Prolonged Absence of Mechanoluminal Stimulation in Human Intestine Alters the Transcriptome and Intestinal Stem Cell Niche

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SUMMARY
The absence of intestinal mechanoluminal stimulation in calorically replete patients decreases proliferation, villus height, crypt depth, and the intestinal stem cell population. Altered genes involved in proliferation, digestion, and inflammation may be targets for enhancing adaptation in short-bowel syndrome patients.

BACKGROUND & AIMS: For patients with short-bowel syndrome, intestinal adaptation is required to achieve enteral independence. Although adaptation has been studied extensively in animal models, little is known about this process in human intestine. We hypothesized that analysis of matched specimens with and without luminal flow could identify new potential therapeutic pathways.

METHODS: Fifteen paired human ileum samples were collected from children aged 2–20 months during ileostomy-reversal surgery after short-segment intestinal resection and diversion. The segment exposed to enteral feeding was denoted as fed, and the diverted segment was labeled as unfed. Morphometrics and cell differentiation were compared histologically.

RNA Sequencing and Gene Ontology Enrichment Analysis identified over-represented and under-represented pathways. Immunofluorescence staining and Western blot evaluated proteins of interest. Paired data were compared with 1-tailed Wilcoxon rank-sum tests with a P value less than .05 considered significant.

RESULTS: Unfed ileum contained shorter villi, shallower crypts, and fewer Paneth cells. Genes up-regulated by the absence of mechanoluminal stimulation were involved in digestion, metabolism, and transport. Messenger RNA expression of LGR5 was significantly higher in unfed intestine, accompanied by increased levels of phosphorylated signal transducer and activator of transcription 3 protein, and CCND1 and C-MYC messenger RNA. However, decreased proliferation and fewer LGR5+, OLFM4+, and SOX9+ intestinal stem cells (ISCs) were observed in unfed ileum.

CONCLUSIONS: Even with sufficient systemic caloric intake, human ileum responds to the chronic absence of mechanoluminal stimulation by up-regulating brush-border enzymes, transporters, structural genes, and ISC genes LGR5 and ASCL2. These data suggest that unfed intestine is primed to replenish the ISC population upon re-introduction of enteral
feeding. Therefore, the elucidation of pathways involved in these processes may provide therapeutic targets for patients with intestinal failure. RNA sequencing data are available at Gene Expression Omnibus series GSE82147. (Cell Mol Gastroenterol Hepatol 2017;3:367–388; http://dx.doi.org/10.1016/j.cjmg.2016.12.008)

Keywords: Intestinal Stem Cell; LGR5; Small Intestine; Calorie Restriction; Enteric Nutrition; Mechanoluminal Flow.

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hort-bowel syndrome (SBS) is a highly morbid and costly disease resulting from insufficient intestinal length or inadequate intestinal absorption. In children, SBS may develop after massive small-bowel resection for a range of diseases including necrotizing enterocolitis (NEC), intestinal atresia, and volvulus. Enteral independence from supplemental parenteral nutrition relies on adaptation of the remaining bowel. In animal models, adaptation is characterized by taller villi, deeper crypts, increased bowel diameter and length, and increased proliferation and apoptosis in the remaining bowel. However, these changes have been inconsistently found in human intestine after resection. Further investigation is necessary to understand regulatory mechanisms that direct adaptation in human intestine.

Several growth factors and hormones have been identified that may enhance adaptation, most notably growth hormone and glucagon-like peptide 2. However, there are many more regulators of intestinal epithelial proliferation, such as immune and inflammatory systems, microbial components, and mechanoluminal stimulation. The mechanisms mediating these effects are incompletely understood. Knowledge of how these diverse mitogens affect proliferation and intestinal stem cell (ISC) activity might help to identify novel therapeutic pathways and agents for SBS.

Prompt and nutritionally appropriate initiation of enteral feeds is a critical component of intestinal rehabilitation programs and is associated with shorter hospitalizations, shorter duration of parenteral nutrition, and increased rates of enteral autonomy. Enteral nutrition may enhance intestinal adaptation via induction of various gastrointestinal hormones and pancreaticobiliary secretions or direct stimulation of intestinal epithelium, but the exact effects induced by mechanoluminal stimulation are unknown.

Animal studies of enteral nutrition often are confounded by differences in caloric status and types of nutrition. In human beings, comorbidities associated with the underlying pathology requiring intestinal resection also must be considered. Thus, we sought to determine the isolated effects of mechanoluminal stimulation on human intestine, independent of such confounders, by comparing closely paired proximal and distal limbs from ileostomy resections in infants with previous NEC or focal intestinal perforation (Figure 1A). To exclude differences caused by regional anatomic variances, we only included matched samples with a minimum of intervening intestinal resection. This allowed us to test a hypothesis that in matched human samples, mechanoluminal flow is a potent mitogen with multiple in vivo effects on cellular differentiation and genetic programming. We found that withdrawing luminal flow decreased common measures of adaptation, including villus height, crypt depth, proliferation, and various ISC populations. In addition, we recognized that in the distal limb of intestine diverted from enteric feeding, up-regulated genes were associated with digestion and metabolism while down-regulated genes were associated with cell proliferation and inflammation. This approach also identified candidate mediators of proliferation that may lead to novel therapeutic targets for enhancing intestinal adaptation.

Materials and Methods

The research protocol was approved by the Children’s Hospital Los Angeles institutional review board (CCI-09-00093). All authors had access to the study data and reviewed and approved the final manuscript.

Patient Population

All patients undergoing small intestinal anastomosis after ileostomy at our institution between August 2012 and
October 2016 were identified by review of surgical records. Inclusion criteria consisted of age younger than 2 years and previous diagnosis of NEC or focal intestinal perforation resulting in ileal resection. The diagnosis was based on clinical presentation, surgical findings, and final pathologic examination of the resected specimen. To minimize any discrepancies resulting from baseline variation in different intestinal segments, patients with long-segment bowel resection resulting in SBS were excluded. Charts were reviewed for demographic and treatment details, including location and amount of intestine resected, duration of intestinal diversion, timing of enteral and parenteral nutrition, ostomy complications, and postreversal complications.

Demographic and clinical characteristics of the 15 included patients are presented in Table 1. Medical chart review showed that 14 patients were premature infants who underwent resection before 5 weeks of age for focal intestinal perforation or NEC. After initial resection, all patients received broad-spectrum antibiotics for at least 10 days. All patients also received total parenteral nutrition and some enteral feeds either orally or via a feeding tube to achieve caloric repletion and appropriate weight gain. Only 5 infants were able to achieve complete enteral independence by the time of ileostomy reversal. Patients were diverted for 7–48 weeks, and afterward most still required total parenteral nutrition until they achieved enteral independence before discharge from the hospital. Ostomy complications occurred infrequently in our patient population. Peristomal hernias without obstruction occurred in 2 patients, and 1 patient underwent an ileostomy revision for stomal retraction.

**Tissue Collection**

Paired human small intestine samples were obtained at the time of small intestinal anastomosis after ileostomy from pathology specimens that otherwise would have been discarded. Ileostomy reversal occurred at least 7 weeks after their primary surgery. Proximal samples, obtained from the resected ileostomy margin that remained exposed to enteral nutrition before reversal, was referred to as fed. Samples obtained from the distal segment of ileum (either a Hartmann’s pouch or mucus fistula), which were diverted from enteric feeding, were designated as unfed (Figure 1A). For fed and unfed samples, tissue was obtained within 1–2 cm of the proximal stoma (fed) and distal diverted (unfed) intestine. Both samples were placed immediately in saline on ice at the time of surgery. Specimens were divided into full-thickness sections for immediate storage in (1) 10% buffered formalin for paraffin-embedded blocks and sections, (2) RNAlater for RNA analyses (Life Technologies, NY) and snap frozen at -80°C, or (3) fresh frozen at -80°C for protein assays. Analyses were limited to the quantity of tissue available for individual specimens.

**Histologic Quantification**

H&E images were captured at 10× magnification by light microscope (DM 1000; Leica, Wetzlar, Germany) and quantified using ImageJ software [image.nih.gov](http://image.nih.gov); National Institutes of Health, Bethesda, MD). A blinded, trained observer analyzed only sections with full-thickness, non-obliqued epithelium. For patients with high-quality epithelium, a minimum of 10 villi and crypts from 2 different sections were quantified.

**Immunofluorescence Microscopy**

Sections (5 μm) of paraffin-embedded tissue were deparaffinized in Histochoose (Sigma-Aldrich, St. Louis, MO) and rehydrated in a graded ethanol series. Heat-induced antigen retrieval was performed with either a citrate-based pH 6.0 solution or a TRIS-based pH 9.0 antigen unmasking solution (Vector Laboratories, Inc, Burlingame, CA). Sections were blocked with 2% goat serum (Sigma-Aldrich, St. Louis, MO) and incubated with primary antibody (Table 3) overnight at 4°C. Secondary antibodies (Table 3) were incubated at room temperature for 1 hour.

For staining of LGR5 protein expression, we performed the protocol as reported by Dame et al.23 Briefly, sections first were blocked with 3% hydrogen peroxide, followed by a donkey serum block. After incubation with primary antibody overnight, sections were incubated with biotinylated secondary antibody for 1 hour, then Vectastain Elite ABC Reagent (Vector Laboratories). Signal then was developed with tyramide signal amplification reagent (Dako, Carpinteria, CA) followed by fluorescein-conjugated streptavidin (Jackson ImmunoResearch Laboratories, Inc, West Grove, PA) for 30 minutes.

All slides were mounted in Vectashield with 4,6-diamidino-2-phenylindole (Vector Laboratories) and imaged on an upright Leica DM5500B immunofluorescence microscope using Leica Suite Advanced Fluorescence (LAS AF) 6000 software. Positive cells then were quantified by a blinded, trained observer. Quantification of immunofluorescence was obtained by evaluating a minimum of 10 villi or crypts per sample from more than 6 different fields of view.

**RNA Sequencing and Analysis**

Total RNA was extracted using TRIzol reagent (Life Technologies) followed by Qiagen column purification with on-column DNase digestion (Qiagen, Valencia, CA). RNA concentration was measured with Nanodrop (ThermoFisher Scientific, Waltham, MA).

Total RNA from 5 paired samples were selected to make complementary DNA libraries for RNA sequencing. RNA integrity was determined using a bioanalyzer (Agilent Technologies, Santa Clara, CA). All samples had an RNA integrity number of at least 7. Before library construction, samples were spiked with Ex-Fold External RNA Controls Consortium controls (Ambion, Foster City, CA). Mix 1 was added to RNA from fed intestine and mix 2 was added to RNA from unfed intestine. Libraries initially were sequenced to 10 million reads for power analysis via the Scotty algorithm.24 Based on the results of the power analysis, libraries then underwent deep sequencing to 40 million base pairs. Sequences were assayed for quality using FastQC,25 and adapter sequences as well as poor-quality sequences were removed with Trimmmomatic.26 By using ENCODE
Table 1. Patient Characteristics

| Patient | Sex | Diagnosis | Gestational age, wk | Birth weight, kg | Age at resection, wk | Weight at resection, kg | Location of resection | Amount resected, cm | Diversion duration, wk | Nutrition after resection | Age at reversal, mo | Weight at reversal, kg | Weight gain, % | Nutrition after reversal | Ostomy complications | Postreversal complications | Comorbidities |
|---------|-----|-----------|---------------------|------------------|---------------------|------------------------|----------------------|-------------------|----------------------|-------------------------|----------------|------------------------|-------------|------------------------|-------------------|--------------------------|--------------|
| A       | F   | NEC       | 25/47               | 0.770            | 2.0                 | 0.750                  | ileum: 7.5 cm from ICV| 4                 | 9.0                  | TPN + formula until ostomy reversal | 2             | 1.785                  | 138.00       | TPN                    | None              | Anastomotic leak          | CLD, IVH, ROP, cholestasis of PN |
| B       | F   | FIP       | 24                  | 0.700            | 2.5                 | 0.835                  | ileum: 5.0 cm from ICV| 2                 | 19.0                 | TPN + formula first 6 wk, then full formula | 5             | 2.93                   | 250.90       | TPN until POD 5, formula started POD 6 | Peristomal hernia without obstruction | None | CLD, IVH, ROP |
| C       | F   | FIP       | 24/57               | 0.705            | 5.0                 | 1.040                  | ileum: 15 cm from ICV | 7                 | 13.5                 | TPN + formula/BM until ostomy reversal | 4.5           | 3.20                   | 257.69       | TPN until POD 23, formula started POD 7 | None | ileus | CLD, BPD, anemia, PS |
| D       | F   | FIP       | 28                  | 0.810            | 1.0                 | 0.800                  | ileum: 45 cm from ICV | 4                 | 11.5                 | TPN + formula/BM until ostomy reversal | 2             | 2.655                  | 231.88       | TPN until POD 27, Peristomal abscess ileus | CLD, PDA, anemia |
| E       | M   | NEC       | 26/3/7              | 0.900            | 1.5                 | 0.900                  | Mid-ileum            | 1.5               | 31.0                 | TPN + formula first 4 wk, then full formula | 7.5           | 5.1                    | 466.67       | TPN POD 8–11, formula started POD 10 | None | None | PDA, ROP, GERD, apnea, anemia |
| F       | M   | FIP       | 26                  | 0.810            | 1.0                 | 0.810                  | Mid-ileum            | 9                 | 15.0                 | TPN + formula until ostomy reversal | 4             | 2.675                  | 230.25       | TPN until POD 15, None | Wound infection Twin, CLD, PDA, cholestasis of PN |
| G       | F   | NEC       | 35                  | 2.240            | 4.0                 | 3.550                  | ileum: 15 cm from ICV| 20                | 7.0                  | TPN + formula until ostomy reversal | 2.5           | 3.62                   | 1.97         | TPN until POD 14, Peristomal hernia without obstruction | None | Twin, rotavirus, thrombosis |
| H       | M   | FIP       | 25/1/7              | 0.655            | 1.0                 | 0.680                  | ileum just proximal to ICV | 0                 | 13.0                 | TPN + fortified BM/formula until ostomy reversal | 3             | 2.415                  | 255.15       | TPN until POD 14, formula started POD 7 | None | Fulminant pneumatisis CLD, meningitis, sepsis, osteopenia |
| I       | M   | NEC       | 25                  | 0.760            | 1.0                 | 0.900                  | ileum: 2 cm from ICV | 4                 | 44.0                 | TPN + formula first 6 wk, then full formula | 10            | 6.54                   | 626.67       | TPN until POD 74, formula started POD 37 | Stomal retraction | Adenovirus diarrhea CLD, PDA cerebrovascular shunt, sepsis |
| J       | M   | FIP       | 26                  | 0.650            | 1.5                 | 0.685                  | ileum: 22 cm from ICV| 2                 | 16.0                 | TPN + BM until ostomy reversal | 4             | 2.655                  | 287.59       | TPN until POD 11, Peristomal skin breakdown | None | CLD, IVH, anemia, osteopenia, ROP |
| K       | M   | FIP       | 25/1/7              | 0.885            | 1.5                 | 0.995                  | ileum                 | 0                 | 11.5                 | TPN + BM for first 4 wk, then full BM | 13            | 3.012                  | 202.71       | TPN until POD 22, EC fistula, peristomal skin breakdown | None | CLD, ROP |
| L       | M   | FIP       | 22/6/7              | 0.487            | 1.0                 | 0.470                  | ileum                 | 8                 | 19.0                 | TPN + BM until ostomy reversal | 20            | 2.95                   | 527.66       | TPN until POD 18, None | Urosepsis, pneumonia PDA, sepsis, ROP |
| M       | F   | FIP       | 24/5/7              | 0.705            | 5.0                 | 1.040                  | ileum                 | 7                 | 13.5                 | TPN + formula/BM until ostomy reversal | 8             | 3.20                   | 207.69       | TPN until POD 26, None | ileus | CLD, BPD, ROP, anemia, PS |
recommended parameters, the remaining high-quality sequences were aligned using the RNA-star short-read aligner to the Gencode human genome version 23, which corresponds to the Genome Research Consortium human genome version GRCh38.p3 (Genome Reference Consortium). Read counts per transcript were obtained using the HTSeq-count Python script. Reads per kilobase per million mapped reads were generated using the edgeR R/Bioconductor software package. Relative log expression graphs and principle component graphs were generated using the plotting functions of the EDASeq R/Bioconductor software package.

Differential gene expression was analyzed based on the Ex-Fold External RNA Controls Consortium probes with the Remove Unwanted Variation R/Bioconductor software package combined with edgeR. Genes with a false-discovery rate-corrected P value less than .05 were considered significant. Gene Ontology enrichment analysis for biological pathways was performed with the Gene Ontology stats R/Bioconductor software and Gene Ontology Consortium (geneontology.org). Ontologic trees were created with BiNGO through Cytoscape. A threshold of a log2 fold change ≥1.5 was selected for choosing genes of interest for further evaluation.

RNA sequencing raw data and processed data were deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus. They can be accessed through GEO series accession number GSE82147. The top 100 up-regulated and down-regulated genes have been provided (Table 2).

For comparison of our RNA sequencing data with previously published RNA sequencing analysis of intestine with active NEC, all differentially expressed genes with a P value less than .05 were downloaded with published fold changes. Pathway analyses were performed as described earlier. Human genes involved in pathways of interest were identified from Gene Ontology human annotation lists. Overlapping pathways and genes were compared to determine depth of similarity.

Quantitative Real-Time Polymerase Chain Reaction

Quantitative real-time polymerase chain reaction (PCR) was performed on several genes of interest to validate the RNA sequencing data. Primers (Table 4) were designed on the Roche (Indianapolis, IN) website (lifescience.roche.com) and purchased from Eurofins Operon (www.operon.com). A total of 1 μg of RNA was reverse-transcribed into complementary DNA with the iScript Reverse Transcription Supermix for real-time-PCR (Bio-Rad, Hercules, CA). Quantitative PCR (qPCR) was run in 96-well plates with SYBR Green (Roche) and analyzed with Roche Lightcycler 480 software release 1.5.0. Expression levels were calculated by the comparative threshold cycle method with delta delta threshold cycle values normalized to human glyceraldehyde-3-phosphate dehydrogenase.

PCR validation first was performed on the same 5 paired samples that underwent RNA sequencing analysis. If a
significant fold change was found on qPCR, 3 additional pairs were evaluated by PCR. Outliers were identified with the ROUT test, Q = 0.1%, and excluded from statistical analysis. This resulted in 6–7 pairs included in the PCR analyses.

**In Situ Hybridization**

The RNAscope 2.5 HD Reagent Kit-RED (322350; Advanced Cell Diagnostics, Newark, CA) was performed on 5-μm, formalin-fixed, paraffin-embedded sections according to the manufacturer’s instructions. The RNAscope probes used were LGR5 (NM_003667.2, region 560–1589, cat no: 311021) and negative control probe DapB (EF191515, region 414–862, cat no: 310043). Crypt expression of LGR5 messenger RNA (mRNA) was quantified according to a modified 5-grade scoring system recommended by the manufacturer (0 = no staining or <1 dot to every 10 cells [63× magnification]; 1 = 1–3 dots/cell [visible at 40–63× magnification]; 2 = 4–10 dots/cell, very few dot clusters [visible at 40–63× magnification]; 3 = more than 10 dots/cell, <10% positive cells have dot clusters [visible at 40–63× magnification]; and 4 = >10 dots/cell). More than 10% positive cells have dot clusters (visible at 40–63× magnification). At least 15 crypts from 4 individual patient samples were quantified.

**Western Blot**

Protein was extracted from 5 pairs of homogenized full-thickness samples of intestine in RIPA buffer, protease inhibitor cocktail (Roche), and phosphatase inhibitor cocktail (Roche). Concentrations were assessed with a BCA protein assay (ThermoFisher Scientific). Samples were run on 4%–12% Bis-Tris Plus gels (ThermoFisher Scientific) in Bolt MES sodium dodecyl sulfate running buffer (ThermoFisher Scientific). After blocking with Odyssey blocking buffer (LI-COR, Lincoln, NE), primary antibodies were incubated overnight at 4°C (Table 3). Secondary antibodies (Table 3) were incubated at room temperature for 1 hour. Blots then were scanned digitally and densitometry-quantified with ImageStudio (LI-COR). Densitometry was quantified relative to actin.

**Statistical Analyses**

Histologic measurements and cell counts were compared with paired 2-tailed Wilcoxon rank-sum tests. To confirm differences noted on RNA sequencing, PCR gene expression values were compared with a paired 1-tailed Wilcoxon rank-sum test. All calculations were performed on Prism (GraphPad Software, San Diego, CA). Values are expressed as medians (95% confidence interval). P values less than .05 were considered significant. Outliers were identified with the ROUT test, Q = 0.1%, and excluded from statistical analysis.37

**Results**

**Mechanoluminal Deprivation Decreases Intestinal Epithelial Proliferation**

Morphometry from pairs with high-quality epithelium was compared to define histologic changes associated with loss of mechanoluminal stimulation. In unfed intestine, villi were 10%–66% shorter and crypts were 22%–62% deeper (Figure 2A–D) (n = 7; P = .02). The muscularis mucosae was 5%–55% thinner (Figure 1C) (n = 5; P = .03), although the majority of stromal elements within the unfed limb by H&E analysis remained similar to fed intestine (Figure 1B). There was no difference noted in crypt width or density, defined as the number of crypts per millimeter of tissue. There was no significant correlation between the length of

**Table 2. Primary and Secondary Antibodies**

| Antibody                  | Species | Company                                | Catalog no | Dilution |
|---------------------------|---------|----------------------------------------|------------|----------|
| Primary                   |         |                                        |            |          |
| Mucin 2 (MUC2)            | Rabbit  | Santa Cruz Biotechnology (Dallas, TX)  | sc-15334   | 1:100    |
| Chromogranin A (CHGA)     | Rabbit  | Abcam (Cambridge, MA)                  | ab15160    | 1:100    |
| Lysozyme (LYZ)            | Rabbit  | Dako Cytomation (Lake Success, NY)    | A0099      | 1:100    |
| E-cadherin (E-CAD)        | Mouse   | BD Transduction (San Jose, CA)         | 610181     | 1:100    |
| Ki67                      | Rabbit  | Thermo Scientific (Waltham, MA)        | RM-9106-S1 | 1:100    |
| Proliferating cell nuclear antigen | Mouse | Vector Laboratories                   | VP-P980    | 1:100    |
| Cleaved caspase 3 (CC3)   | Rabbit  | Cell Signaling Technologies (Danvers, MA) | 9661-01-001 | 1:50     |
| Phosphorylated STAT3 (pSTAT3) | Rabbit | Cell Signaling Technologies           | 9145       | 1:1000   |
| STAT3                     | Rabbit  | Cell Signaling Technologies           | 4904       | 1:1000   |
| Actin (clone AC-15)       | Mouse   | Sigma (St. Louis, MO)                  | A1978      | 1:10000  |
| OLFM4                     | Rabbit  | Abcam                                  | AB85046    | 1:200    |
| SOX9                      | Rabbit  | Millipore (Billerica, MA)              | AB5535     | 1:200    |

**Secondary**

| Antibody                  | Species | Company                                | Catalog no | Dilution |
|---------------------------|---------|----------------------------------------|------------|----------|
| Alexa Fluor anti-rabbit 488 | Goat    | Life Technologies (Carlsbad, CA)       | Z25302     | 1:200    |
| Alexa Fluor anti-rabbit 555 | Goat    | Life Technologies (Carlsbad, CA)       | A21429     | 1:200    |
| Biotinylated anti-rabbit  | Goat    | Jackson ImunoResearch (West Grove, PA) | 111-066-047 | 1:1000   |
| DTAF-conjugated streptavidin | Goat  | Jackson ImunoResearch                  | 016-101-048 | 1:500    |
| IRDye 680LT anti rabbit   | Donkey  | LI-COR (Lincoln, NE)                   | 926-68023  | 1:10,000 |
| IRDye 800CW anti-mouse    | Donkey  | LI-COR                                 | 926-32212  | 1:10,000 |

**NOTE.** The name, source, catalog number, and dilution are provided for the primary and secondary antibodies included in the study. DTAF, dichlorotriazinyl amino fluorescein.
| Gene name   | Description                                                                 | logFC  | LR       | P value       | Ensembl gene ID     |
|------------|------------------------------------------------------------------------------|--------|----------|---------------|---------------------|
| FMO1       | Flavin containing monoxygenase 1                                            | 1.935answer | 74.803answer   | 5.20085E-18     | ENSG00000010932     |
| UPK3A      | Uroplakin 3A (source: HGNC symbol)                                          | 2.1475answer | 66.6548518     | 6.86813E-12     | ENSG0000100373      |
| CXCL5      | Chemokine (C-X-C motif) ligand 5                                             | -2.73answer | 41.69828868 | 1.06582E-10     | ENSG0000163735      |
| RNF224     | Ring finger protein 224                                                     | 1.89answer | 41.87209741 | 9.74433E-11     | ENSG00000233199     |
| CYSRT1     | Cysteine-rich tail protein 1                                                | 1.725answer | 39.9409415   | 2.61759E-10     | ENSG0000197911      |
| SLCO5A2    | Solute carrier family 5 (sodium/monocarboxylate cotransporter), member 1   | 2.132answer | 38.74586262 | 4.83048E-10     | ENSG0000148942      |
| SULT2A1    | Sulfotransferase family, cytosolic, 2A, dehydroepiandrosterone-prefering, member 1 | 2.094answer | 37.8205594 | 7.75006E-10     | ENSG0000105398      |
| MRO        | Maestro                                                                      | 1.84answer | 36.97909256 | 1.19403E-09     | ENSG0000134042      |
| LINC01595  | Long intergenic nonprotein coding RNA 1595                                  | 2.165answer | 36.0616015  | 1.91171E-09     | ENSG00000259108     |
| SHBG       | Sex hormone-binding globulin                                                | 1.73answer | 35.47000772 | 2.59008E-09     | ENSG0000129214      |
| SLCO2A1    | Solute carrier family 23 (ascorbic acid transporter), member 1              | 1.603answer | 35.1428148 | 0.000000003     | ENSG0000170482      |
| SLCO3A3    | Solute carrier family 34 (type II sodium/phosphate cotransporter), member 3 | 1.53answer | 34.51159913 | 4.23719E-09     | ENSG0000198569      |
| PDZK1      | PDZ domain containing 1                                                     | 1.58answer | 34.29271495 | 4.74153E-09     | ENSG0000174827      |
| TREH       | Trehalase (brush-border membrane glycoprotein)                              | 1.194answer | 33.81359331 | 6.06536E-09     | ENSG0000118094      |
| SULT1C2    | Sulfotransferase family, cytosolic, 1C, member 2                            | 1.907answer | 33.14193541 | 8.56702E-09     | ENSG0000198203      |
| ENPP7      | Ectonucleotide pyrophosphatase/phosphodiesterase 7                         | 1.30answer | 32.5690058  | 1.13395E-08     | ENSG0000182156      |
| F7         | Coagulation factor VII (serum prothrombin conversion accelerator)           | 1.780answer | 31.7925485 | 1.71567E-08     | ENSG0000005793      |
| CYP4F2     | Cytochrome P450, family 4, subfamily F, polypeptide 2                       | 1.63answer | 31.31912349 | 2.18913E-08     | ENSG0000186115      |
| SLCO13A1   | Solute carrier family 13 (sodium/sulfate symporter), member 1               | 2.53answer | 30.8168591 | 2.83662E-08     | ENSG0000081800      |
| SLCO5A9    | Solute carrier family 5 (sodium/sugar cotransporter), member 9             | 1.53answer | 30.9081275 | 2.70548E-08     | ENSG0000117834      |
| PRKG2      | Protein kinase, cGMP-dependent, type II                                     | 1.278answer | 30.94340641 | 2.65663E-08     | ENSG0000138669      |
| RP11-798K3.2 | NA                                                                              | 1.855answer | 30.7019997 | 3.00855E-08     | ENSG00000259347     |
| FRMD1      | FERM domain containing 1                                                    | 1.696answer | 30.15783587 | 3.98275E-08     | ENSG00000153303     |
| NAALADL1   | N-acetylated α-linked acidic dipeptidase-like 1                             | 1.672answer | 30.09099621 | 4.12421E-08     | ENSG0000168060      |
| MS4A8      | Membrane-spanning 4-domains, subfamily A, member 8                          | 1.779answer | 29.9263181 | 4.4878E-08      | ENSG0000166959      |
| FADS6      | Fatty acid desaturase 6                                                     | 1.92answer | 29.9663597 | 4.32858E-08     | ENSG0000172782      |
| SLCO7A9    | Solute carrier family 7 (amino acid transporter light chain, bo,+ system), member 9 | 1.481answer | 29.7595574 | 4.89089E-08     | ENSG0000021488      |
| CDHR5      | Cadherin-related family member 5                                           | 1.515answer | 29.50960252 | 5.56404E-08     | ENSG00000273572     |
| CEL        | Carboxyl ester lipase                                                      | 1.63answer | 28.99145378 | 7.26983E-08     | ENSG0000170835      |
| NAT8       | N-acetylaspartate 8 (GCN5-related, putative)                                | 2.675answer | 28.7107357 | 8.4061E-08      | ENSG0000144035      |
| ATP13A4    | ATPase type 13A4                                                           | 1.939answer | 28.12477638 | 1.1374E-07      | ENSG0000127249      |
| UNC93A     | Unc-93 homolog A (Caenorhabditis elegans)                                  | 1.601answer | 28.04197919 | 1.18712E-07     | ENSG0000112494      |
| SLC2A2     | Solute carrier family 2 (facilitated glucose transporter), member 2        | 1.566answer | 27.87085693 | 1.29688E-07     | ENSG0000163581      |
| TMIGD1     | Transmembrane and immunoglobulin domain-containing 1                       | 1.967answer | 27.7059208 | 1.41295E-07     | ENSG0000182271      |
| TFC        | Transcription factor EC                                                    | 1.3215answer | 27.5716133  | 1.51382E-07     | ENSG0000105967      |
| CUBN       | Cubilin (intrinsic factor-cobalamin receptor)                              | 2.34answer | 27.18194662 | 1.8518E-07      | ENSG0000107611      |
| Gene name | Description | logFC | LR     | P value   | Ensembl gene ID |
|-----------|-------------|-------|--------|-----------|-----------------|
| SLC19A3   | Solute carrier family 19 (thiamine transporter), member 3 | 1.552887155 | 27.26057746 | 1.778E-07 | ENSG00000135917 |
| SMLR1     | Small leucine-rich protein 1 | 1.612876682 | 27.21140528 | 1.823E-07 | ENSG00000256162 |
| NNMT      | Nicotinamide N-methyltransferase | -1.472271231 | 26.94985142 | 2.080E-07 | ENSG00000166741 |
| NAT8B     | N-acetyltransferase 8B (GCN5-related, putative, gene/pseudogene) | 1.718975423 | 26.9959476 | 2.03882E-07 | ENSG00000204872 |
| SLC28A1   | Solute carrier family 28 (concentrative nucleoside transporter), member 1 | 1.407436527 | 26.62926909 | 2.46481E-07 | ENSG00000156222 |
| AC007325.2 | NA | 1.46829846 | 26.64878695 | .000000244 | |
| GGT1      | γ-Glutamyltransferase 1 | 1.080943383 | 26.48751824 | 2.65246E-07 | ENSG00000100031 |
| TRPM6     | Transient receptor potential cation channel, subfamily M, member 6 | 1.828527681 | 26.32603584 | 2.88375E-07 | ENSG00000119121 |
| KHK       | Ketohexokinase (fructokinase) | 1.251604031 | 26.35745455 | 2.83722E-07 | ENSG00000138030 |
| SLC6A4    | Solute carrier family 6 (neurotransmitter transporter), member 4 | 1.399303921 | 25.97462634 | 3.45934E-07 | ENSG00000108576 |
| SLC28A2   | Solute carrier family 28 (concentrative nucleoside transporter), member 2 | 2.441573348 | 25.9860024 | 3.43902E-07 | ENSG00000137860 |
| ENPP3     | Ectonucleotide pyrophosphatase/phosphodiesterase 3 | 1.084760034 | 26.01060946 | 3.39546E-07 | ENSG00000154269 |
| TMEM236   | Transmembrane protein 236 | 1.492276653 | 25.93225942 | 3.5361E-07 | ENSG00000148483 |
| NR1H4     | Nuclear receptor subfamily 1, group H, member 4 | 1.530681737 | 25.66165786 | 4.0683E-07 | ENSG00000125040 |
| COL2A1    | Collagen, type II, α 1 | 2.7427092 | 25.57237858 | 4.26095E-07 | ENSG00000139219 |
| DPEP1     | Dipeptidase 1 (renal) | 2.012090209 | 25.51498355 | .000000439 | ENSG00000154134 |
| SLC16A10  | Solute carrier family 16 (aromatic amino acid transporter), member 10 | 1.57457146 | 25.44844819 | 4.54363E-07 | ENSG00000112394 |
| CLIC5     | Chloride intracellular channel 5 | 1.133190018 | 25.28347044 | 4.94932E-07 | ENSG00000112782 |
| PRLR      | Prolactin receptor | 1.441337236 | 25.30981848 | 4.88217E-07 | ENSG00000113494 |
| MCOLN3    | Mucolipin 3 | 1.595364418 | 25.21959734 | 5.11597E-07 | ENSG00000155732 |
| RP11-1193F23.1 | NA | 2.58543625 | 25.20976053 | 5.14213E-07 | ENSG00000279024 |
| PKIB      | Sp8 transcription factor | 6.674019267 | 25.17393696 | 5.23854E-07 | ENSG00000164651 |
| MAMDC4    | MAM domain containing 4 | 1.277939128 | 24.86218203 | 6.15787E-07 | ENSG00000177943 |
| SLC3A1    | Solute carrier family 3 (amino acid transporter heavy chain), member 1 | 1.781625122 | 24.80772832 | 6.3343E-07 | ENSG00000138079 |
| PKIB      | Protein kinase (CAMP-dependent, catalytic) inhibitor δ | 1.549061572 | 24.60352117 | 7.04229E-07 | ENSG00000135549 |
| CYP3A4    | Cytochrome P450, family 3, subfamily A, polypeptide 4 | 1.922951569 | 24.62844524 | 6.9518E-07 | ENSG00000160686 |
| CDRH5     | Cadherin-related family member 5 | 1.430921311 | 24.46118573 | 7.5822E-07 | ENSG00000099834 |
| SOAT2     | Sterol O-acyltransferase 2 | 1.220274465 | 24.30393855 | 8.22707E-07 | ENSG00000167780 |
| SFP5      | Secreted frizzled-related protein 5 | 1.286605732 | 24.05353528 | 9.36941E-07 | ENSG00000120057 |
| DAB1      | Dab, reelin signal transducer, homolog 1 | 1.317550733 | 24.100991 | 9.14132E-07 | ENSG00000173406 |
| LRRC19    | Leucine-rich repeat containing 19 | 1.489115345 | 24.05613315 | 9.35668E-07 | ENSG00000184434 |
| TYRP1     | Tyrosinase-related protein 1 | -1.664137392 | 23.96832679 | 9.79336E-07 | ENSG000000170165 |
| CCDCC108  | Coiled-coil domain containing 108 | 2.035404412 | 23.98451824 | 9.71135E-07 | ENSG00000181378 |
| TRHDE-AS1 | TRHDE antisense RNA 1 | 2.045816171 | 23.98498662 | 9.7088E-07 | ENSG00000236333 |
| AQP7      | Aquaporin 7 | 1.601418775 | 23.74146929 | 1.10183E-06 | ENSG00000165269 |
| MYO7A     | Myosin VIIA | 1.042540585 | 23.61253694 | 1.17187E-06 | ENSG00000137474 |
| SLC26A2   | Solute carrier family 26 (anion exchanger), member 2 | 1.088822376 | 23.62142755 | 1.17274E-06 | ENSG00000155850 |
| Gene name  | Description                                                                 | logFC       | LR            | P value          | Ensembl gene ID   |
|------------|------------------------------------------------------------------------------|-------------|---------------|-----------------|------------------|
| SLC30A2    | Solute carrier family 30 (zinc transporter), member 2                         | 1.335008616 | 23.60508117   | 1.18275E-06     | ENSG00000158014  |
| SMIM24     | Small integral membrane protein 24                                           | 1.25301892  | 23.47494696   | 1.26551E-06     | ENSG0000095932   |
| RORC       | RAR-related orphan receptor C                                                | 1.621731103 | 23.41211224   | 1.30753E-06     | ENSG00000143365  |
| CLEC4F     | C-type lectin domain family 4, member F                                     | 1.905689525 | 23.13627604   | 1.50916E-06     | ENSG00000152672  |
| FOLH1      | Folate hydrolase (prostate-specific membrane antigen) 1                      | 1.593863663 | 23.09609472   | .000001541      | ENSG0000086205   |
| PGC        | Progastricin (pepsogen C)                                                   | -1.923972415| 22.9906472    | 1.62765E-06     | ENSG0000096088   |
| FOSL1      | FOS-like antigen 1                                                           | -2.400350289| 22.8706113    | 1.73282E-06     | ENSG00000175592  |
| ABCC6      | ATP-binding cassette, subfamily C (CFTR/MRP), member 6                      | 1.701645886 | 22.7420918    | 1.85234E-06     | ENSG0000091262   |
| SLC52A1    | Solute carrier family 52 (riboflavin transporter), member 1                  | 1.33048729  | 22.76413548   | 1.83152E-06     | ENSG00000132517  |
| ERCH4      | Glutamate-rich 4                                                             | 1.152189244 | 22.7799852    | 1.81646E-06     | ENSG00000204978  |
| PNPLPRP2   | Pancreatic lipase-related protein 2                                           | 1.430493624 | 22.75890815   | 1.83651E-06     | ENSG00000266200  |
| ASAH2      | N-acylsphingosine amidohydrolase (nonlysosomal ceramidase) 2                 | 1.367297002 | 22.67467775   | 1.9188E-06      | ENSG00000188611  |
| PDZD7      | PDZ domain containing 7                                                      | 1.19335086  | 22.6056947    | 1.98894E-06     | ENSG00000186862  |
| DCSTAMP    | Dendrocyte-expressed 7 transmembrane protein                                 | 4.290165257 | 22.3551009    | 2.2661E-06      | ENSG00000164935  |
| ABCG8      | ATP-binding cassette, subfamily G (white), member 8                          | 1.334210188 | 22.32450691   | 2.30249E-06     | ENSG00000143921  |
| RAB17      | RAB17, member RAS oncogene family                                            | 1.246564272 | 22.2498867    | 2.39371E-06     | ENSG00000124839  |
| CA7        | Carbonic anhydrase VII                                                       | 1.453047121 | 22.28269301   | 2.35318E-06     | ENSG00000168748  |
| KCNH6      | Potassium channel, voltage-gated eag-related subfamily H, member 6          | 1.685088587 | 22.2554876    | 2.38679E-06     | ENSG00000173826  |
| ESPN       | Espin                                                                       | 1.133210251 | 22.0639674    | 2.63714E-06     | ENSG00000187017  |
| C10orf99   | Chromosome 10 open reading frame 99                                          | 2.465624328 | 22.05917803   | 2.64373E-06     | ENSG00000188373  |
| CLDN23     | Claudin 23                                                                  | 1.045566126 | 22.05403209   | 2.65082E-06     | ENSG00000253958  |
| SLC26A3    | Solute carrier family 26 (anion exchanger), member 3                         | 1.49472698  | 22.01805961   | .000002701      | ENSG0000091138   |
| BTN1L3     | Butyrophilin-like 3                                                          | 1.26562259  | 21.8690556    | 2.9182E-06      | ENSG00000186903  |
| B4GALNT2   | β-1,4-N-acetyl-galactosaminyl transferase 2                                  | 2.20301248  | 21.8177065    | 2.9982E-06      | ENSG00000167080  |
| MEP1B      | Meprin A, β                                                                 | 1.442428396 | 21.74734893   | 3.11019E-06     | ENSG00000141434  |
| ENTPD8     | Ectonucleoside triphosphate diphosphohydrolase 8                            | 1.275503513 | 21.74791285   | 3.10928E-06     | ENSG00000188833  |
| OSBP2      | Oxysterol binding protein 2                                                  | 1.07307668  | 21.62906243   | .000003308      | ENSG00000184792  |

**NOTE.** RNA sequencing analysis identified the top 100 up- and down-regulated genes that were affected by prolonged absence of mechanoluminal stimulation in human patients with ileostomies. ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; CFTR/MRP, cystic fibrosis transmembrane conductance regulator/multidrug resistance-associated protein; cGMP, cyclic guanosine monophosphate; EC, classic E basic helix-loop-helix protein; FERM, 4.1 protein/ezrin/radixin/moesin; FOS, Fos proto-oncogene, AP-1 transcription factor subunit; GCN5, gene control of amino acid synthesis protein 5-like 2; HGNC, HUGO Gene Nomenclature Committee; MAM, meprin/A5 protein/receptor protein-tyrosine phosphatase mu; RAR, retinoic acid receptor; RAS, retrovirus-associated DNA sequences; TRHDE, thyrotropin releasing hormone degrading enzyme.
Enteric Diversion Increases LGR5 mRNA Expression and Alters Downstream Wnt/β-Catenin Target Genes in the Intestine Lacking Mechanoluminal Flow

Given the diminished villus height/crypt depth, reduced cellular division within the transit-amplifying zone, and broad decrease in cell proliferation genes in unfed intestine, we sought to elucidate the effect of mechanoluminal deprivation on the ISC population. Several ISC markers recently were identified for both proliferative, more damage-sensitive crypt-based cells (LGR5, ASCL2, SOX9, OLFM4, MSI1, and SMOC2) and a class of more damage-resistant +4 stem cells (BMI1, LRIG1, HOPX, and TERT). Only LGR5 mRNA had a more than 1.5 log2-fold change difference in expression on RNA sequencing analysis, with increased expression in the unfed limb (NCBI Gene Expression Omnibus GSE82147, log2-fold change = 1.68; P = .01). In situ hybridization confirmed increased expression of LGR5 mRNA within ISCs within the crypts of unfed intestine compared with

Absence of Mechanoluminal Stimulation in Diverted Small Intestine Is Associated With Increased Expression of Digestion, Metabolism, and Transport Genes

RNA sequencing genome-wide analysis (NCBI Gene Expression Omnibus, GSE82147) was performed on 5 pairs (patients F–I) to identify biological processes affected by the absence of mechanoluminal stimulation. The top 100 up-regulated and down-regulated genes were identified (Table 2). On principal component analysis of all sequenced genes, unfed limbs clustered more closely than fed limbs, although there still was overlap of the fed and unfed limbs (Figure 3A). Of the 22,007 genes sequenced, 648 had significant differences in expression between paired fed and unfed limbs, with a false-discovery rate corrected P value less than .05 (Figure 3B). This included protein-coding transcripts and non–protein-coding transcripts, for example, long non-coding RNA, processed and polymorphic pseudogenes. Eighty-five percent of these significantly different genes were up-regulated in the unfed limb. A total of 191 had a log2-fold change greater than 1.5 or less than -1.5. Validation of selected target genes (LGR5, COL2A1, NAT8, FMO1, IL22, REG1B, and IL1B) with qPCR confirmed significant differences in expression with high correlation between the RNA sequencing and qPCR data (Figure 3C) (r > 0.9; P < .01).

Significantly different genes were compared with the Homo sapiens gene annotations of biological processes in the Gene Ontology Consortium database to determine which processes were over-represented and which were under-represented. A process was over-represented if significantly more of our differently expressed genes annotated to that process than in the background database. In the unfed limb, Gene Ontology analysis of significantly up-regulated genes showed that digestion, nutrient transport, and absorption, particularly of fatty acids and cholesterol, were over-represented pathways (Figure 4A and Supplementary Table 1). Conversely, nucleic acid synthesis and transcription were under-represented pathways (Figure 4B and Supplementary Table 2). Significantly down-regulated genes were involved in immune system processes, inflammation, angiogenesis, cell proliferation, and apoptosis (Figure 4C and Supplementary Table 3). No processes were significantly under-represented by these down-regulated genes.

time of diversion and the percentage difference in villus height and crypt depth. There was not any difference between infants who received breast milk vs formula, or between infants who weaned off or continued parenteral nutrition preoperatively vs those who did not. There was no correlation with the duration of total parenteral nutrition after ileostomy reversal and the percentage difference in villus height and crypt depth. The overall decrease in villus height was not associated with a measurable difference in differentiated epithelial cell types in the villus. There was no difference in the number of MUC2+ goblet cells (Figure 2E–G) (n = 5; P = .19) or chromagranin A+ endoendocrine cells (Figure 2H–J) (n = 5; P = .19) per hemivillus between fed and unfed intestine, suggesting that enterocytes were fewer in number in unfed intestine. In the crypts, however, unfed intestine had significantly fewer LYZ+ Paneth cells (Figure 2K–M) (n = 6; P = .03).

Crypt proliferation and villus apoptosis then were examined to explain the differences in villus and crypt size. Ki67+ immunofluorescence staining showed a 0.6%–21% decrease in the percentage of proliferating cells in the crypt of unfed intestine (Figure 2N–P) (n = 5; P = .03). Analysis of apoptosis by CC3+ staining showed that in both limbs, apoptosis was a rare event with no significant difference in the numbers (Figure 2Q–S) (n = 4; P = .41).

Table 4.qPCR Primer List

| Gene   | Primer sequence                           |
|--------|------------------------------------------|
| LGR5   | F: GCCCTTCCAGTGCCAAAG                    |
|        | G: TTGGCACCAGTAGCAGGCTTCAGTGGTC          |
| CCND1  | F: GGCAGGAACTGTGACTC                     |
|        | G: TCCTCTTTTTCTACGGTGTC                  |
| C-MYC  | F: GCCCTTAGCCAGCTGTTA                    |
|        | G: TGCAGTTAGCCAGGCTGGAT                 |
| REG1b  | F: GCCTCCATGACCCAAAAA                    |
|        | G: TTTCAGGTTAGGGCCACGATAG               |
| IL1B   | F: TACCTGTCCTGCCTGTTGAA                  |
|        | G: TCTCTGTCCTGCCTGTTGAA                 |
| COL2A1 | F: GTGAACCTGTGTCCTGGTGC                  |
|        | G: TTCCAGGTTTCAGCTGTC                   |
| NAT8   | F: GGACCAAGGGTACAGTGGAAT                |
|        | G: TGTTAGAGGGCCATAGCA                   |
| IL22   | F: CAAAGGCTAAGCAGACATGTC                |
|        | G: ACTGTCCTGCTGCTGTTG                   |
| FMO1   | F: TTGGACCAAGAAATTACAAAGAG              |
|        | G: TTCCAGGACAGCACCTGTTGAG              |

NOTE. Forward (F) and reverse (R) primer sequences for genes of interest in the study are provided.
Figure 2. Absence of mechanoluminal stimulation decreases villus height, crypt depth, and crypt proliferation. (A–D) H&E staining and morphometric analysis of matched fed vs unfed intestine from each patient. (E–S) Immunofluorescence staining and quantification of (E–G) MUC2+ goblet cells per hemivillus, (H–J) chromogranin A (CHGA)+ enteroendocrine cells per hemivillus, (K–M) LYZ+ Paneth cells per crypt with E-cadherin (Ecad) counterstain, (N–P) percentage of Ki67+ cells in crypts, and (Q–S) CC3+ cells per 10 villi. Images are representative pairs from different patients (n=5–7 pairs of intestine for quantification). Images were obtained on an upright Leica DM5500B immunofluorescence microscope using Leica Suite Advanced Fluorescence (LAS AF) 6000 software, processed with ImageJ software. Scale bars: 100 μm. *P < .05. Grey bars on plots indicate median with 95% confidence interval.
LGR5 regulates the Wnt/β-catenin pathway,39 which promotes transcription of cell-cycle regulators CCND1 and MYC. qPCR confirmed significantly increased mRNA expression for these genes in the unfed limb (Figure 5C and D) (n = 6; P = .03 for CCND1 and P = .02 for MYC). Upstream of LGR5, previous studies have shown the importance of signal transducer and activator of transcription 3 (STAT3) function in the survival and proliferation in intestinal stem cells.40,41 Furthermore, phosphorylation of STAT3 also induces expression of CCND1 and C-MYC.12 Consequently, Western blot analysis of STAT3 phosphorylation was performed and confirmed increased STAT3 phosphorylation in unfed intestine (Figure 5F) (n = 5; P = .03).

**Mechanoluminal Deprivation Decreases Intestinal Stem Cell Populations**

LGR5 had a more than 1.5 log2-fold change difference in expression on RNA sequencing analysis, with increased expression in the unfed limb (NCBI Gene Expression Omnibus, GSE82147). Immunofluorescence staining for LGR5 then was performed to localize LGR5 expression and quantify ISC. Positive immunofluorescence staining for LGR5 was found at the crypt bases in non-Paneth cells (Figure 6A–F). Although RNA sequencing analysis showed that LGR5 mRNA expression was increased in the unfed limb, quantification of ISC actually showed 8%–37% fewer ISCs per crypt in unfed intestine (Figure 6G) (n = 5; P = .03). Co-staining with proliferative marker proliferating cell nuclear antigen (Figure 6A, B, D, and E) also showed that 1%–60% fewer LGR5+ cells were proliferating in unfed intestine (Figure 6H) (n = 5; P = .03). Other ISCs showed changes in expression levels between fed and unfed intestine that were statistically significant; however, their log2-fold change difference was less than 1.5. ASCL2 was up-regulated in unfed intestine (NCBI Gene Expression Omnibus GSE82147, log2-fold change = 1.49; P = .005), whereas HOX1 (log2-fold change = -0.80; P = .02) and LRIG1 (NCBI Gene Expression Omnibus GSE82147, log2-fold change = -0.43; P = .05) were down-regulated. BMI1, OLFM4, TERT, and SOX9 remained unchanged. Given the discordance between mRNA expression and LGR5+ cell numbers in the distal unfed limb, we evaluated SOX9 (Figure 6I and J) and OLFM4 (Figure 6L and M) expression to determine if other ISCs were affected in a similar manner. In the unfed limb, 40%–56% fewer SOX9+ (Figure 6K) (n = 4; P = .03) and 22%–31% fewer OLFM4+ cell numbers (Figure 6N) (n = 4; P = .03) were identified.

**Chronic Absence of Mechanoluminal Stimulation Is Associated With Increased Brush Border Function and Down-Regulation of Cell Proliferation, Inflammation, and Immune Process Genes**

To further investigate differential regulation of biological processes in chronically diverted intestine lacking mechanoluminal flow, we investigated gene families important in brush-border structure and activity, stem cell pathways,
Figure 4. Gene Ontology enrichment analysis of genes up-regulated and down-regulated by absence of mechanoluminal stimulation indicates significantly affected biological processes. Gene Ontology (GO) analysis of up-regulated genes (A) over-represented and (B) under-represented by withdrawal of mechanoluminal stimulation and (C) down-regulated genes over-represented by the absence of mechanoluminal stimulation with a false-discovery rate corrected $P$ value < .05. Over-represented pathways are shown, with circle size proportional to the number of genes included in the GO term. $P$ value is represented by the color scale. Arrows indicate hierarchical relationships with most generalized terms at the base.
immunologic function, and cell proliferation/death. Heatmap representation of these genes shows differential expression between the fed and unfed limbs (Figure 7A–E). Regarding the top 100 up-regulated and down-regulated genes in our data set, a vast majority were up-regulated genes intrinsic to brush-border enzymatic, transporter, or structural function (Table 2). Common brush-border enzymes TREH, MGAM, and SI, and transporters SLC15A1, SLC9A3, ABCG2, and ABCC2, were increased significantly in the unfed limb (P < .001) (Figure 7A).

Although LGR5 was the only stem cell marker gene with a log2-fold change greater than 1.5, we further characterized the changes in RNA expression among common ISCs and downstream targets (Figure 7B). LGR5 (NCBI Gene

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**Figure 5. Absence of mechanoluminal stimulation increases LGR5 mRNA expression and downstream Wnt signaling gene expression.** (A and B) Quantification and in situ hybridization of LGR5 ISCs within the crypts of fed and unfed intestine. *Inset:* High-magnification photographs label LGR5-positive in situ hybridization with purple arrows. qPCR comparison of (C) LGR5, (D) CCND1, and (E) MYC mRNA expression between 6 and 7 pairs of matched fed vs unfed intestine with outliers excluded. (F) Representative images of Western blot analysis of STAT3 phosphorylation from 3 pairs of matched tissue. Images were obtained on an upright Leica DM5500B immunofluorescence microscope using Leica Suite Advanced Fluorescence (LAS AF) 6000 software, processed with ImageJ software. Scale bars: 50 μm. *P < .05. Grey bars on plots indicate median with 95% confidence interval.
May 2017 RNA-Seq Human Matched Fed and Unfed Intestine

Fed and Unfed Intestine

A) PCNA/LGR5
B) PCNA/LGR5
C) ECAD/LYZ
D) PCNA/LGR5
E) ECAD/LYZ

G) LGR5+ Cells per Crypt
H) LGR5+/PCNA+ Cells per Crypt (%)

I) SOX9
J) SOX9

K) SOX9+ Cells per Crypt

L) OLFM4
M) OLFM4
N) OLFM4+ Cells per Crypt

Patient Legend:
A: ● H: ▽
E: △ I: ♦
C: ■ J: ◇
F: △ L: ◇
G: ▽ O: ◇
Expression Omnibus GSE82147, log₂-fold change = 1.68; P = .01 and ASCL2 (NCBI Gene Expression Omnibus GSE82147, log₂-fold change = 1.49; P = .005) were noted in unfed intestine. Conversely, a down-regulation in LRIG1 (NCBI Gene Expression Omnibus GSE82147, log₂-fold change = -0.43; P = .05) and HOPX (NCBI Gene Expression Omnibus GSE82147, log₂-fold change = -0.80; P = .02) was identified. SOX9, BMI1, OLFM4, PTEN, MTOR, and TERT remained unchanged.

Significantly different genes with log₂-fold change greater than 1.5 involved in immune cell processes, cell proliferation, and apoptosis are shown (Figure 7C–E). Because all samples were obtained from children with previous NEC or focal intestinal perforation, we sought to determine whether the significant genes associated with immune and inflammatory processes were chronic changes induced by the initial inflammatory insult. Our data were compared with previously reported RNA sequencing–based gene expression profiles from preterm infants with NEC.26 Comparison of significant pathways in the 2 gene sets showed that inflammation and regulation of inflammation both were over-represented. However, of the 98 genes that were significantly different in both gene expression profiles, only CXCL5 and REG1B had a more than 1.5-fold difference in expression levels in both gene expression profiles.

RNA sequencing identified several growth factors with decreased expression in unfed intestine, such as WNT2 (NCBI Gene Expression Omnibus GSE82147, log₂-fold change = -2.13; P < .001) and FGFR5 (NCBI Gene Expression Omnibus GSE82147, log₂-fold change = -2.09; P < .001). Of interest, several genes involved in cell proliferation also were involved in immune processes and inflammation. IL1B, IL22, and Reg1B are associated with intestinal inflammation and increased epithelial proliferation.11,43–46 qPCR confirmed significantly decreased expression of IL22 (Figure 7F) (n = 7; P = .04) and REG1B (Figure 7G) (n = 6; P = .03) in unfed intestine, but not IL1B (Figure 7H) (n = 7; P = .31).

Discussion

In pediatric human small intestine, we showed that extended absence of mechanoluminal flow in diverted small intestine leads to decreased epithelial cell and ISC proliferation within the crypt, up-regulation of metabolism and transport genes, and down-regulation of inflammatory, immune, and proliferative genes. These effects occur despite adequate caloric intake and provide insight into the importance of mechanoluminal stimulation for normal intestinal adaptation and maintenance of the intestinal epithelium and ISCs. Surgical treatment of acute and chronic intestinal insults often results in diversion of enteral flow. Diversion can vary in location within the gastrointestinal tract and can be variable in time, from short-term withdrawal of enteral feeding, affecting the entire gastrointestinal tract, or partial diversion from an ostomy, resulting in distal deprivation of mechanoluminal flow. Small case series and individual reports have shown improved weight gain and weaning from parenteral nutrition in SBS infants who received enteral nutrition in the distal mucous fistula.47 However, refeeding of the distal diverted intestine is not widely practiced and the timing and method of enteral feeding in surgical patients remains a contested issue because of conflicting evidence and regional variations in accepted protocols.

Our study suggests that in human beings, mechanoluminal stimulation is required to prevent intestinal atrophy and maintain intestinal homeostasis independent of systemic caloric repletion. In Drosophila, enteral feeding increases gut size and cell number, which is absent during fasting.48 Starvation is associated with a decrease in villus size, crypt size, and mitotic activity in rats.49 Calorically restricted mice show decreased mass, villus height, and number of enterocytes within the small intestine.50 Although infants in our study received adequate caloric intake to promote appropriate weight gain, distal diverted intestine still showed cytarchitectural characteristics representative of acute animal models of caloric restriction or starvation, including decreased epithelial cell proliferation, villus height, and crypt depth in unfed intestine (Figure 2).

It is unclear if and how mechanoluminal deprivation affected intestinal diameter, length, and mesenchymal components. Neither initial resection specimens representative of the patient’s baseline nor full circumferential sections from proximal and distal limbs after diversion were available for comparison of these parameters. However, thinner muscularis mucosae and differences in genes encoding mesenchymal components such as type II collagen were found in unfed intestine on RNA sequencing analysis. The intestinal stroma recently has been identified to provide an intestinal stem cell niche in the absence of epithelial Wnts, suggesting that the stroma can fully support murine intestinal homeostasis.49 Mechanical and biochemical signaling from the underlying mesenchyme also is believed to play a crucial role in dynamically altering signaling gradients that promote the formation of villi and restrict the ISC population to the base of the crypts.50 In a porcine model, muscle hypertrophy as a result of massive small-bowel resection potentially helps improve weight gain by slowing

Figure 6. (See previous page). Global decrease of LGR5+, SOX9+, and OLFM4+ intestinal stem cell populations occurs in the absence of mechanoluminal flow. (A–H) Quantiﬁcation and immunofluorescence staining of LGR5+ rapidly cycling intestinal stem cells per crypt with proliferating cell nuclear antigen counterstain in fed vs unfed intestine. (C and F) LYZ + Paneth cells with E-cadherin (Ecad) counterstain of same crypt shown in panels A and D, respectively, for comparison. (I–N) Quantiﬁcation and immunofluorescence staining of SOX9 and OLFM4 intestinal stem cells per crypt in fed vs unfed intestine. Images were obtained on an upright Leica DM5500B immunofluorescence microscope using Leica Suite Advanced Fluorescence (LAS AF) 6000 software, processed with ImageJ software. Scale bars: (B, C, E, and F) 50 μm, and (A, D, I, J, L, and M) 100 μm. *P < .05. Grey bars on plots indicate median with 95% conﬁdence interval. PCNA, proliferating cell nuclear antigen.
gastrointestinal motility and subsequent increase in mucosal absorption.\textsuperscript{51,52} Thus, changes in both the intestinal epithelium and the underlying stroma may affect the adaptive capacity of the proximal and distal limbs of the intestine during chronic diversion. Adaptive mechanisms within the stroma that augment epithelial cell proliferation and function may provide useful clinical targets for treating patients with SBS and intestinal failure.

Our study examined a unique intestinal diversion model in which the effect of mechanoluminal stimulation on

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**Figure 7.** Heatmap representation of biological processes of interest shows differential expression of significant genes between fed and unfed intestine. (A–E) Heatmap representation of significantly different genes within biological processes of interest for the 5 pairs analyzed by RNA sequencing. One gene is graphed in each row and one intestinal sample is graphed in each column. Color represents the expression level with increased expression in green and decreased expression in magenta. (F–H) qPCR comparison of pro-proliferative inflammatory genes IL22, REG1B, and IL1B mRNA expression between 6 and 7 pairs of matched fed vs unfed intestine with outliers excluded. \*P < .05. Grey bars on plots indicate median with 95\% confidence interval.
intestinal adaptation could be examined without the confounding factor of systemic caloric status, showing an unexpected intestinal stem cell marker expression pattern potentially linked to the relative plasticity and interconversion of various ISCs. Immunofluorescence staining showed a decrease in proliferation that affected differentiated cells in the crypt more than in the villus. Significantly fewer Paneth cells and ISCs were noted in unfed segments, but neither goblet cell nor enteroendocrine cell numbers changed (Figures 2 and 6). Overall, this may represent a shift in differentiation toward a secretory lineage and away from the absorptive lineage. Paneth cells secrete several proliferative signals, including Notch ligand Dll4, epidermal growth factor, transforming growth factor-α, and WNT3 to regulate neighboring ISC activity in the intestinal crypt. Acute caloric restriction in mice has been shown to increase Paneth cell and ISC number while also increasing Paneth cell ability to enhance ISC proliferation and regeneration, leading to intestinal adaptation. In contrast, we found fewer Paneth cells and ISCs in chronically unfed intestine, although, curiously, LGR5 expression, as shown by qPCR and in situ hybridization, showed increased expression within individual LGR5+ cells. Notably, there appeared to be a decrease in numerous ISC types, including SOX9, OLFM4, and LGR5 stem cells, as shown by immunofluorescence staining (Figure 6). RNA sequencing also showed significant increases in ASCL2 with a concomitant down-regulation of LRIG1 and HOPX.

The adaptability of the intestine during states of injury and acute loss of rapid-cycling ISCs may relate to the relative plasticity of several reserve or dormant stem cell populations that reside within the crypt. Cells within the transit-amplifying zone that are in direct contact with Paneth cells can transform into LGR5+ ISCs during damaged states to repopulate this population. LGR5+ cells can be restored from conversion of HOPX+ cells during acute injury. BM1, TERT, HOPX, and LRIG1 have shown significant expression levels within LGR5+ cells, suggesting that all 4 of the +4 markers do not appear to define a single class of ISCs and could play potential roles in interconversion to repopulate lost rapid-cycling ISCs. Given the significant decrease in HOPX and LRIG1 shown in our RNA sequencing analysis, these ISC populations could be contributing to restoration of rapid-cycling LGR5+ stem cells.

On RNA sequencing analysis, we also observed a significant increase in expression of ASCL2, a direct Wnt pathway target. Wnt signaling is well described as a modulator of intestinal stem cell identity. However, how the crypt to villus signaling gradient is sensed to define epithelial cell types is less understood. ASCL2 has been reported recently to control ISC stemness by acting as a bimodal switch, forming an autoactivating loop in response to the Wnt signaling gradient, and thereby determining transcriptional activation or repression of downstream Wnt targets. Ectopic expression of ASCL2 induces hyperproliferation of crypts and expansion of SOX9+ and LGR5+ cells within the crypts. In addition, spatial positioning of LGR5+ stem cells within the crypt may affect their availability to enter the cell cycle and influence their propensity to undergo symmetric vs asymmetric division. Given the discordance identified in increased LGR5 mRNA expression and decreased cell number (Figures 5 and 6), it is plausible that ASCL2, another rapid-cycling ISC, regulates LGR5 downstream effectors in a similar fashion in chronically diverted intestine lacking luminal flow and acts as a master regulator to prime rapid-cycling ISCs in a ready state to contribute to ISC proliferation and epithelial cell differentiation upon the re-introduction of luminal contents.

How ISCs respond to acute changes in intraluminal nutrient sensing and re-feeding remains relatively unknown. In Drosophila, feeding activates ISCs, accelerates division rates, and favors symmetric rather than asymmetric division so that the number of stem and total cells increase. In contrast, an increased number of LGR5+ intestinal progenitors and Paneth cells are found in calorically restricted mice when compared with calorically replete mice. Moreover, crypts from these calorically restricted mice show enhanced proliferation and regeneration in the formation of organoids. Richmond et al recently showed that acute nutrient deprivation in mice induces phosphorylation of PTEN in mTert+ ISCs, which release inhibitory signaling of the phosphoinositide 3-kinase/mechanistic target of rapamycin pathway and promotes significant proliferation of the dormant ISC population. We did not observe significant differences in the expression of TERT, BM1, PTEN, MTO1, or PI3KCA in the distal limb on RNA sequencing analysis. However, our tissues were obtained from nutritionally replete patients who had undergone diversion for more than 7 weeks, compared with the 48-hour fasting period in the murine model described previously. Although the observations identified by Richmond et al likely may have occurred very early during initial diversion in our patient population, our results reflect mechanisms of intestinal homeostasis and adaptation that occur during chronic absence of mechanoluminal stimulation in a nutritionally replete population. Therefore, enteric feeding and whole-body caloric status may reflect 2 distinct but important activators of ISC proliferation, and controlled animal models investigating chronic mechanoluminal diversion may begin to allow for greater elucidation of luminal signaling mechanisms that could regulate ISC proliferation and intestinal homeostasis. Given our results, we hypothesize that intraluminal cues provided by mechanoluminal stimulation may independently drive intestinal adaptation and regeneration and that absence of mechanoluminal stimulation, particularly in the setting of adequate caloric intake, drives ISC into a less-proliferative, hibernative state.

Numerous biological processes significantly up-regulated and down-regulated by the absence of mechanoluminal stimulation indicate that nutrient sensing, biliary secretions, and immune and inflammatory signals all likely play a role in intestinal adaptation. Without mechanoluminal stimulation, unfed intestine increases the expression of genes involved in digestion, transport, and metabolism, perhaps as a feedback loop to optimize and prime nutrient extraction to rapidly accommodate future refeeding.
Interestingly, although protein and carbohydrate pathways are up-regulated, more up-regulated pathways are involved in fatty acid and cholesterol absorption and metabolism. In our RNA sequencing analysis, we identified up-regulation of oxoicosanoid-receptor 1, a G-protein-coupled receptor, which binds long-chain polyunsaturated fatty acids, in the unfed limb. In embryonic stem cells, unsaturated lipids are crucial for maintenance. Embryonic stem cells express a unique lipid profile high in unsaturated lipids and fatty acids that decrease in differentiated cell states. Upon inhibition of the eicosanoid pathway, pluripotency in embryonic stem cells is maintained as a result of retained levels of unsaturated fatty acids.65 High-fat diets have been shown to enhance stemness by increasing the number and function of LGR5+ cells through activation of peroxisome proliferator-activated receptor-δ signaling.66 High-fat diets, and omega-3 fatty acids in particular, also have been shown to enhance adaptation after massive small-bowel resection in rodent models.68–70 In infants with SBS, fish oil-based intravenous lipid emulsions help ameliorate or reverse cholestasis associated with parenteral nutrition.70,71 However, no studies have addressed the effect of high-fat enteral diets on adaptation, enteral independence, or weight gain. Because unfed intestine seems primed to absorb and metabolize fats, the effect of high-fat enteral diets should be investigated further.

Although biliary secretions were diverted from unfed intestine, several up-regulated genes also were involved in bile acid metabolism or are associated with hepatic biological processes. For example, NAT8 and FMO1 encode enzymes that have been reported previously in fetal liver, but not intestine.72,73 In vivo, NAT8 transfection into hepatocytes increases resistance to apoptosis after injury.73 Little is known about the function of FMO1 in human intestine, but the adult isoform FMO2 in nematodes can be activated by dietary restriction and hypoxia to increase nematode life span.74 Thus, absence of mechanoluminal stimulation induces the expression of genes not previously identified in human intestine. These enzymes may serve a protective role but the function and effect of these novel factors requires further investigation.

The down-regulation on RNA sequencing analysis of genes such as IL22 involved in inflammation and immune system processes in unfed intestine was somewhat surprising because, clinically, diversion can be associated with inflammation. Except for REG1B and CXCL5, none of the significantly different genes with fold change greater than 1.5 involved in immune cell processes and inflammation also was significant in active NEC transcriptome analysis.75 Thus, although NEC resolution seems to be associated with induction of distinct immune process genes in fed intestine, absence of mechanoluminal stimulation in unfed intestine may prevent the same immune process activation. This difference in immune process activation may contribute further to the decreased proliferation in unfed intestine. Interleukin 22 via STAT3 signaling has been shown to promote ISC-mediated regeneration in vivo and increase pancreatic expression of REG1, which itself promotes cell-cycle progression and regeneration.44,76 Several other significant genes also are involved in both immune processes and cell proliferation, such as CXCL5, IL1B, and EDN2. Up-regulated genes also were involved in xenobiotic responses and there is evidence that the microbiome is a major contributor to epithelial integrity and homeostasis.76,77

We previously studied whether there was a difference in the pediatric microbiome between fed and unfed intestine. We observed no consistent difference; in fact, the microbiota of the unfed intestine most resembled its paired fed intestine.78 Thus, mechanoluminal stimulation also may be required for immune recovery, cross-talk with the microbiome, and/or maintenance and proliferation of ISCs after disease insult. Multiple growth factors have been identified that enhance adaptation in animal models, such as growth hormone, insulin-like growth factor-1, epidermal growth factor, glucagon-like peptide 2, and steroids.10 Of these, growth hormone and glucagon-like peptide 2 have shown promising results in improving nutrient absorption and weight gain in human patients with SBS.11–13 In our study, RNA sequencing analysis did not show any significant difference in levels of mRNA for growth hormone receptor, epidermal growth factor, insulin-like growth factor-1, epidermal growth factor receptor, or glucagon-like peptide 2 receptor. Growth hormone and glucagon-like peptide 2 were not among the sequenced genes. Genes involved in steroid secretion and response to steroids were significantly down-regulated in unfed intestine. Thus, the factors and pathways affecting adaptation after small-bowel resection may differ from those affected by mechanoluminal stimulation. Alternatively, different factors may peak at variable times and after several weeks of diversion, expression of these previously identified growth factors already may have waned. However, it is possible that the RNA sequencing analysis may not have been powered adequately to detect differences in these humoral factors, or that protein expression and pathway activation may have been significantly different without differences in mRNA expression. As with most studies of complex processes in human tissue, these data are limited by the small sample size, finite tissue specimens, and the inability to define the cellular location of the transcriptional events. To minimize variability and confounding resulting from patient factors, a very narrow set of patients was studied and internally controlled by accessing paired specimens in close anatomic approximation to avoid regional physiologic and genetic differences. Because intestine is undergoing rapid development during infancy and childhood, the mechanisms driving and regulating proliferation and adaptation in adult intestine may be different.

Nonetheless, these data provide new insight into the human intestinal response to chronic absence of luminal stimulation and the signaling that governs small intestine proliferation and adaptation. The broad effects of mechanoluminal deprivation on intestinal structure and function emphasize the importance of enteral nutrition in the treatment of short-bowel syndrome. Overall, nutrition status and enteral flow provide 2 distinct environmental cues for the intestine with important effects on intestinal homeostasis.
and adaptation. As we seek to better understand the mechanisms driving intestinal adaptation and regeneration, these data may enable future identification of novel therapeutic targets for treatment of patients with SBS and intestinal failure.

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Supplementary Figure 1. (A) H&E analysis of full-thickness intestinal tissue sections verified that the stroma between fed and unfed intestine remained otherwise nonreactive with no marked changes in cytoarchitecture outside of (B) a significant decrease in the thickness of the muscularis mucosae. Scale bars: 200 μm. *P < .05.