Orthotopic transplantation of intestinal mucosal organoids in rodents

Jeffrey R. Avansino, MD,[a] David C. Chen, MD,[b] Vicki D. Hoagland, BS,[a] Jacob D. Woolman, BA,[a] *and* **Matthias Stelzner, MD,[a,b]** *Seattle, Wash, and Los Angeles, Calif*

Background. *Orthotopic transplantation of intestinal mucosal organoids that contain putative mucosal stem cells serves as an important step toward implementing intestinal gene therapy and treatment for malabsorption syndromes in animals and humans. We hypothesized that intestinal mucosal organoids can be transplanted along the axis of the small bowel giving rise to a neomucosa expressing proteins of its donor origin.*

Methods. *Epithelial organoids were harvested from neonatal mice or rat small intestine with the use of a combination of enzymatic digestion with dispase and collagenase, and gravity sedimentation. In adult syngeneic recipients, a 7-cm segment of midjejunum was isolated, leaving its vascular pedicle intact. The remaining proximal and distal segments were anastomosed to restore intestinal continuity. The isolated segments were randomly subjected to surgical or chemical mucosectomy with a chelator solution for 30, 45, or 60 minutes and then compared. Histologic examination was used to confirm the presence of enterocytes, goblet cells, enteroendocrine cells, and Paneth cells in the neomucosal segments. To confirm the presence of ileal bile acid transporter (IBAT) gene message and function, we measured sodium-dependent bile acid uptake and IBAT-messenger RNA. Immunohistochemical examination using anti-IBAT antibodies was performed to demonstrate the expression of IBAT in the neomucosal segments. Experiments were repeated in a murine model transgenic for the green fluorescent protein to verify donor origin of the engrafted mucosa expressing IBAT.*

Results. *The area of peak IBAT function was found to be located in the terminal ileum. Organoid units harvested from this region were capable of generating a small-bowel neoileal mucosa after being seeded into the jejunum. This mucosa was histologically confirmed to differentiate into all 4 intestinal lineages and to express IBAT signal, confirming its donor-derived origin. Optimal engraftment of mucosa expressing the IBAT protein was found in isolated jejunal segments debrided for 45 minutes. Sodium-dependent bile acid uptake was 5-fold higher in the neoileum, compared with the jejunum. IBAT-mRNA levels in the neoileum were 18-100–fold higher than those in the jejunum. Areas of green fluorescent protein–positive mucosa stained positively with anti-IBAT antibody in adjacent sections, suggesting that the regenerated mucosa is from transplanted ileal stem cells.*

Conclusions. *Orthotopic transplantation of epithelial organoids containing ileal stem cells was used to generate a neoileal mucosa that expressed all 4 intestinal lineages along with a new zone of active bile acid uptake and IBAT expression in a recipient jejunal segment.*

(Surgery 2006;140:423-34.)

From the Department of Surgery, University of Washington, VA Puget Sound Health Care System,[a] and the Department of Surgery, VA Greater Los Angeles Health Care System, University of California at Los Angeles[b]

Supported by a VA Merit Review Grant and a G. H. A. Clowes Research Career Development Award from the American College of Surgeons.

Accepted for publication March 16, 2006.

Reprint requests: David C. Chen, MD, Dept of Surgery, University of California at Los Angeles, 10833 Le Conte Ave, Los Angeles, CA 90024. E-mail: dcchen@mednet.ucla.edu.

0039-6060/$ - see front matter

doi:10.1016/j.surg.2006.03.012

ORTHOTOPIC TRANSPLANTATION of small intestinal mucosal organoids that contain putative mucosal stem cells offers therapeutic promise for congenital and acquired malabsorptive syndromes such as glucose-galactose malabsorption or bile acid malabsorption. Moreover, it represents an important step toward intestinal gene therapy directed at conditions other than malabsorption. In addition, methods to transplant mucosal stem cells may enable studies of epithelial/mesenchymal interactions and signals responsible for stem cell differentiation in the intestine in vivo.

Intestinal mucosal stem cells are believed to occupy the lowest positions at the base of the crypt[1-3] and give rise to daughter cells that generate all 4 lineages of the small intestinal mucosa (Paneth, absorptive, goblet and enteroendocrine cells).[4] Intestinal epithelial stem cells appear to require the presence of mesenchymal cells to proliferate and divide in vitro.[5] Successful transplantation of the putative stem cells requires isolation in the form of stem cell/mesenchymal cell aggregates ("epithelial organoids") rather than as individual stem cells.[6]

Transplantation into the small bowel has broad clinical and scientific applications. These applications prompted us to investigate whether orthotopic engraftment of intestinal mucosal organoids in the small intestine is feasible and whether expression of specific small intestinal proteins is maintained in the resulting mucosa. Ileocytes feature most of the main membrane transport systems found in jejunocytes but uniquely express the ileal bile acid transporter (IBAT).[7,8] Since IBAT is not expressed in the jejunal mucosa even if the ileum has been removed, surgical resection of the ileum results in a syndrome of bile acid malabsorption (BAM).[9,10] BAM can occur in patients with inflammatory conditions of the ileum or with cystic fibrosis but is most common after ileal resection. It causes diarrhea and other serious conditions such as gallstones, nephrolithiasis, growth retardation and weight loss.[10-12] In addition, elevated levels of bile acids in the colon have been associated with increased risk of colon cancer.[13,14] Successful transplantation of ileal organoids into the jejunum can be used to treat BAM by generating a segment with neoileal mucosa. Such a segment can act as a substitute ileum.[15]

Intestinal stem cell grafting has been applied in rat studies by using colon segments as the recipient site after surgical mucosal stripping in addition to seeding of biodegradable polymers.[16-18] Grafting of cells into the colon and biodegradable polymers is disadvantageous for clinical application since the colon and tissue-engineered intestine have very different motility patterns, compared with the small intestine. Normal propulsive activity is important for optimal bile acid absorption.[19] Stasis of fluid in the lumen results in bacterial overgrowth, deconjugation of bile acids, and diarrhea.[20]

Ileal organoid transplantation provides a potential means of correcting BAM and proof of the principle that transplantation is feasible. Finally, a sound methodology for orthotopic intestinal mucosal organoid transplantation may be an important step toward intestinal gene therapy.

MATERIAL AND METHODS

Animals. Male Lewis rats weighing 220 to 250 gm and mature females with litters were obtained from Harlan Laboratories (Indianapolis, Ind). Wild-type and GFP-transgenic mice (wild type = C57BL/6J; transgenic = C57 BL/6-TgN (ACTbEGFP) 1Osb) were taken from an in-house breeding colony in the Seattle VA Medical Center. The founder mice had been obtained from Jackson Laboratories (Bar Harbor, Me). Rats received regular rodent chow (Nestle Purina, St. Louis, Mo) and water ad libitum, while mice received microstabilized liquid rodent diet (Test Diet, Richmond, Ind) for the first week and high-fat breeder chow (Nestle Purina) and water ad libitum thereafter. All animals were kept in a 12-hour light-dark cycle (light from 6 AM until 6 PM). All animals were acclimatized to the vivarium environment and were allowed to adjust for at least 2 days before experimentation commenced. All procedures were approved by the institutional review committees at the Seattle and West Los Angeles VA medical centers.

Mapping of IBAT expression in neonatal rat small intestine. Two 5- to 7-day old neonatal Lewis rats were sacrificed, and IBAT expression was examined by using a "Swiss roll" technique previously described by Stelzner et al.[21] In brief, the small intestine was harvested from the ligament of Treitz to the ileocecal valve and immediately rinsed with cold mammalian Ringer solution. In 1 animal, the intestine was subsequently opened along its antimesenteric side. The entire small intestine was then rolled, beginning at the jejunum, onto a center rod forming a Swiss roll. The ileal end was lightly marked with black ink to identify it on tissue sections. The rolled-up tissue was fixed in 70% alcohol and paraffin-embedded for permanent sections. In the second animal, the intestine was cut in cross-section at 1-mm intervals, and the sections were placed in tissue cassettes in series. These sections were fixed in 70% alcohol and paraffin-embedded for permanent sections.

For immunohistochemistry studies, polyclonal antibodies raised in rabbits against IBAT were obtained from a commercial source and purified as described.[28] Slides were deparaffinized in xylene, dehydrated in 100% ethanol, and then rehydrated. They were subsequently incubated in citrate buffer at pH 6.8 (10 mmol/L sodium citrate in distilled water [pH 6.8] with NaOH) at 95°C for 1 hour. Sections were then washed briefly in phosphate-buffered saline (PBS) and incubated for 5 minutes with 5% goat serum in PBS to reduce background staining. A 1:10 dilution of affinity-purified anti-IBAT antibody was applied to tissue sections; the sections were incubated at room temperature for 3 hours. Sections were washed in PBS and incubated with a 1:100 dilution of a secondary goat antirabbit Cy3-IgG antibody (Jackson Immuno-Research Laboratories, West Grove, Pa) for 30 minutes, and then washed in PBS. Slides were mounted with the use of an aqueous medium containing Hoechst 33258 dye as a nuclear stain (Sigma). The slides were evaluated for distribution of the fluorescence signal under a fluorescence microscope (Axioscope II; Zeiss, Thornwood, NY) equipped with a digital imaging and an intensity-measuring program (Axiocam, Zeiss). Sections of adult ileum and jejunum were used as positive and negative controls respectively. Preimmune serum was also used as a negative control.

Mapping of bile acid uptake in the rat small intestine. Five male Lewis rats were sacrificed; their small intestines were harvested and transferred in oxygenated, 2°C cold Ringer solution (128 mmol/L NaCl, 4.7 mmol/L KCl, 2.5 mmol/L $CaCl_2$, 1.2 mmol/L KH_2PO_4, 1.2 mmol/L $MgSO_4$, and 20 mmol/L $NaHCO_3$; pH = 7.30-7.40 at 37°C, 290 mOsm, gassed with continuous 95% O_2/5% CO_2). In each rat, the small intestine was divided into 1-cm sections from 0 to 10 cm by proceeding backwards from the ileocecal valve to the oral direction. Then, 2 segments, each 1-cm long, were obtained at 15-17, 25-27, 40-42, and 70-72 cm. In an alternating manner, segments adjacent to each other were then used for measurements of either sodium-dependent or sodium-independent uptake rates (ie, segment 0-1 cm = sodium-dependent; segment 1-2 cm = sodium-independent; segment 2-3 cm = sodium-dependent; and so forth).

Tritium-labeled taurocholate uptake was measured in each tissue section by using a standardized everted sleeve technique as described previously.[22,23] Briefly, the 1-cm tissue specimens were taken from the 2°C cold oxygenated mammalian Ringer solution. Specimens were everted and secured onto 4-mm-thick stainless steel rods with the use of silk ties and stored in oxygenated ice-cold Ringer solution. All uptake measurements were performed between 1 and 2 hours after excision. Mounted tissues were preincubated for 5 minutes in 37°C oxygenated Ringer solution and then incubated for 2 minutes in either sodium-containing or sodium-free Ringer solution with 1 mmol/L taurocholate. Sodium-dependent transport was defined as the difference between uptake rates in sodium-containing and sodium-free taurocholate-Ringer solutions.

To measure sodium-independent uptake, we prepared sodium-free Ringer solution by replacing NaCl with choline-Cl and $NaHCO_3$ with $KHCO_3$. Taurocholate in the sodium-free solutions was added as a potassium salt. To prepare potassium-taurocholate, we performed ion exchange of a 1 mol/L sodium-taurocholate solution with potassium-loaded AmberLyte (Biorad Laboratories, Hercules, Calif). The potassium salt was subsequently recrystallized. Absence of sodium ions after the ion exchange was confirmed by flame-photometry. Tracer amounts of radiolabeled taurocholic acid, [^{3}H-(G)] (PerkinElmer Life Sciences, Boston, Mass) were added to the uptake solutions. Polyethylene glycol, [1,2-^{14}C] (molecular weight, 4000 Da), a marker substance not subject to carrier-mediated transport and with a very low transmembrane diffusion coefficient, was also added to these solutions to account for ^{3}H-radiotracer in the adherent fluid.[23-25]

The length of the incubation period was chosen on the basis of initial validation experiments by using mouse and rat jejunal and ileal tissues as suggested by Karasov and Diamond.[25] These validation studies showed that, at 2 minutes, the uptake of radioactive taurocholate still showed linear increases over time while complete equilibration of radioactive PEG had occurred (data not shown).

After incubation, tissues were removed from the rods, placed in scintillation vials, weighed, and solubilized in 730 μL TS-2 tissue solubilizer (Research Products International, Mount Prospect, Ill). Vials were incubated at 58°C until the tissues were completely dissolved, and 5 mL acidified Safety-Solve scintillation cocktail (Research Products International) was added. Beta emissions (in disintegrations per minute) from tritium and ^{14}C were counted in a liquid scintillation counter (Tricarb 2200 CA; Packard Instrument Co, Downers Grove, Ill).

Uptake ratios were normalized to true mucosal surface area with the use of a surface amplification index (see details below) to account for differences in villus and crypt morphology along the axis of the

small intestine. Values were expressed as follows: pmol/min × mm^2.

Isolation of organoids. Intestinal organoids were isolated as described by Evans et al.[26] In brief, 5- to 7-day-old rats and mice were sacrificed by cervical dislocation, and the entire neonatal small bowel was resected. For ileal organoids, the terminal one fifth of the small bowel was harvested. The harvested intestine was opened longitudinally and cut into 2-mm long segments. These intestinal pieces were copiously washed 8 times in 50 ml of Hanks' buffered saline solution (HBSS). The washed segments were minced into pieces smaller than 1 mm^3, transferred into 20 ml HBSS with 0.1 mg/mL dispase type 1 (Roche, Indianapolis, Ind) and 300 units/mL collagenase type XI-S (Sigma), and rocked continuously for 25 minutes at 22°C. The suspension was shaken vigorously for 1 minute and allowed to sediment out for 1 minute. The supernatant was carefully transferred to a new tube and the pellet discarded. This process was repeated 3 times, clearing the suspension of larger debris. The cleared solution was then mixed with an equal amount of Dulbecco modification of Eagle medium-sterile (DMEM-S) tissue culture medium. The following was added to the 0.45% glucose DMEM-S (Gibco, Gaithersburg, Md): 2% D-Sorbitol (Sigma), 2.5% fetal bovine serum (Hyclone, Logan, Utah), 100 IU/mL penicillin + 100 μg/mL streptomycin (Gibco). The modified DMEM-S/supernatant solution was centrifuged (10 g) for 2 minutes at 300 rpm at room temperature. Contents were allowed to sediment at normal gravity for 30 seconds and the supernatant discarded. The pellet was washed with 50 mL of DMEM-S and centrifuged as described previously for 6 times, or until the supernatant was no longer turbid and the pellet was well defined. The pellet was then resuspended in DMEM and the organoids used for engraftment.

Surgical procedures. *Rats.* Adult male Lewis rats were used as recipients of rat ileal organoids. The animals were anesthetized with inhaled isoflurane. In 4 rats, a midline laparotomy was performed. An organoid suspension was prepared by mixing 5600 organoids in 0.1 mL of DMEM with an equal amount of extra cellular matrix gel at 4°C (Sigma). Two boluses of 0.1 mL of the mixture were injected between the anterior and posterior leaves of the omentum in different locations. The abdomen was closed, and the animals were allowed to recover. The segments of omentum were harvested after 2 weeks. In all other rats, a laparotomy was performed and the small intestine exteriorized on moist gauze.

In 8 rats, two 7-cm segments of midjejunum were isolated by proximal and distal transection of the bowel, and kept in the abdomen with blood circulation intact. The isolated segments were randomly subjected to surgical or chemical mucosectomy and compared in the same animal. For surgical mucosectomy, segments were opened at the antimesenteric border, and the mucosa was removed sharply with a scalpel under magnification. Suspended ileal organoids were then seeded onto the denuded areas of the jejunal segment. The jejunal segment was loosely approximated with 6-0 silk sutures (Ethicon, Somerville, NJ) and the abdomen closed. For chemical stripping, we developed a perfusion method of continuous vigorous flushing that resulted in a partial or complete removal of the epithelial cell layer with little damage to the muscularis propria and serosal layers. We modified a technique first described by Weiser[26] for in vitro studies. We determined that 40 mL/min was the fastest achievable flush rate we could use with our system without damaging the gut. The mucosa of the jejunal segment was cannulated on either end and first flushed for 5 minutes at that rate with a 0.9% NaCl containing 1 mmol/L dithiothreitol (DTT) to remove the mucus. The bowel was then flushed with isotonic citrate buffer (96 mmol/L NaCl, 1.5 mmol/L KCl, 27 mmol/L sodium citrate, 8 mmol/L KH_2PO_4; pH = 7.3) followed by 30, 45, or 60 minutes isotonic ethylenediamine tetra-acetic acid (EDTA) buffer (133 mmol/L NaCl, 2.7 mmol/L KCl, 1.7 mmol/L Na_2HPO_4, 3 mmol/L EDTA, 1 mmol/L DTT; pH = 9). The resulting chelation of calcium and magnesium ions facilitates the detachment of the epithelial cells from the basement membrane. During perfusions, the vasculature of the jejunal segment was cross-clamped to prevent systemic EDTA toxicity. The segments were ultimately flushed with 300 mL HBSS containing calcium and magnesium. Representative biopsies were taken from the jejunal segments immediately after perfusion. The segments were then seeded with 3000 ileal organoids in 300 μL DMEM and tied off at either end. Over the ensuing 2 weeks, jejunal segments were kept out of continuity of the gastrointestinal tract to prevent loss of the seeded cells in the fecal stream. After 2 weeks, the jejunal segments were harvested. Three additional control rats received HBSS in place of organoids after 45 minutes of debridement. In an additional 6 rats, the jejunal segment was flushed as described above for 45 minutes and the segments were seeded with organoids and harvested after 6 weeks.

Mice. Adult male wild-type mice were used as recipients of the organoids in the murine experiments. Mice were anesthetized as described

above, and a 3-cm segment of midjejunum was isolated and debrided chemically by perfusing the intestine for 5 minutes with 0.9% NaCl/1 mmol/L DTT, for 10 minutes with isotonic citrate buffer/1 mmol/L DTT, for 10, 20, or 30 minutes with isotonic EDTA buffer, and then with 300 mL HBSS (same compositions as for rate described above). Mice were immediately sacrificed, and the segments were examined with hematoxylin-eosin (H&E) staining for degree of debridement. After we had established that an EDTA perfusion for 20 minutes resulted in partial debridement similar to a 45-minute EDTA treatment in rats, all further experiments were carried out with 20 minutes of EDTA perfusions. The proximal and distal small bowel was re-anastomosed with 9-0 nylon (Ethicon) in an end-to-end fashion to restore bowel continuity. In 5 mice, two 1.5-cm debrided segments were seeded with 10,000 clusters per segment or HBSS. The ends of each segment were tied off with 6-0 silk (Ethicon). The segments were placed into the abdomen for a 3-week period. The mice were then sacrificed and the segments recovered.

Tissue processing after surgery. The dimensions of the jejunal segments were measured, and the intestine was then opened longitudinally and immediately transferred into 2°C cold mammalian Ringer solution as described above. The small intestine was divided into random sections; adjacent pieces from each segment were used for bile acid uptake studies, histology, and immunohistochemistry. Pieces that had been located immediately next to each other in the specimen were used to measure sodium-dependent and sodium-independent bile acid uptake rates, respectively.

Histology and immunohistochemistry. Omental and small intestinal tissues were mounted in optimal cutting temperature compound (Pella, Redding, Calif) and frozen on dry ice. Other neomucosal sections were fixed in 70% ethanol and paraffin-embedded for histologic analysis. Frozen tissues were sectioned on a cryostat (Leica, Wetzlar, Germany) and mounted on Superfrost/Plus glass slides (Fisher Scientific, Pittsburgh, Pa). Tissue sections were stained with H&E for light microscopy or processed for immunohistology.

For immunohistochemistry studies, polyclonal antibodies against IBAT were used as previously described.[28] These anti-IBAT antibodies were first tested on paraffin sections by using different preparation techniques. Initially, these tests did not yield satisfactory results, and IBAT staining of adult neoileal tissues were performed with the use of frozen sections. Frozen tissue sections were air-dried at room temperature, fixed with acetone for 15 minutes at 20°C, and allowed to completely air dry. They were then washed briefly in PBS, and IBAT immunostaining was performed as previously described on neonatal tissues. Slides were mounted wit the use of an aqueous medium containing Hoechst 33258 dye as a nuclear stain (Sigma). The slides were evaluated for distribution of the fluorescence signal under a fluorescence microscope (Axioscope II) equipped with a digital imaging and an intensity-measuring program for fluorescence systems (Axiocam).

A surface amplification index ratio of neomucosa was determined with the use of image analysis software (Axiovision; Zeiss). This ratio was created to account for the increase in surface attributable to the presence of villi, which amplify the surface of mucosa in comparison with a flat geometric plane. The ratio was derived by tracing the brush border perimeter of 5 consecutive individual villi and dividing this value by the length of the bases of these 5 villi. This procedure was performed in 5 random sections of neoileum (mean, 5.03), ileum (mean, 4.08), and jejunum (mean, 7.99) in each animal. In this manner, small-diameter control segments of ileum and jejunum can be compared with the dilated neoileum segments that have foreshortened villi. Analysis of variance was used to compare the mean values between the different experimental groups. In comparing means between the 2 groups of interest, Student *t* test was used as a post-hoc test to confirm the significance of the statistical findings.

Paraffin-embedded slides of neoileal tissue were used to histologically confirm the presence of intestinal epithelial lineages in donor-derived neomucosal segments. IBAT signal was confirmed in adjacent paraffin sections wit the use of 70% ethanol fixation. H&E-stained sections were used to demonstrate intestinal crypt/villus architecture with enterocytes and goblet cells under light microscopy. Antilysozyme immunostaining for Paneth cells, periodic acid Schiff-light green (PAS-LG) staining for goblet cells, and antisynaptophysin immunostaining for enteroendocrine cells were all performed by the West Los Angeles VA Pathology Department.

Quantification of IBAT messenger RNA. Small sections of intestinal tissues (100-250 mg) from wild-type animals and seeded segments were used to prepare total RNA[29] with the use of the RNeasy Extraction Kit (Qiagen, Santa Clarita, Calif). The total RNA was incubated with RQ1 DNase (Promega, Madison, Wis) and further purified by being applied to RNeasy-Mini columns (Qiagen). One microgram of purified total RNA from each

sample was reverse-transcribed with reverse transcriptase (RT; Gibco) and the complementary DNA (cDNA) used as templates in subsequent polymerase chain reactions (PCRs). A 582-bp fragment from the coding region of rat-IBAT was amplified with the use of the primer pair rIBAT 1: 5′ ATCTTCGTGGGCTTCCTCTGTCAG 3′ and rIBAT2: 5′ TTCCAAGGCAACTGTTCGGC 3′. As a normalizing control, a 618-bp fragment from the coding region of rat–glyceralde-3-phosphate dehydrogenase (GAPDH) was amplified with the primer pair rGAPDH1: 5′ ATGGGGTGATGCTGGTGCTGA 3′ and rGAPDH2: 5′ GAATGGGAGTTGCTGTTGAAG 3′ as described by Kim.[30] We first determined the number of PCR cycles at which the 2 PCR products were accrued by linear increases from these cDNAs. To this end, we varied the number of PCR cycles from 20 to 40. Linear increases in all samples were observed with 31 PCR cycles for rIBAT and 21 cycles for rGADPH (data not shown). After running all the samples for these cycles, PCR products were electrophoresed in a 1.2% agarose gel in 0.5× TRIS borate EDTA buffer (44.6 mmol/L TRIS base, 44.5 mmol/L boric acid, 0.5 mmol/L EDTA; pH = 8.3) and the rat-IBAT and rat-GAPDH bands quantified by gel densitometry on a Nucleotech 5000 densitometry unit (Nucleotech, San Diego, Calif).

Statistical analysis. Data were collected and results expressed as mean ± SEM. All quantitative experiments were carried out in triplicate. Mean values of the various groups of samples obtained along the longitudinal axis of the small intestine were compared by means of single-factor analysis of variance, followed by the Student *t* test as a post-hoc test.[31] Significance was assumed at a level of $P < 0.05$. The data points from the proximal jejunum (70 cm proximal to the ileocecal valve) were considered "jejunal baseline" values.

RESULTS

Mapping IBAT expression in the small intestine. Five rats showed a characteristic distribution of IBAT function with a long steady increase of sodium-dependent bile acid absorption toward the ileocecal valve. The area of peak IBAT function was located in the terminal ileum, 0 to 20 cm proximal to the ileocecal valve (Fig 1, *A*).

Mapping of IBAT expression in neonatal rat small intestine. Immunohistochemical studies of the neonatal rat tissues using anti-IBAT antibody demonstrated no fluorescent signal for IBAT protein throughout the length of the small intestine, both in the Swiss roll specimens and in serial cross-sections. Control slides of adult rat ileum were positive for apical IBAT signal, while control jejunal slides were negative for IBAT staining. Neojejunal controls (n = 3) were also performed in which jejunal cells were seeded into jejunum, which remained negative for IBAT signal.

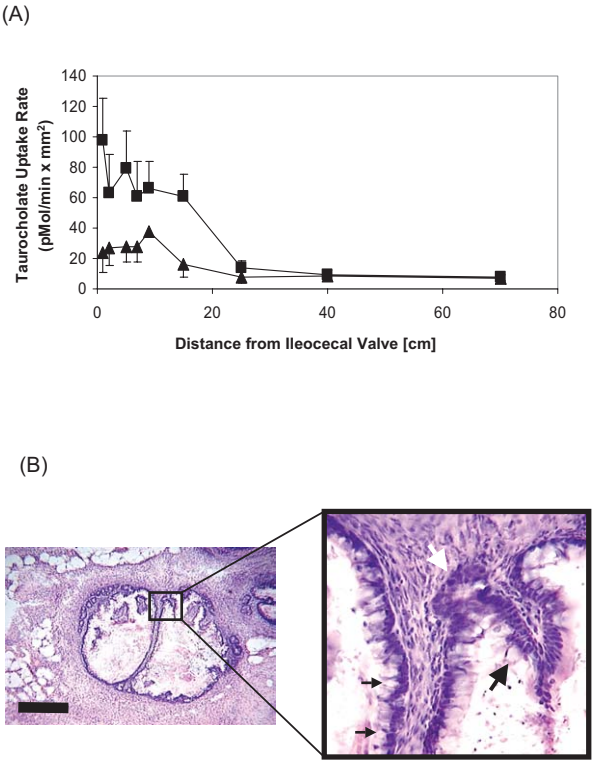

Fig 1. Bile acid uptake in rat intestine and functional testing of intestinal organoids. **A,** Bile acid uptake rates were measured in everted mucosal sleeves at different locations in rat small intestine with the use of sodium-containing (*squares*) and sodium-free (*triangles*) solutions of radiolabeled taurocholate (n = 5 rats). Taurocholate uptake measurements were based on distance from ileocecal valve. Sodium-dependent uptake mediated by IBAT was observed only in the terminal 25 cm of the small intestine. **B,** Organoid units derived from neonatal small intestine were implanted into the omentum (n = 4 rats). They gave rise to cysts lined with small intestinal mucosa that featured typical crypt (*large white arrow*) and villus (*large black arrow*) formations. Goblet cells are also present (*small black arrows*). Scale bar = 500 μm.

Verification of isolation methods. To test our isolation methods, intestinal mucosal organoids were injected into the omentum of 4 adult Lewis recipients. After 2 weeks, growth of several epithelialized cysts in the omentum was observed. The inner lining of these cysts had features characteristic of rat intestinal mucosa with goblet cells and enterocytes that were organized in crypt and villus formations (Fig 1, *B*).

Optimization of mucosal debridement. Depending on the debridement method, we observed either the development of a partial "neomucosa" expressing

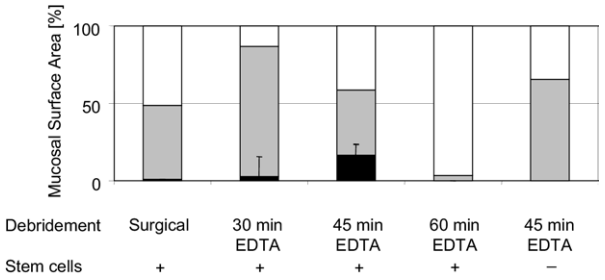

Fig 2. Mucosal engraftment with different jejunal debridement methods. The surface of the engrafted segments was examined for neoileal mucosa (*black*), residual jejunal mucosa (*grey*), and scarring (*white*), and quantified histomorphometrically for each method of debridement. The highest engraftment of ileal mucosal cells was found after 45 minutes of EDTA treatment. Only scarring and jejunal mucosa was seen after 45 minutes of EDTA treatment without instillation of organoid units (*far right bar*). *EDTA,* Ethylenediamine tetra-acetic acid.

IBAT protein, regrowth of jejunal epithelium, or formations of extensive connective tissue without epithelium consistent with scarring (Fig 2). Surgical stripping led to uneven debridement. Examination of the resulting mucosa 2 weeks after grafting showed significant scarring of about half of the inner surface of the segments. In other areas there was restituted native intestinal mucosa without detection of mucosa that stained positive with anti-IBAT antibodies. Chemical debridement for 30 minutes resulted in debridement of most villus enterocytes but not in removal of resident jejunal cells in the crypts (Fig 3, *A*). The resulting mucosal surface consisted of about 80% of restituted IBAT-negative mucosa with a few patches of IBAT-positive ("neoileal") mucosa (Fig 3, *C* and *E*). In segments undergoing chemical stripping with 45-minute EDTA flushes, removal of all jejunal villus cells and most of the jejunal crypt cells occurred (Fig 3, B). Two weeks after grafting, about 60% of the resulting surface was epithelialized, and about 30% of this mucosa stained positive with anti-IBAT antibodies (Fig 3, *D* and *F*). Chemical mucosal debridement for 60 minutes resulted in removal of all jejunal villus and crypt cells, and of submucosa in some areas, which resulted in excessive scarring and minimal restitution of native jejunal epithelium (Fig 2). In 3 additional rats, control experiments were performed in which jejunal segments were chemically debrided for 45 minutes with EDTA and "grafted" with vehicle only (containing no organoids). These segments did show regrowth of IBAT-negative mucosa on about 60% of the surface (Fig 2).

In additional experiments, it was shown that IBAT-expression persisted for at least 6 weeks in

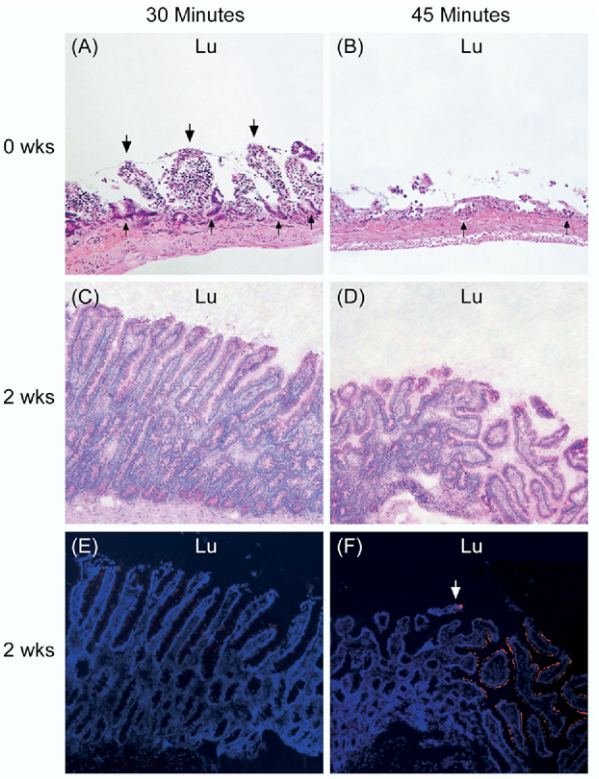

Fig 3. H&E and immunofluorescent sections of jejunum with 30 minutes of EDTA debridement (*left column*) and 45 minutes of EDTA debridement (*right column*) immediately after chemical mucosectomy (**A** and **B**) and 2 weeks after chemical mucosectomy (**C-F**). **A,** Thirty minutes of EDTA debridement resulted in loss of villus enterocytes (*thick arrows*) with a few remaining native crypts (*thin arrows*). **B,** Forty-five minutes of EDTA debridement resulted in the removal of most crypts. However, a few crypts remained (*thin arrows*). **C** and **E,** Mucosal regeneration after 2 weeks resulted in regeneration of native jejunal mucosa with no IBAT-positive mucosa in the 30-minute EDTA group. **D** and **F,** IBAT-positive mucosa (*red signal* = IBAT positive, *to right of arrow*) is present in those segments of jejunum that were adequately debrided with 45-minute EDTA treatment. *Lu,* Lumen.

the engrafted segments with little change in morphology and engraftment (data not shown). The IBAT-positive ileal neomucosa and the regrown recipient-derived mucosal epithelium showed mucosal atrophy with a relative paucity or blunting of villi (Fig 3, *C-F*). Surface area index ratio in the jejunum and ileum was 7.2- and 4.0-fold higher than in the neoepithelium, respectively.

Development of intestinal epithelial lineages from donor-derived epithelial organoids. Paraffin-embedded slides of neoileal tissue were used to histologically confirm the presence of the 4 intestinal epithelial lineages in donor-derived neo-

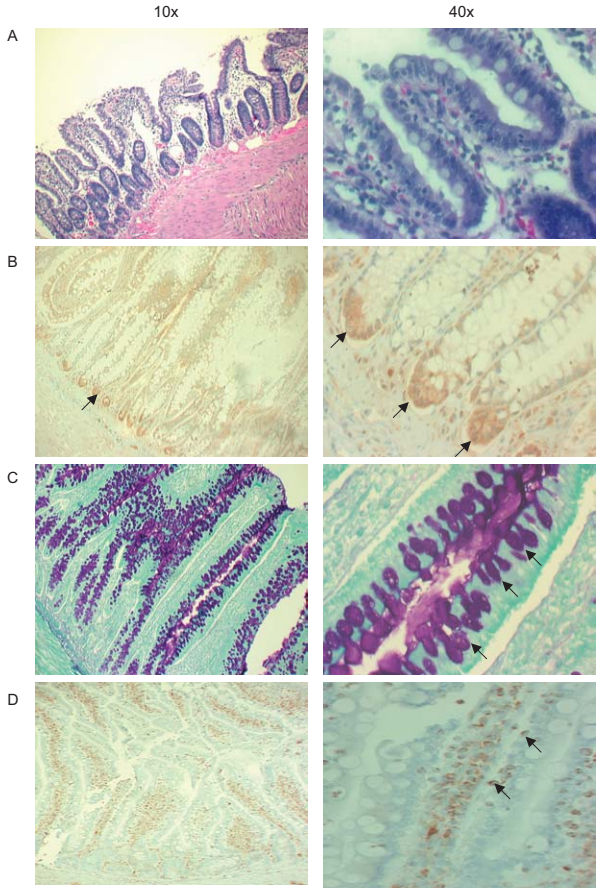

Fig 4. H&E-stained sections of neoileal mucosal segments with enterocytes and goblet cells at ×10 and ×40 original magnification (**A**). Antilysozyme immunostaining displaying Paneth cells localized to the crypt base at ×10 and ×40 original magnification (*arrows*) (**B**). PAS-LG staining for goblet cells at ×10 and ×40 original magnification (*arrows*) (**C**). Antisynaptophysin immunostaining for enteroendocrine cells at ×10 and ×40 original magnification (*arrows*) (**D**).

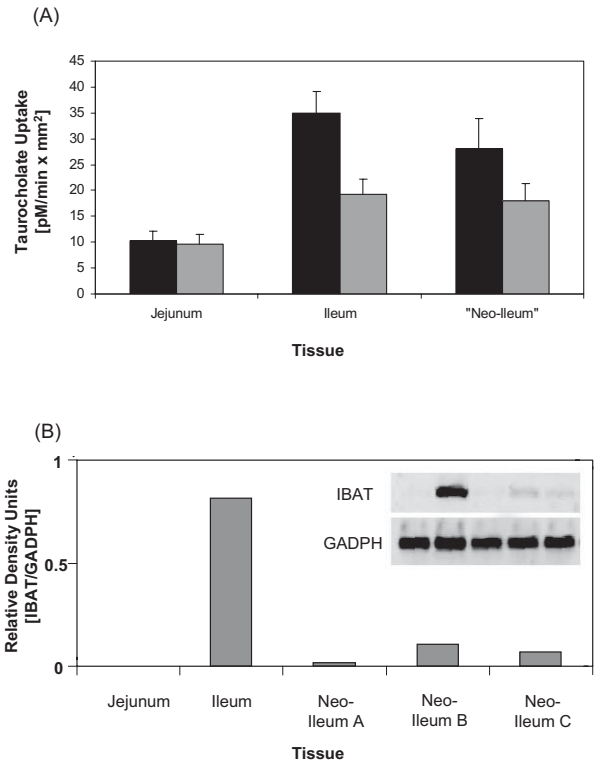

Fig 5. A, Bile acid uptake measurements in neoileal mucosa and normal gut. Total (*black columns*) and sodium-independent (*grey columns*) taurocholate absorption rates were measured in everted mucosal sleeves from engrafted segments (n = 16 sleeves), control jejunum (n = 12 sleeves), and ileum (n = 30 sleeves) (* $P < .05$ vs jejunum). The surface amplification index ratio was used to account for mucosal atrophy in the neoileal mucosa. **B,** Quantification of IBAT mRNA in neoileal mucosa. IBAT cDNA was produced from 3 segments of neoileal mucosa and from control intestinal tissues ("Jejunum" was taken from 5-10 cm distal to the ligament of Treitz; "Ileum" from 5-10 cm proximal to ileocecal valve). PCR runs and densitometry measurements were performed in triplicate. *GADPH,* Glyceraldehyde-3-phosphate-dehydrogenase; *IBAT,* ileal bile acid transporter.

mucosal segments at 6 weeks postengraftment. H&E-stained sections examined under light microscopy demonstrated intestinal crypt/villus architecture with enterocytes and goblet cells (Fig 4, A). Paneth cells were identified on H&E sections by their eosinophilic granules and location at the base of the intestinal crypts. Specific staining with an antilysozyme antibody confirmed the presence of Paneth cells localized to the crypt base (Fig 4, *B*). PAS-LG staining clearly demonstrated mucin-containing goblet cells (Fig 4, *C*). Antisynaptophysin immunostaining was used to identify enteroendocrine cells (Fig 4, *D*). Adjacent sections of these neoileal slides demonstrated an IBAT signal, confirming that this differentiated neomucosa demonstrating all 4 of the intestinal epithelial lineages was derived from ileal stem cells contained in donor epithelial organoids.

Sodium-dependent bile acid uptake and IBAT-mRNA. To confirm the presence of IBAT gene message and function, we examined several segments containing neoileal mucosa for the presence of sodium-dependent bile acid uptake and the presence of IBAT- mRNA. Active taurocholate uptake was demonstrated in segments with neoileal mucosa; however, rates were lower than those seen in the native ileum (Fig 5, *A*). Active uptake was absent in the jejunum. RT-PCR was performed with rat-specific primers to detect rat IBAT-mRNA and

GADPH-mRNA as a control. It was demonstrated that IBAT-mRNA was transcribed in the neoileum at levels 18- to 100-fold above the trace amounts normally expressed in the jejunum (Fig 5, *B*).

GFP transgenic mice. We chose syngeneic C57BL/6 mice that were transgenic for GFP to demonstrate that enterocytes expressing IBAT were from the transplanted population of ileocytes. Initially we optimized debridement times for the mouse by examining the extent of the debridement after ion chelator perfusions of 10, 20, and 30 minutes using methods analogous to the previous rat experiments. A debridement time of 20 minutes appeared to provide optimal removal of jejunal villi and crypts without debridement into the submucosa. Organoids were subsequently harvested from the entire small intestine of neonatal transgenic GFP mice as described above and seeded into a segment of debrided midjejunum (n = 5 mice). The seeded jejunal segments remained dormant for a period of 3 weeks before being harvested and examined with fluorescent microscopy. Segments filled slowly with mucus and cell debris, but no significant dilation was observed. Segments contracted longitudinally by about 20% because of scarring. Engraftment of GFP (+) epithelium varied from individual crypts to entire segments of mucosa (Fig 6, *A*). Mucosal epithelium with a mosaic distribution of GFP (+) and native jejunal crypts and small villi was identified (Fig 6, *A*). Engrafted segments contained areas of GFP (+) subepithelial tissue that ranged in size from small foci to the entire submucosa (Fig 6, *B*). Average engraftment in each segment (mean ± SEM) was 0.9% ± 0.2% of GFP (+) epithelium and 5.9% ± 1.0% of GFP (+) subepithelial tissue. Areas of GFP (+) mucosa stained positively with anti-IBAT antibody in adjacent sections, giving further support that the regenerated mucosa was derived from transplanted ileal organoids (Figs 6, *C* and *D*). Dormant segments that had not been seeded with ileal organoids showed some nonspecific staining of intraluminal debris with anti-IBAT antibodies. However, successfully seeded segments were found to contain debris that stained strongly anti-IBAT positive, likely resulting from the presence of desquamated neoileal enterocytes.

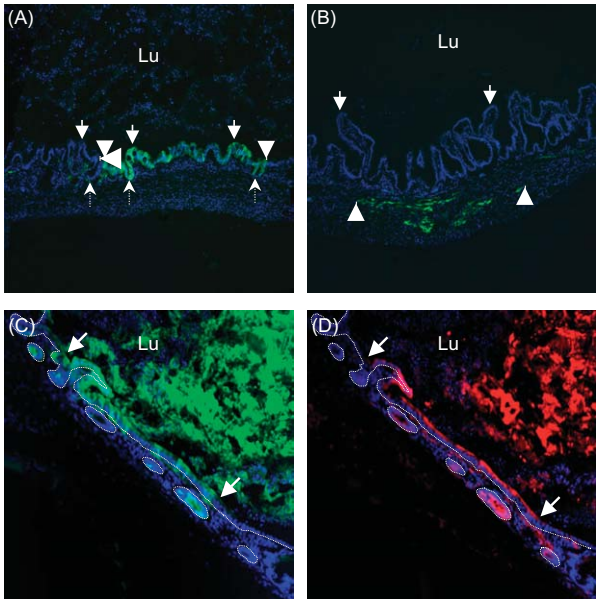

Fig 6. Transplantation of intestinal mucosal organoids from GFP (+) transgenic mice into wild-type recipients. **A,** Three weeks after transplantation, a neomucosa (*between white arrowheads*) developed and integrated completely into the host jejunal mucosa. Rudimentary villi (*white arrows*) were derived from both GFP (+) and native jejunal crypt cells (*thin dotted arrows*). **B,** Engrafted segments contained areas of subepithelial engraftment (*between white arrowheads*). In some areas the entire submucosa was replaced by GFP (+) tissue. Native jejunal GFP (–) villi (*white arrows*) are present over the GFP (+) subepithelium. **C** and **D**) Transplantation of ileal organoids of GFP (+) mice gave rise to mucosa (*outlined*) that showed colocalization of GFP (**C,** *green signal*) and IBAT (**D,** *red signal*) (GFP-positive and IBAT- positive mucosa, *between the white arrows*, with native jejunal mucosa *outside the arrows*). *Lu,* Lumen.

DISCUSSION

Intestinal epithelial organoids have been postulated to contain intestinal stem cells capable of regenerating an intestinal epithelium. To test feasibility of orthotopic ileal organoid transplantation, we mapped the sites of maximal IBAT expression and function in the small intestine, isolated mucosal organoids that give rise to a neoileal mucosa, developed a method to free the jejunum from its mucosal lining to generate an engraftment bed for the ileal organoids, and, finally, demonstrated the capacity of the neomucosa to absorb bile acids in a sodium-dependent manner. The latter would be evidence for the presence of IBAT, the only known sodium-dependent bile acid transporter in the small intestine.

To identify the optimal harvest area containing ileal cells with the highest bile acid absorption capacities in the small intestine, we mapped the axial distribution of active bile acid transport capacity in Lewis rats at high resolution. In rats, as is demonstrated in several other species, maximum sodium-dependent bile absorption steadily increases toward the ileocecal valve. These data confirm previous mapping studies at lower resolution by others.[10,32]

The mechanisms controlling ontogenic regulation of ileal bile acid transport are not well understood. It is known that the expression and function of IBAT rises abruptly around the time of weaning. Endogenous surges in corticosteroids and thyroid hormones may be involved as evidenced by their capacity to induce precocious expression in suckling animals.[34,35] Development of IBAT may be stimulated by expansion of the bile acid pool, and presentation to the terminal ileum as gavage administration of bile salts has also been shown to induce precocious expression of IBAT. Using molecular probes to IBAT, Shneider et al[33] demonstrated that developmental regulation of IBAT was partially controlled at the level of transcription with abrupt increases in IBAT mRNA and protein levels at the time of weaning. IBAT is thus unlikely to be expressed in neonatal organoids at the time of harvest.

To characterize IBAT expression in the neonatal donors, we performed immunohistochemical studies using anti-IBAT antibody. These studies demonstrated no significant IBAT staining, confirming this lack of IBAT expression in the normal preweaned neonatal rodent. These findings are consistent with previously reported literature findings that IBAT expression is not seen until weaning (day of life, 19-28).[36] However, earlier expression of IBAT can occur even before weaning. Using a hypoxia-hypothermia–induced model of necrotizing enterocolitis, precocious IBAT expression has been demonstrated with alterations in intestinal integrity.[34] At the time of harvest, our transplanted organoids would be approximately 21 days old. However, on the basis of the abrupt surge in IBAT expression and function seen in normal rats at the time of weaning and in response to physiologic and noxious stimuli, IBAT expression in the recipient animal would be expected to abruptly increase postengraftment, as many of the factors that signal the postweaning state should be present in the adult recipient rat. Thus, although IBAT expression is not seen in donor neonates at the time of organoid harvest, factors that physiologically would induce its expression would immediately have been present in the host environment and accounts for the IBAT expression in the neomucosal segments.

Next, we wanted to determine if organoids from these areas of maximal IBAT expression and the highest rates of bile acid absorption could develop a mucosa with small-bowel morphology. A combination of gentle enzymatic digestion with collagenase and dispase, and gravity sedimentation has become a standard method for the isolation of intestinal organoids from intestinal mucosa of rodents.[16,26,37] Previous investigators have demonstrated the successful engraftment of intestinal organoids isolated by a similar method in the subcutaneous region of severe combined immune deficiency mice and in a segment of colon from which the mucosa had been removed surgically.[16,33] We verified our isolation method of intestinal organoids by seeding into the omentum of syngeneic recipients. This procedure resulted in small cysts with features characteristic of intestinal mucosa, consisting of crypts and villi.

Before transplantation of ileal organoids into the jejunum, a method to remove native jejunal villi and crypts, and maintain an intact submucosa for engraftment had to be developed. The isolated segments were randomized to surgical or chemical mucosectomy: For the former, the jejunal mucosa was scraped off with a scalpel, and for the latter; we developed a novel perfusion method. Surgical mucosectomy was found to be an inconsistent method of debridement. Areas of inadequate debridement appeared to yield regrowth of native mucosa; other areas showed deep debridement and subsequent scar formation. For the chemical debridement, we adapted a previously described in vitro method to harvest enterocytes from the villi and crypts of the gut.[27] As reported in those studies, we observed that the extent of debridement was time-dependent and varied with EDTA treatments for 30, 45, or 60 minutes. Short debridement times resulted in inadequate removal of native jejunal crypts and eventual restitution of native jejunum. Longer debridement times resulted in the presence of very few or absent native crypts and, in some areas, in visible damage of the underlying subepithelium. Debridement times of 60 minutes resulted in scar formation with minimal mucosal restitution. Such profound debridement appeared to create an unfavorable environment for the attachment and growth of seeded organoids. In contrast, an intermediate time period of 45 minutes seemed to provide a balance between these extremes. Removal of most—but not all—of the resident jejunal mucosa promoted successful engraftment. An intact submucosa may be necessary to provide intestinal organoids with a microenvironment suitable for engraftment and differentiation.

In additional experiments, the IBAT expression persisted after 6 weeks. This finding suggests that the engrafted ileal organoids produce a mature ileal mucosa. Histologic staining of adjacent sections revealed that all 4 epithelial intestinal lineages were present in this ileal organoid–derived neomucosa. All IBAT-positive ileal neomucosa and resident mucosa demonstrated a relative blunting

of villi, suggesting some atrophy. This finding was expected as enterocytes of the isolated segments were deprived of nutrients and growth factors provided by normal enteric flow. Reversal of such atrophic changes was not tested here but has been described in bioengineered intestinal mucosa that is grown on biodegradable scaffoldings when it is left dormant at first and then exposed to small intestinal chyme.[17]

To confirm that the neomucosa-expressing IBAT protein was not derived from the recipient jejunum that had undergone unexpected transdifferentiation as a consequence of the debridement and seeding process, we carried out an additional experiment in which neojejunal segments were created by using jejunal-derived organoids. These segments were uniformly negative for IBAT expression. In addition, we tested whether our results in rats were reproducible in a transgenic mouse model. Seeding of GFP (+) intestinal organoids into the isolated segment of a wild-type recipient resulted in a mosaic mucosal epithelium of GFP (+) and resident jejunal crypts and small villi. Previous studies by others[38] showed generation of a mosaic intestinal mucosa when intestinal organoids from transgenic ROSA26 mice (transgenic for the beta-galactosidase gene) and from beta-galactosidase-negative wild-type mice were mixed and implanted subcutaneously in severe combined immune deficiency mice. Expression of normal transport proteins was not tested in this chimeric neomucosa.

To verify that IBAT-positive neomucosa is derived from ileal organoids, we harvested organoids from the terminal ileum of neonatal GFP (+) mice. These mice were seeded into an isolated segment of jejunum and the jejunal segments were harvested as described above. Adjacent sections of tissue were examined with fluorescent microscopy to identify GFP (+) mucosa and stained with anti-IBAT antibodies by using immunofluorescent microscopy. Areas of GFP (+) mucosa stained positively with anti-IBAT antibody in adjacent slide sections, suggesting that the regenerated mucosa is derived from transplanted ileal organoids.

Percent engraftment of neomucosa varied between models. In Lewis rats, the neoileal mucosa covered approximately 16% of the mucosal surface area, whereas, in the GFP mouse, engraftment rates were much lower. Attempts by our group and others (Stelzner M, 2004, unpublished data) to transplant ileal organoids in a fashion similar to that of the studies described here but using Fischer rats were repeatedly unsuccessful Thus, the success of intestinal organoids transplantation varies considerably between different rodent strains and species. Methods to improve debridement and enhance engraftment will need to be further studied.

CONCLUSION

We have demonstrated that intestinal stem cells derived from intestinal epithelial organoids may be transplanted into a denuded segment of jejunum to produce a neomucosa comprised of all 4 intestinal epithelial lineages. The denuded jejunum provides a natural matrix to allow for regenerative mucosal development. Neoileal mucosa demonstrates sodium-dependent bile acid uptake activity similar to that of native ileum and distinct from jejunal mucosa. Similar techniques are being used to bioengineer intestinal mucosa with the use of biodegradable polyglycolic acid polymers.[18] Further studies will have to focus on a more-complete evaluation of these functions and on clinical testing of this novel methodology.

The authors are indebted to Sum P. Lee, MD, PhD, Anna Mae Diehl, MD, and Patrick Tso, PhD, for helpful discussions, generous advice, and encouragement.

REFERENCES

1. Potten CS, Loeffler M. Stem cells: attributes, cycles, spirals, pitfalls and uncertainties. Lessons for and from the crypt. Development 1990;110:1001-20.
2. Potten CS, Booth C, Tudor GL, et al. Identification of a putative intestinal stem cell and early lineage marker; musashi-1. Differentiation 2003;71:28-41.
3. Wright N, Alison M. The biology of epithelial cell population. The Clarendon Press; 1984.
4. Cheng H, Leblond CP. Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine, V: unitarian theory of the origin of the four epithelial cell types. Am J Anat 1974;141:537-61.
5. Sanderson IR, Ezzell RM, Kedinger M, et al. Human fetal enterocytes in vitro: modulation of the phenotype by extracellular matrix. Proc Natl Acad Sci U S A 1996;93:7717-22.
6. Campbell FC, Tait IS, Flint N, Evans GS. Transplantation of cultured small bowel enterocytes. Gut 1993;34:1153-5.
7. Hofmann AF. Intestinal absorption of bile acids and biliary constituents. In: Johnson LR, editor. Physiology of the gastrointestinal tract. Vol 2. New York: Raven Press, 1994. p. 1845-65.
8. Henning SJ, Rubin DC, Schulman RJ. Ontogeny of the Intestinal Mucosa. In: Johnson LR, editor. Physiology of the gastrointestinal tract. Vol 1. New York: Raven Press, 1994. p. 571-610.
9. Coppola CP, Gosche JR, Arrese M, et al. Molecular analysis of the adaptive response of intestinal bile acid transport after ileal resection in the rat. Gastroenterology 1998;115:1172-8.
10. Hofmann AF. Bile acid malabsorption caused by ileal resection. Arch Intern Med 1972;130:597-605.
11. Weber AM, Roy CC, Morin CL, Lasalle R. Malabsorption of bile acids in children with cystic fibrosis. N Engl J Med 1973;289:1001-5.
12. Fromm H, Malavolti M. Bile acid-induced diarrhoea. Clin Gastroenterol 1986;15:567-82.

13. Kanamoto R, Azuma N, Suda H, Seki T, Tsuchihashi Y, Iwami K. Elimination of Na+-dependent bile acid transporter from small intestine by ileum resection increase colonic tumorigenesis in the rat fed deoxycholic acid. Cancer Lett 1999;145:115-20.
14. Morvay K, Szentleleki K, Torok G, Pinter A. Effect of small bowel resection on fecal bile acid excretion and on experimental colon tumour in rats. Acta Chir Hung 1990;31:25-31.
15. Avansino JR, Chen DC, Hoagland VD, Woolman JD, Haigh WG, Stelzner M. Treatment of bile acid malabsorption using ileal stem cell transplantation. J Am Coll Surg 2005;201(5):710-20.
16. Tait IS, Evans GS, Flint N, Campbell FC. Colonic mucosal replacement by syngeneic small intestinal stem cell transplantation. Am J Surg 1994;167:67-72.
17. Kim SS, Kaihara S, Benvenuto MS, et al. Effects of anastomosis of tissue-engineered neointestine to native small bowel. J Surg Res 1999;87:6-13.
18. Tavakkolizadeh A, Berger UV, Stephen AE, et al. Tissue-engineered neomucosa: morphology, enterocyte dynamics, and SGLT1 expression topography. Transplantation 2003;75:181-5.
19. Armstrong DN, Krenz HK, Modlin IM, Ballantyne GH. Bile salt inhibition of motility in the isolated perfused rabbit terminal ileum. Gut 1993;34:483-8.
20. Feurle GE. Pathophysiology of diarrhea in patients with familial amyloid neuropathy. Digestion 1987;36:13-7.
21. Stelzner M, Hoagland V, Somasundaram S. Distribution of bile acid absorption and bile acid transporter gene message in the hamster ileum. Eur J Physiol 2000;440:157-62.
22. Stelzner M, Hoagland V, Somasundaram S. A simple method for measuring of intestinal solute transport in mucosal biopsy specimens. Dig Dis Sci 2001;46:451-61.
23. Stelzner M, Fonkalsrud EW, Buddington RK, Phillips JD, Diamond JM. Adaptive changes in ileal mucosal nutrient transport following colectomy and endorectal ileal pull-through with ileal reservoir. Arch Surg 1990;125:586-90.
24. Stelzner M, Buddington RK, Phillips JD, Diamond JM, Fonkalsrud EW. Changes in mucosal nutrient transport in small and large ileal reservoirs after endorectal ileal pull-through. J Surg Res 1990;49:344-9.
25. Karasov WH, Diamond JM. A simple method for measuring intestinal solute uptake in vitro. J Comp Physiol 1983;152:105-16.
26. Evans GS, Flint N, Somers AS, Eyden B, Potten CS. The development of a method for the preparation of rat intestinal epithelial cell primary cultures. J Cell Sci 1992;101:219-31.
27. Weiser M. Intestinal epithelial cell surface membrane glycoprotein synthesis. I. An indicator of cellular differentiation. J Biol Chem 1973;248:2536-41.
28. Stelzner M, Hoagland VD, Woolman JD. Identification of optimal harvest sites of ileal stem cells for treatment of bile Acid malabsorption in a dog model. J Gastrointest Surg 2003;7:516-22.
29. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1986;162:156-9.
30. Kim YI, Shirwadkar S, Choi SW, Puchyr M, Wang Y, Mason JB. Effects of dietary folate on DNA strand breaks within mutation-prone exons of the p53 gene in rat colon. Gastroenterology 2000;119:151-61.
31. Armitage P. Statistical methods in medical research. Malden, MA: Blackwell Scientific Publications; 1971.
32. Baker R, Searle G. Bile acid absorption at various levels of rat small intestine. Proc Soc Exp Biol Med 1960;105:521-3.
33. Shneider BL, Dawson PA, Christie DM, Hardikar W, Wong MH, Suchy FJ. Cloning and molecular characterization of the ontogeny of a rat ileal sodium-dependent bile acid transporter. J Clin Invest 1995;95:745-54.
34. Barnard JA, Ghishan FK. Methylprednisolone accelerates the ontogeny of sodium-taurocholate cotransport in brush border membrane vesicles from rat ileum. J Lab Clin Med 1986;108:549-55.
35. Heubi JE. Role of thyroxine on postnatal development of ileal bile salt transport. Am J Physiol 1986;251:237-42.
36. Halpern MD, Holubec H, Saunders TA, Dvorak K, Clark JA, Doelle SM, Ballatori N, Dvorak B. Bile acids induce ileal damage during experimental necrotizing enterocolitis. Gastroenterology 2006;130(2):359-72.
37. Booth C, O'Shea JA, Potten CS. Maintenance of functional stem cells in isolated and cultured adult intestinal epithelium. Exp Cell Res 1999;249:359-66.
38. Slorach EM, Campbell FC, Dorin JR. A mouse model of intestinal stem cell function and regeneration. J Cell Sci 1999;112(Pt 18):3029-38.