhibitor, another molecular effector pathway of intestinal adaptation, EGFR, was evaluated for downstream changes within the proximal intestine of our zebrafish SBS model. The EGFR pathway is largely conserved between humans and zebrafish (9, 36). Zebrafish homologues have been isolated for direct EGFR ligands, including egf, btc, hb-egf, and tgfα. In the zebrafish SBS model, the proximal intestine demonstrated a significant increase in egf, btc, and egfr compared with the sham fish. These changes are consistent with data from rodent models of adaptation, further validating our model.

Shortening the bowel in zebrafish through the creation of a proximal stoma overcomes many of the limitations of mammalian models of SBS and intestinal adaptation such as high animal mortality, operative complexity and time, high cost, and low experimental numbers secondary to these factors. The model closely resembles the human disease of SBS with the possibility for more rapid investigations of key morphogens that may eventually be translational targets. Our model can easily be modified to create more distal ostomies. Histological and biochemical changes may differ based on the level of ostomy creation and percentage of bowel resected and will be important to further understand intestinal adaptation.

This model demonstrates the feasibility and survivability of performing intraperitoneal microsurgery in the adult zebrafish. With advanced genetic and pharmacological tools now available, this approach also allows for drug and small molecule administration and may lead to progress in understanding the mechanism of SBS as it is identified in human patients who have severely truncated small intestines. All of the SBS fish had an increased inner epithelial perimeter of the intestine with greater villus ridge complexity, dilated girth of the intestine, and increased serosal circumference. Linked to the increase in BrdU-positive cells identified in conjunction with these histological findings when not exposed to inhibitor, a stem/progenitor cell mechanism is a likely candidate.

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GRANTS
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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES


