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Engraftment of Mucosal Stem Cells into Murine Jejunum is Dependent on Optimal Dose of Cells

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Background. **Transplantation of intestinal mucosal stem cells is an important step in the development of intestinal gene therapy and treatment of intestinal mucosal diseases. We hypothesized that engraftment rates increase proportionally with increasing doses of seeded stem cells and increasing jejunal débridment.**

Materials and methods. **Intestinal mucosal organoids were harvested from neonatal mice carrying a green-fluorescent protein (GFP) transgene and transplanted into adult GFP(−) mice (n = 66). In recipients, two jejunal segments (1.5 cm) were isolated with their blood circulation left intact with anastomosis of the distal and proximal segments to restore continuity. Debridement of native enterocytes was performed by perfusing luminally with ethylene diamine tetraacetc acid solutions for 20 min. A total of 5,000, 10,000, or 25,000 organoids were then seeded. Three weeks later, cross sections (n = 398) of the segments were evaluated for the presence of GFP(+) neomucosa using fluorescence microscopy. Additional segments were débrided for 30 and 40 min (n = 83). Other conditions were not tested because of the rate of high mortality in these experiments.**

Results. **The group seeded with 10,000 organoid units at 20 min showed the highest engraftment of GFP(+) epithelium. Engraftment was improved by increasing débridment times at this seeding density. Overall mortality was 70%.**

Conclusions. **These findings suggest that there is an optimal seeding density of stem cell clusters for enhanced engraftment in this model. Mortality prohibited complete testing of all combinations of seeding density and debridement times.**

Key Words: **intestinal stem cell transplantation; GFP; neo-mucosa; mucosal restitution; intestinal stem cell seeding density; jejunal débridment time.**

INTRODUCTION

In the small intestine, mucosal stem cells occupy the lower third of each crypt. This location has been based on studies using ^{3}H-thymidine labeling and, more recently, investigations with antibodies against the Mushashi-1 mRNA binding protein, which binds to intestinal stem cells [1, 2]. Intestinal stem cells divide and give rise to both new stem cells and daughter cells that generate distinct epithelial lineages (Paneth, absorptive, goblet and enteroendocrine cells). After a cytotoxic insult, stem cells and their immediate progeny are capable of dividing to regenerate an entire intestinal mucosa [3].

Epithelial cells require the presence of mesenchymal cells to proliferate and survive *in vitro* [4]. Thus, the successful transplantation of stem cells requires isolation of stem cells in the form of stem cell/mesenchymal cell aggregates ("epithelial organoid units") rather than individual stem cells. These organoids can be harvested using standard method of gentle enzymatic digestion with collagenase and dispase followed by gravity sedimentation [5–8]. These organoid units have the capacity to generate all lineages of normal small bowel mucosa when injected into the subcutaneous tissue of nude mice [5, 7].

Our group first reported successful transplantation of ileal stem cells into denuded jejunal segments in

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2000 [9]. In those experiments, the neoileum was identified using antibodies to the ileal bile acid transporter (IBAT), which predominantly is located on mature ileocytes and is responsible for sodium-dependent uptake of bile acids. However, IBAT may be expressed partially on immature ileocytes that have successfully been engrafted, thus limiting accurate quantitation of engrafted epithelium. For the present studies, we chose a mouse transgenic for the green fluorescent protein (GFP) as a stem cell donor, to accurately quantitate epithelial engraftment. Larger animal models that are syngeneic and transgenic for a suitable marker protein are currently not available. We hypothesized that the area of engraftment would increase proportionally with increasing doses of seeded mucosal stem cells and increasing débridment times in an isolated segment of jejunum.

MATERIALS AND METHODS

Animals

Five to seven-day-old neonatal mice carrying a GFP transgene were used as donors for intestinal stem cell isolation. Wild-type C57BL/6 mice that weighed 30–50 g served as syngeneic recipients. Wild-type and eGFP-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. All animals were housed in accordance with the National Institutes of Health guidelines for the care of laboratory animals, maintained under a 12-h light/dark cycle (6 a.m. to 6 p.m.), and received high-fat breeder chow (Nestle Purina, St. Louis, MO) and water ad libitum.

Isolation of Stem Cell Organoids

Intestinal stem cell clusters were isolated as described by Evans *et al.* [6]. In brief, 5- to 7-day-old GFP-positive mice were sacrificed by cervical dislocation and stem cells were harvested from the entire small bowel from the ligament of Treitz to the ileocecal valve. The intestine was opened longitudinally and cut into 2-mm long segments. The intestinal pieces were washed copiously in Hanks buffered saline solution (HBSS). The washed segments were minced into pieces smaller than 1 mm^3 and then transferred into 20 mL of HBSS, with 0.1-mg/ml dispase type 1 (Roche, Indianapolis, IN) and 300 U/mL collagenase Type 11 (Sigma, St. Louis, MO) and rocked continuously for 25 min at 22°C. The suspension was shaken vigorously for 1 min and allowed to sediment out for 1 min. The supernatant was carefully transferred to a new tube and the pellet discarded. This was repeated three times, clearing the suspension of larger debris. The cleared suspension was then mixed with an equal amount of Dulbecco's modification of Eagle's medium–sterile (DMEM-S). The following was added to the 0.45% glucose DMEM-S (Gibco, Gaitersburg, MD): 2% D-Sorbitol (Sigma, St. Louis, MO), 2.5% FBS (Hyclone, Logan, UT), and 100 IU/mL penicillin +100 μg/mL streptomycin (Gibco; Gaitersburg, MD). The modified DMEM-S/supernatant solution was centrifuged for 2 min at 6g (300 rpm) at room temperature. Contents were allowed to sediment at normal gravity for 30 s and the supernatant discarded. The pellet was washed with 50 mL of DMEM-S and centrifuged six times or until the supernatant was clear and the pellet well defined. The pellet was then resuspended in DMEM.

Surgical Procedure

Adult wild-type C57BL/6 mice were used as recipients of the stem cell clusters. Mice were anesthetized with inhaled isoflurane. A 3-cm segment of mid-jejunum was isolated in each mouse on its mesenteric pedicle, maintaining perfusion to the segment. The remaining proximal and distal thirds were anastomosed with a 9-O polypropylene suture (Ethicon, Somerville, NJ) in an end-to-end fashion to restore bowel continuity, under 20x magnification. The mucosal epithelium from the isolated jejunum was stripped by cannulating the jejunal segment on either end. The segment was first flushed for 5 min with 0.9% NaCl containing 1 mM dithiothreitol (DTT) to remove the mucus. The bowel was then flushed 10 min with isotonic citrate buffer (96 mM NaCl, 1.5 mM KCl, 27 mM sodium citrate, 8 mM KH_2PO_4, pH 7.3) followed by isotonic ethylene diamine tetraacetic acid (EDTA) buffer (133 mM NaCl, 2.7 mM KCl, 1.7 mM Na_2HPO_4, 3 mM EDTA, 1 mM DTT, pH 9) for 20 min at 50 cc/min flow and 37°C [10]. This resulted in a partial or complete dislodgment of the mucosal cells from the basement membrane with little damage to the underlying tissue. Before the administration of the chelators, the mesenteric pedicle was clamped for the duration of the irrigation to prevent the absorption of large quantities of perfusate. After débridment, the isolated segment of intestine was divided into two 1.5-cm subsegments. Initially, each 3-cm segment was débrided for 20 min and each subsegment was seeded randomly with 5,000, 10,000 or 25,000 clusters or vehicle only (HBSS). In additional experiments, these segments were further débrided for 30 or 40 min and seeded with the optimal number of organoid units determined at 20 min. The ends of each segment were tied with a 6-O silk (Ethicon, Somerville, NJ). The segments were placed in the abdomen for a 3-week period. After this time, the mice were sacrificed and the segments recovered. At the time of harvest, the transplanted segments were measured and the surface area was calculated based on the cylindrical shape of the segment, using the formula $2\pi r^2 + 2\pi rl$ (r = radius, l = length).

Histology

Small intestine tissues were mounted in OCT compound (Ted Pella Inc., Redding, CA) and sectioned at 10 μm thickness. The sections were stained with hematoxylin and eosin, and alkaline phosphatase. Alkaline phosphatase staining was done using a Vector Red Alkaline Phosphatase Substrate Kit (Vector Laboratories, Inc., Burlingame, CA).

Fluorescent Microscopy

The harvested segments were initially placed in 4% phosphate buffered formalin overnight and mounted with OCT (Ted Pella Inc., Redding, CA) on dry ice the following day. Tissues were sectioned (10 μm thickness) on a cryostat (Leica, Wetzlar, Germany) and mounted on Superfrost/Plus glass slides (Fisher Scientific, Pittsburgh, PA). Vectashield hard mount with DAPI (4′,6-diamidino-2-phenylindole; Vector Laboratories, Inc., Burlingame, CA) was used to mount the tissue and stain the nuclei. The seeded segments were evaluated for the presence of neomucosa with a strong GFP fluorescent signal. A mean increase fluorescence intensity of 3-fold over background was considered a positive test in all experiments. The extent of GFP-positive epithelium and subepithelial tissues were divided by the circumference to determine the percentage of GFP-positive mucosa in each cross-section. The circumference was determined on hematoxylin and eosin stained adjacent sections, using a computerized imaging device (Nucleo Vision; Nucleo Tech, Westport, CN).

Statistical Analysis

All data are expressed as mean ± SEM. The statistical analysis was performed using an analysis of variance and two-tailed test to compare individual groups as a post hoc test. A correction for mul-

tiple comparisons was performed using a Bonferroni adjustment. A P value <0.05 was considered significant. In all groups (5,000, 10,000, and 25,000 stem cell clusters), cross sections were treated as independent data points. The areas featuring implantation of GFP-positive cells were calculated separately for both epithelial and subepithelial tissues, respectively.

RESULTS

Gross Findings

Intestinal stem cell transplantation was performed on 66 mice. Twenty mice survived the 3-week study period and the two 1.5-cm seeded segments from each mouse were harvested and evaluated. Findings at the time of sacrifice varied for each mouse. Most segments were dilated to varying degrees; some showed an inflammatory infiltrate with multiple adhesions to adjacent small intestinal segments and others had minimal peritoneal inflammation. These findings did not correlate with the débridement times or conditions. The transplanted segments were somewhat dilated compared with the pretransplant segments. The average surface area of the dormant segments at harvest was calculated as 321 mm^2 ± 59 mm^2 (average ± SEM), which was larger than the calculated pretransplant surface area of 214 mm^2 (length of 15 mm and diameter 2 mm).

Forty-six of the 66 mice died prematurely, mainly as the result of obstruction, infection, anastomotic leak, and complications of anesthesia. Mice with débridement times of 30 or 40 min more commonly died of infection/sepsis than those mice débrided for 20 min (Table 1).

TABLE 1

Cause of Mortality of Mice at Different Débridement Times for All Stem Cell Cluster Seeding Densities (Pre/Post-Op = Death During or Immediately After Surgery): Infection Was Defined as Abscess Formation or Anastomotic Leak

Time	Cause of death	Individual segments (n)	%
20 min	Sacrifice	14	39
	Obstruction	13	36
	Infection	6	17
	Pre/post op	3	8
	Unknown	0	0
30 min	Sacrifice	2	11
	Obstruction	5	28
	Infection	6	33
	Pre/post op	4	22
	Unknown	1	6
40 min	Sacrifice	4	33
	Obstruction	2	17
	Infection	6	50
	Pre/post op	0	0
	Unknown	0	0

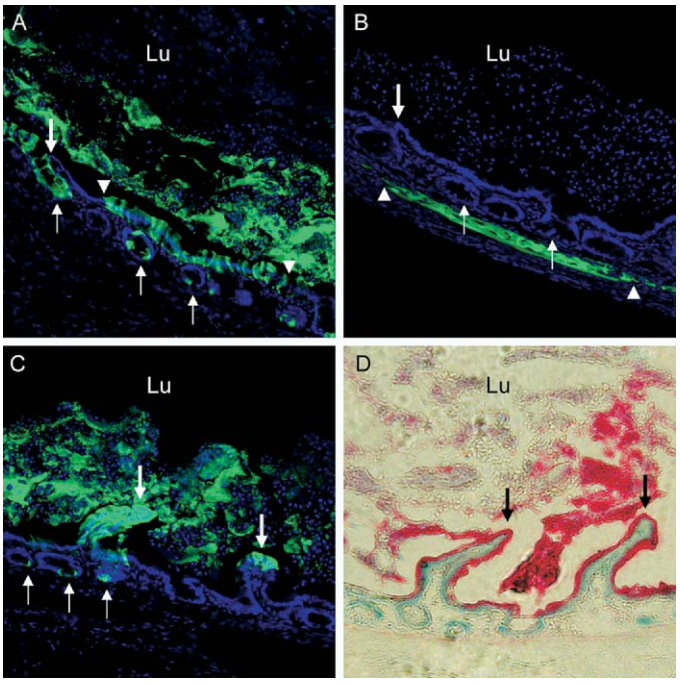


FIG. 1. (A) Representative florescent microscopic section of an isolated jejunal segment that has been débrided and seeded with GFP-positive stem cells. Successfully engrafted GFP-positive donor stem cells create a neo-mucosa (GFP-positive cells = green, left of large arrow and between white arrowheads) compared with the native jejunum (blue nuclear stain only). GFP-positive crypts (thin white arrows) underlie the GFP-positive mucosa. (B) Subepithelial engraftment of GFP-positive tissue (between white arrowheads) that underlies rudimentary villi (thick white arrow) and crypts (thin white arrows) of the native jejunal mucosa. Lumen (Lu). Nuclear stain: DAPI = blue. (C) GFP-positive rudimentary villi (thick arrows) with GFP-positive crypts in close proximity (thin arrows). (D) An adjacent section is stained with alkaline phosphatase. Arrows show rudimentary villi.

Microscopic Findings

The transplanted segments were sectioned in their entirety. Cross sections were evaluated at 500-μm intervals resulting in 481 representative cross-sections. The epithelial layer was intact on all cross sections. The control group showed only nonfluorescent mucosa in restitution. Engraftment of epithelium for seeding densities of 5,000, 10,000, and 25,000 groups varied from single crypts to larger areas comprising maximally 60% epithelial engraftment of a single cross section (in the group débrided for 40 min and seeded with 10,000 stem cell clusters). The mucosa of the transplanted segments showed some epithelial atrophy with a relative paucity or blunting of villi (Fig. 1A). Subepithelial engraftment of GFP-positive tissue also was observed (Fig. 1B). Engraftment of both GFP-positive epithelium and subepithelial tissues in the same cross section ranged from 7% to 48% in each individual group. In these sections, overlap of GFP-positive epithelium and subepithelial tissues represented 1% of total GFP engraftment. Engraftment of GFP-positive subepithe-

lial tissue was 1.5 to 4.5 times more common than GFP-positive epithelium. Alkaline phosphatase staining of the restituted mucosa verified maturity of the neo-mucosa (Fig. 1C and D). Alkaline phosphatase staining was confirmed in all experimental groups.

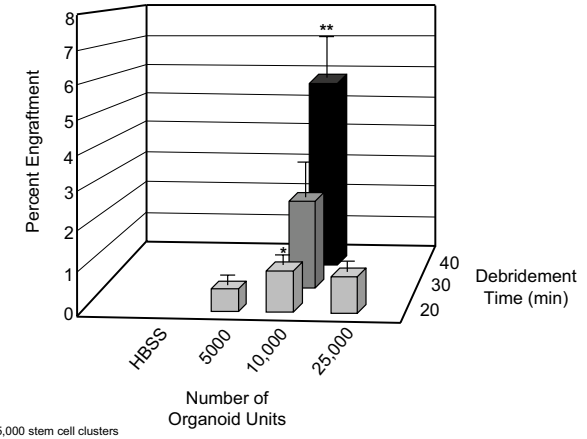
The group seeded with 10,000 organoid units demonstrated engraftment of GFP-positive epithelium and subepithelial tissue that was significantly greater at the 20-min débridement time point. There was a trend toward decreased epithelial engraftment and a significant decrease in engraftment of subepithelial tissue in the group seeded with 25,000 stem cell clusters compared with the group seeded with 10,000 stem cell clusters at 20 min débridement (Fig. 2A). Engraftment of GFP-positive epithelium was significantly increased in the group seeded with 10,000 organoid units and debrided for 40 min compared with the group débrided for 20 min. There was a trend toward increased engraftment of GFP-positive epithelium in the group débrided for 30 min compared to those débrided for 20 min. Engraftment of GFP-positive subepithelial tissue was significantly increased in the groups seeded with 10,000 organoid units and débrided for either 30 or 40 min compared with the group débrided for 20 min (Fig. 2B). There was a trend toward increased engraftment in the groups débrided for 40 min compared to those debrided for 30 min. Further studies of combinations of debridement times and stem cell cluster seeding densities were discontinued because of high mortality rates (Fig. 2C).

DISCUSSION

In these experiments, we demonstrated successful engraftment of GFP-positive intestinal stem cells into the partially denuded segment of jejunum in adult syngeneic GFP-negative recipients. Our data suggest that there is likely an optimal seeding density of intestinal stem cell clusters and débridement time for the recipient intestine. However, because of the high mortality rate in the murine model, we were not able to prove this conclusively for all tested combinations.

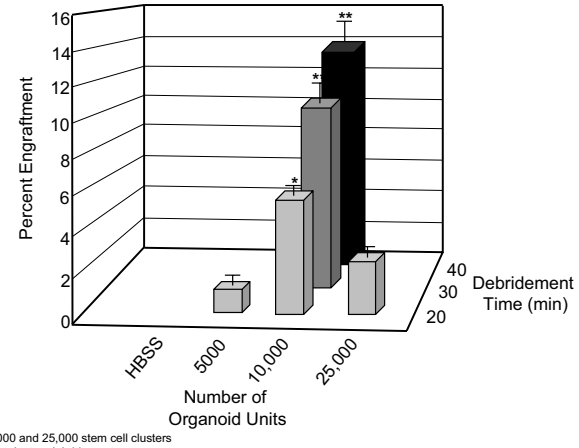
The neomucosa expressed typical small bowel morphology that is indistinguishable from the adjacent native mucosa. Identification of mature absorptive enterocytes was verified in all groups with neomucosa using alkaline phosphatase staining. Stem cells from donor and native crypts contribute to mosaic villi containing mucosa arising from both types of crypts. This integrated growth of transplanted stem cells has been described in the literature in which mixed populations of engrafted cells differentiated by transgene markers

A



*p < 0.05 vs. 5,000 stem cell clusters
**p < 0.05 vs. 20 minutes debridement

B



*p < 0.05 vs. 5,000 and 25,000 stem cell clusters
**p < 0.05 vs. 20 minutes debridement

C

Debridement Time [Minutes]	Seeding Density [Number of Stem Cell Clusters]		
	5,000 [# of cross-sections]	10,000 [# of cross-sections]	25,000 [# of cross-sections]
20	n = 69	n = 183	n = 146
30	Specimen not obtained	n = 31	Specimen not obtained
40	Specimen not obtained	n = 52	Specimen not obtained

FIG. 2. (A) Engraftment of GFP-positive epithelium for various organoid unit seeding densities and débridement times. (B) Engraftment of GFP-positive subepithelial tissue for various organoid unit seeding densities and débridement times. (C) Different combinations of stem cell seeding density and débridement times studied and the number of cross-sections examined in each group. (Color version of figure is available online.)

form a neomucosa with crypt clonality but villous polyclonality [7]. This chimerism is similarly observed in our finding of GFP-positive neomucosa integrated within native, GFP-negative neomucosa derived from regrowth of native stem cell clusters. The mucosa in our dormant transplant segment was atrophic as a result of isolation from normal enteric flow. Our group and others have demonstrated an increase in villus surface area when an isolated segment of intestine is placed in continuity with the intestine (Avansino, Manuscript in Preparation), [11].

There was also engraftment of GFP-positive subepithelial tissue. It was previously reported that the stem cell clusters have approximately 10 percent mesenchymal cells as determined by antibody staining [6]. Thus, mesenchymal cells contained in the GFP-positive clusters likely resulted in engraftment of subepithelial tissue. Engraftment of subepithelial tissues may have occurred more commonly than epithelial tissue despite the reported small mesenchymal component in the stem cell clusters. Alternatively, engrafted mesenchymal progenitors may simply exhibit greater viability than their epithelial counterparts.

Seeding Density

We found that engraftment did not increase proportionally with increasing stem cell cluster dose in the groups undergoing 20 min of débridement. There was a significantly higher engraftment of GFP-positive epithelium in the group seeded with 10,000 stem cell clusters compared to the group seeded with 5,000 stem cell clusters at the 20-min débridement time. A lower seeding density may not fully repopulate the vacated spaces provided by the débridement. Thus, regeneration of native jejunal mucosa from a few remaining crypts may predominate. There was also a trend toward decreased engraftment of GFP-positive epithelium and subepithelial tissue with an increased seeding density compared to the group seeded with 10,000 stem cell clusters. Under these conditions, the number of seeded stem cell clusters may exceed the available space and nutrients in the microenvironment. This may result in death of the stem cell clusters, stimulating an inflammatory response and creating unfavorable conditions for growth.

Mucosal Débridement

Engraftment improved by increasing the débridement time. Prolonged exposure to divalent ion chelators increased enterocyte dissociation, removing native crypts that may compete for space and nutrients in the microenvironment. While we were not able to examine this further due to the high mortality in the present model, it appears unlikely that still longer debridement times would necessarily have further increased engraftment rates. Longer débridement times will have deleterious effects by prolonging mesenteric ischemia secondary to vascular occlusion that has to be maintained during the perfusion phase to prevent chelator toxicity. In addition, we have found in studies of rats that prolonged exposure to chelators in excess of 60 min results in destruction of the mucosal stroma. (Avansino, manuscript in preparation).

Mortality

Helmrath *et al.* found mortality rates in mice to be approximately 15% after 50% bowel resection with anastomosis [12]. Mortality in our population was higher and it was most commonly the result of obstruction or sepsis. The confounding factor resulting in increased mortality in our population appears to be the débridement of the jejunal segment. The warm ischemia created by clamping the mesentery may render the intestine susceptible to ischemia–reperfusion injury, inflammation, adhesions, anastomotic leak, translocation of bacteria, and ultimately sepsis. At necropsy, some transplant segments were found to be dilated with inflammation, although the severity of these changes did not correlate with débridement times or seeding densities.

Future Direction

This research demonstrates that intestinal stem cell transplantation is feasible and that altering the experimental conditions can enhance engraftment. This therapy could be used to treat intestinal mucosal diseases and as a first step in intestinal stem cell gene therapy. The high mortality makes this murine model not well suited for further investigations. Future research should focus on a larger animal model to further study optimal intestinal stem cell engraftment. Engraftment of the stem cells should be optimized by altering stem cell cluster concentrations and débridement conditions. Modifications of the débridement solutions, the perfusion flow rate, the perfusion pressure, and débridement times are all possible parameter that may determine the success of débridement and engraftment. Other studies focusing on the process of stem cell engraftment and subsequent development and differentiation are also being explored.

REFERENCES

1. Booth, C., and Potten, C. S. Gut instincts: Thoughts on intestinal epithelial stem cells. *J. Clin. Invest.* **105:** 1493, 2000.
2. Potten, C. S., Booth, C., Tudor, G. L., Booth, D., Brady, G., Hurley, P., Ashton, G., Clarke, R., Sakakibara, S., and Okano, H. Identification of a putative intestinal stem cell and early lineage marker; musashi-1. *Differentiation* **71:** 28, 2003.
3. Campbell, F. C., Tait, I. S., Flint, N., and Evans, G. S. Transplantation of cultured small bowel enterocytes. *Gut* **34:** 1153, 1993.
4. Sanderson, I. R., Ezzell, R. M., Kedinger, M., Erlanger, M., Xu, Z. X., Pringault, E., Leon-Robine, S., Louvard, D., and Walker,

W. A. Human fetal enterocytes in vitro: Modulation of the phenotype by extracellular matrix. *Proc Natl Acad Sci USA.* **93:** 7717, 1996.

5. Booth, C., O'Shea, J. A., and Potten, C. S. Maintenance of functional stem cells in isolated and cultured adult intestinal epithelium. *Exp Cell Res.* **249:** 359, 1999.
6. Evans, G. S., Flint, N., Somers, A. S., Eyden, B., and Potten, C. S. The development of a method for the preparation of rat intestinal epithelial cell primary cultures. *J Cell Sci.* **101:** 219, 1992.
7. Slorach, E. M., Campbell, F. C., and Dorin, J. R. A mouse model of intestinal stem cell function and regeneration. *J Cell Sci.* **112:** 3029, 1999.
8. Tait, I. S., Evans, G. S., Flint, N., and Campbell, F. C. Colonic mucosal replacement by syngeneic small intestinal stem cell transplantation. *Am J Surg.* **167:** 67, 1994.
9. Stelzner, M. Ileal bile acid transport protein is expressed in the jejunum following transplantation of ileal mucosal stem cells. *J Surg Res.* **93:** 337, 2000.
10. Weiser, M. M. Intestinal epithelial cell surface membrane glycoprotein synthesis. I. An indicator of cellular differentiation. *J Biol Chem.* **248:** 2536, 1973.
11. Kim, S. S., Kaihara, S., Benvenuto, M. S., Choi, R. S., Kim, B. S., Mooney, D. J., and Vacanti, J. P. Effects of anastomosis of tissue-engineered neointestine to native small bowel. *J Surg Res.* **87:** 6, 1999.
12. Helmrath, M. A., VanderKolk, W. E., Can, G., Erwin, C. R., and Warner, B. W. Intestinal adaptation following massive small bowel resection in the mouse. *J Am Coll Surg.* **183:** 441, 1996.