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Transplantation of Human Intestinal Organoids into the Mouse Mesentery: A More Physiologic and Anatomic Engraftment Site

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Abstract

Background—We previously described the development of human intestinal organoids (HIOs) from pluripotent stem cells, as well as their *in vivo* maturation when transplanted into the mouse kidney capsule. While sufficient for certain aspects of study, this model has limitations. Herein, we describe an alternative model of HIO transplantation into the mouse mesentery. We hypothesize that efficient engraftment and marked differentiation of HIOs will be similar to our kidney model yet in a more anatomically appropriate location allowing for improved *in vivo* modeling.

Methods—HIOs were generated by directed differentiation of H1 embryonic stem cells. HIOs were then transplanted into the mesentery of immunosuppressed mice. Gross and histologic analysis of tissue was performed.

Results—HIOs were transplanted into the mouse mesentery and allowed to grow for 10 weeks. Mouse survival was 85% and among the surviving mice, 82% of transplanted HIOs successfully engrafted. Upon graft harvest, transplanted HIOs were larger than *in vitro* HIOs (1.75mm vs 6.27mm, $p<0.0001$) and grew along a vascular pedicle allowing for interventions and reconstructive surgeries to access the HIO lumen. Histologic analyses of transplanted HIOs confirmed the presence of major cell types, as well as stem cell activity.

Conclusions—The mouse mesentery is a viable location for the transplantation of HIOs, yielding grafts of reproducible size and quality. This improved model serves to advance functional and translational studies of HIOs.

Keywords

human intestinal organoids; pluripotent stem cells; animal models; intestinal development; intestinal maturation

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Background

Stem-cell based therapies offer great promise towards the future of patient care and regenerative medicine. Within the domain of stem-cell based research are human organoids, which are self-organizing 3D structures grown *in vitro* from human induced pluripotent stem cells (iPSC) and embryonic stem cells (ESC) that form human tissue. Organoids allow for the study of organogenesis, pathogenesis, and potential treatment options including pharmacologic development, gene manipulation, and therapeutic transplantation.

The rapid development of pluripotent stem cells has enriched the body of knowledge on the development of the gastrointestinal tract. Significant progress towards generating patient-specific human bowel occurred with our group's published methods to direct pluripotent stem cells into human intestinal organoids (HIO).[1] Subsequently, we developed an *in vivo* transplantation model involving transplantation of HIOs under the kidney capsule of immunodeficient mice.[2] These transplanted HIOs (tHIOs) recapitulate the human intestine both in their architectural features (e.g. crypt-villus structures) and observed cell types (e.g. Paneth and goblet). These early studies highlighted the potential for *in vivo* models to provide insight into new aspects of intestinal development and maturation.

Our work also supported the notion that at the present time *in vitro* culturing systems are limited to producing early developmental intestinal tissues, thereby making translation to human conditions challenging. In contrast, additional *in vivo* cues result in marked intestinal maturation and functional capability following transplantation. Animal models are therefore invaluable to study human intestinal development and function and to model GI diseases. Previous work from our laboratory also demonstrated the ability to both incorporate healthy and diseased enteric nervous systems into HIOs to study the effect of a severe phenotype of Hirschsprung's disease.[3] This type of modeling may prove beneficial for both congenital and acquired infant conditions such as necrotizing enterocolitis (NEC). Ultimately, a major application of organoids is to develop functional tissue that can replace lost bowel as therapy for patients with short bowel syndrome.

Based on our experience, we recognized that in order to further study the human intestines, models to expose the tHIO to luminal contents are required. As a result, this study set out to improve our *in vivo* transplantation model to enable access for HIO manipulation to achieve this goal. While our kidney transplantation model is sufficient for certain aspects of study (e.g. developmental biology), its retroperitoneal location presents limitations for disease modeling and translational studies that will be needed for the future clinical application of HIOs. A significant barrier with our existing model is the inability to transpose the matured tHIO intra- abdominally and place in continuity with the host's intestine. Herein, we describe an alternative model of HIO transplantation into the mesentery of immunodeficient mice. We hypothesize that this model will result in growth of tHIOs compared to *in vitro,* similar to our kidney model. More importantly, we propose that this more anatomically appropriate engraftment site will provide vascular ingrowth within the splanchnic circulation, thereby lending improved functional HIO studies and may offer a potential way for placement of the tHIO into the host's luminal content stream.

Methods

Generation of human intestinal organoids

The H1 embryonic stem cell (ESC) line was directed into definitive endoderm and subsequently HIOs as previously described by our groups.[1, 4] Briefly, H1 ESCs (WiCell research Institute, Inc) were grown in feeder-free conditions in Matrigel (BD Biosciences) and cultured in mTESR1 media (Stem Cell Technologies). Upon differentiation, the ESCs were harvested and treated with Activin A to induce the formation of definitive endoderm (DE).[5] The subsequent DE was then treated with midgut/hindgut differentiation media containing FGF4 (R&D) and Chiron 99021 (Tocris) to induce the formation of mid-hindgut spheroids. These spheroids were maintained in Mini-Gut Media with EGF (R&D) for 14 days to generate human intestinal organoids (HIOs). After 14 days, the HIOs were "split" and individually plated in Matrigel. HIOs were cultured for an additional 14 days in Mini-Gut Media and then were either processed and embedded for analysis (*in vitro* group) or prepared for transplantation *(in vivo* group). The HIOs used in this experiment were of the same batch for both the groups to ensure biological replicates of comparable size. Of note, work by our group and our collaborators has established that HIOs grown *in vitro* for a prolonged period do not have enhanced growth, maturation, or engraftment (data not shown).[1, 2]

Animals

Immunodeficient nonobese diabetic (NOD) severe combined immunodeficiency (SCID) interleukin-2Rγ^{null} (NSG) mice 5-8 weeks old were used in all experiments. Mice were obtained from the Comprehensive Mouse and Cancer Core Facility at our institution. Although males and female NSG mice equally engraft HlO's, all mice used in the current study were male due to the availability of litters at the time when females were dedicated toward enhanced internal breeding strategies.[3] All mice were housed in the pathogen-free animal facility at the Cincinnati Children's Hospital Medical Center (CCHMC). Handling was done humanely in accordance with the NIH *Guide for the Care and Use of Laboratory Animals.* All experiments were performed with the prior approval of the Institutional Animal Care and Use Committee of CCHMC (Signaling Pathways associated with Intestinal Stem Cell Expansion, Protocol No. 2016-0014).

Transplantation of human intestinal organoids

NSG mice were kept on antibiotic chow (275 p.p.m. Sulfamethoxazole and 1,365 p.p.m. Trimethoprim; Test Diet). Food and water were provided *ad libitum* before and after surgery. A single HIO matured *in vitro* for 28 days was transplanted into the mouse mesentery.[6] Briefly, mice were anesthetized with 2% inhaled isoflurane (Butler Schein) and the abdominal wall was prepped in a sterile fashion with isopropyl alcohol and povidine-iodine. A 1-2 cm midline incision was made to gain access to the abdominal cavity. The cecum was identified and gently pulled out with the colon and small intestine following. The mesentery was splayed out with identification of the distal ileum and the ascending colon. At a location with bifurcating mesenteric vessels 1-2 arcades from the ileocecal junction, a single drop of octyl/butyl cyanoacrylate adhesive glue (GLUture, Abbott Laboratories) was placed and the HIO was dropped onto the glue and allowed to dry in place for a minimum of 3 minutes

(Figure 2B). The intestines were then returned to the abdominal cavity and the mice were given an intraperitoneal flush of piperacillin/tazobactam (100 mg/kg; Pfizer Inc.). The skin was closed in a double layer and the mice were given a subcutaneous injection with Buprenex (0.05 mg/kg; Midwest Veterinary Supply). Survival of mice was followed out to 10 weeks at which time the mice were humanely euthanized. The percent engraftment and size of HIO were measured in millimeters following excision of the graft and volume calculated using the equation $V = 4/3\pi r^3$.

For mice that underwent an organoid-to-intestine anastomosis, the same operative protocol was followed except that an *ad libitum* diet was provided 48 hours prior to surgery. At the second operation, the organoid and adjacent small bowel were identified and removed from the abdominal cavity. A side-to-side anastomosis was performed using 9-0 nylon an in interrupted fashion.[6] Upon completion, the anastomosis was evaluated for gross leakage and the intestines were replaced into the abdominal cavity, taking care to avoid torsion.

Tissue processing, immunohistochemistry, and microscopy

Transplanted HIOs were harvested and fixed overnight in 4% paraformaldehyde (PFA), then processed and embedded in paraffin. Slides of 5 μm thick sections of tissue were made and deparaffinized, followed by heat-induced epitope retrieval and staining. Incubation for both primary and secondary antibodies took place at 4°C overnight in 1% bovine serum albumin in phosphate buffered saline (PBS). The following primary antibodies and their respective dilutions were used: goat anti-villin (1:100), mouse anti-HuMuc2 (1:1250), mouse anti-DEFA5 (1:500), mouse anti-CHGA (1:500) and rabbit anti-OLFM4 (1:400). The following secondary antibodies were used: horse anti-goat biotin (1:1000), horse anti-mouse biotin (1:1000) and goat anti-rabbit biotin (1:1000). A peroxidase-based detection system was used followed by nuclear fast red as counterstain (Vector Labs; Polysciences, Inc). Images were captured on a Nikon Eclipse T*i* and analyzed using Nikon Elements Imaging Software (Nikon).

Human Tissue Collection

Transplanted HIO grafts were histologically compared to human intestinal tissue. Human tissue specimens were collected with the prior approval of CCHMC's Institutional Review Board. In order to utilize the samples, informed consent was obtained from all patients and/or parent/legal guardians as appropriate. Samples of human jejunum were obtained from full thickness surgical specimens from adolescent/young adult patients (between 16 and 25 years of age) undergoing bariatric operations. The patients did not have known intestinal pathology and the tissues were pathologically normal in appearance.

Statistical analysis

JMP Pro Version 12.0 (SAS Institute, Cary, NC) was used to perform Student's t-test for statistical analysis. A p-value <0.05 was considered statistically significant.

Results

Transplantation into the mouse mesentery is feasible and successful

HIOs following 28 days of differentiation *in vitro* were transplanted into the mesentery of immunosuppressed mice (n=20) and allowed to grow for 10 weeks as depicted in Figure 1A. The mice tolerated the surgery well with a survival rate of 85% (n=17/20) (Figure 1B). Moreover, 82% of the tHIOs (n=14/17) successfully engrafted within the host mesentery (Figure 1C).

HIO size increases following in vivo growth into the mouse mesentery

Following *in vivo* growth of tHIOs for 10 weeks, the grafts (n=14) were harvested and compared directly to *in vitro* HIOs (n=15). The tHIOs had grown notably compared to their *in vitro* counterparts, as expected (Figure 2A-D), and were significantly larger (diameter = 6.27 mm ± 1.43 vs 1.75 mm ± 0.44, $p<0.0001$) (Figure 2E). This translates to a tHIO volume approximately 46 times larger than *in* vitro HIOs (2.81 mm^3 vs 129.06 mm^3). The graft sizes following maturation in the mesentery were comparable to those observed in our published work using the kidney capsule transplantation model.[2]

Transplanted HIOs resemble human intestines

Visual inspection of the tHIOs was notable for normal intestinal morphology with ingrown vasculature from the host. When incised, a majority of the tHIOs contained a single lumen with mucous debris (Figure 4H). Histologic analyses of tHIOs confirmed native appearing mesenchyme with subepithelial elements and muscular layers, as well as expansion of the epithelium with the presence of the major cell lineages including enterocytes (VIL1), enteroendocrine cells (CHGA), goblet cells (MUC2), and Paneth cells (DEFA5), similar to that of human intestine (Figure 3). In addition, this staining for the primary epithelial cell types is regularly observed with *in vitro* HIOs prior to transplantation.[1] However, Paneth cells in tHIOs were located to the intestinal crypts, which is a hallmark of early maturation. In addition, tHIOs stained positively for olfactomedin-4 (OLFM4), which is associated with the intestinal stem cells and is an additional marker of early intestinal maturation.[7] These two markers of early maturation are not uniform among *in vitro* HIOs[7], but are seen when matured *in vivo* with this model, as well as following transplantation under the kidney capsule.[2]

Mesentery transplantation has multiple advantages for in vivo study

Following *in vivo* growth in the kidney model, the tHIO grows to a significant size but as a result of the kidney's attachment the retroperitoneum, subsequent access and manipulation of the tHIO is difficult (Figure 4A). In contrast, tHIOs following *in vivo* growth in the mesentery are distinct from adjacent bowel and can be easily accessed and manipulated (Figure 4B-D). When tHIOs grow in the mesentery, they receive vascular ingrowth from the host's mesenteric vessels and as a result can be mobilized along its vascular pedicle (Figure 4E-F). As a result, tHIOs can be accessed at a later date for further interventions such as injections or direct placement of contents intraluminally (Figure 4G-H). As a proof of concept, pilot work was also performed to study the feasibility of performing an organoid-

to-intestine anastomosis (n=6). An anastomosis was able to be performed in all mice and 50% survived to 21 days at the time of harvest (Figure 5).

Discussion

In this study, we present a practicable and successful mesentery transplantation model for the *in vivo* growth and maturation of human intestinal organoids. Similar to our previously described kidney transplantation model, HIOs grow considerably larger when transplanted into the mesentery of immunosuppressed mice and form fully laminated human tissue. More importantly, this improved engraftment site takes advantage of the host's intestinal blood supply and allows for vascular ingrowth into the splanchnic circulation along a vascularized pedicle. The intraabdominal location facilitates easy access and mobilization of the tHIO for surgical manipulation allowing for interventions and reconstructive surgeries that enable placement of contents, such as nutrients and microbiota, into the HIO lumen, as well as exposure to the host fecal stream.

Our initial model used the kidney as the engraftment site because it is an isolated, immune-privileged location that is easily accessible and is well tolerated by the mice. As reported by Watson *et al.,* marked changes in the HIO were noted following transplantation into the kidney capsule with maturation of both epithelial and mesenchymal elements of the tissue. In addition, the tissue was noted to have functional capacity including barrier function and absorption of sugars and amino acids.[2] Our group recently showed that a functional enteric nervous system can be developed with the HIO, resulting in increased activity of the intestinal stem cell/transit-amplifying compartment and electromechanical coupling that regulates propagating contractions.[3] As our experience advanced, however, we encountered limitations with the kidney model when developing studies to assess the role of luminal contents. Namely that when the HIO grew under the kidney capsule, it did not have its own vascular pedicle and is constrained by the kidney's retroperitoneal attachment. As such, subsequent surgical manipulation of the HIO for direct luminal studies or future surgical modeling to place in continuity with the host intestinal tract proved difficult.

The mesentery transplant model, in contrast, is not only feasible and reproducible, but moreover meets our needs for improved *in vivo* modeling. This includes performing an organoid-to-intestine anastomosis, as well as other interventions such as direction injection or placement of an infusion catheter into the HIO lumen. Through our experience with this procedure in establishing an appropriate methodology for this model, we have learned a number of important considerations. Deaths in the early postoperative phase are typically related to the graft being directly adjacent to bowel resulting in obstruction with growth or intestinal perforation by a sharp edge of excessive glue in the mesentery. Pilot work with this model to assess engraftment included placement of the HIO into a small mesenteric pocket secured with a purse string suture, but this was challenging for routine use whereas the use of glue allowed for the procedure to be easily performed by all laboratory members. Early bowel obstruction due to kinking of the mesentery can be resolved by allowing sufficient time for drying (minimum 3 minutes) prior to returning the bowel to the abdominal cavity. Whereas the kidney capsule can accommodate extremely large HIO growth without consequence, we have encountered the occasional extremely large HIO (up to 2 cm), as well

as inflammation from the glue, can cause intestinal obstruction. To avoid this, we ensure placement of the HIO approximately 8-12mm away from the mesenteric border on the bowel.

Organoids introduce a new mechanism by which the intestines can be studied using patient-specific tissue. Notable advancements have occurred through the use of *in vitro* models to study various genetic (e.g. cystic fibrosis), infectious (e.g. clostridium difficile colitis and rotavirus gastroenteritis), and inflammatory (e.g. inflammatory bowel disease) pathologies.[8-11] While these models provide insight into the physiologic response of the intestines, they are limited by their *in vitro* nature which does not fully recapitulate what occurs *in vivo* at the patient level. To improve the translational impact of organoids then, *in vivo* models are necessary. *In vivo* models offer a significant benefit by allowing for the study of intestinal development and maturation in a more natural and physiologic environment. The completeness of *in vivo* models was elegantly demonstrated by Fumagalli *et al.* in which they manipulated human organoids through CRISPR/Cas9 to mimic common mutations seen in colorectal cancer and following transplantation into immunodeficient mice, modeled the adenoma-carcinoma sequence with subsequent metastasis that is observed in humans.[12]

Traditionally, animal models have been used as a surrogate to study developmental biology, physiology, and pathogenesis of human disease. The obvious limitation is the translation of animal processes to humans. Organoids, however, allow for the study of human tissue directly. When HIOs are then studied *in vivo,* the complex interaction of the intestines with its host remain intact to provide a deeper understanding into how the intestines respond to factors such as ischemia, infection, and surgery. One of the main goals of organoid systems is to develop functional organs and as such, translational studies are required to increase their clinical relevance. In order to achieve this, large animal models will likely be required before organoids are used in humans. Moreover, the immune component of HIOs and their *in* vivo engraftment play an important consideration and will require either syngeneic animals or humanizing the host. Nonetheless, we believe our mouse model serves as a step along the continuum to achieving this goal. If pluripotent stem cell technology will one day be used for transplantation, they must grow with a blood supply that is physiologic, which we demonstrate in this study. Our work therefore supports the concept that this can be done in humans. Further work to demonstrate increased function is needed over time and this is the focus of ongoing work in our lab. Ultimately, we anticipate this model will be used to study several GI conditions of congenital etiology such as intestinal atresias, cystic fibrosis, and NEC.

We recognize that there are limitations to this study. First, it was not designed to directly compare the two models of transplantation and therefore we cannot conclude whether one model is superior to the other. Rather, we believe they are complementary and serve different purposes depending on the biologic question being explored. Second, we sought out to evaluate this model as a proof-of-concept and measured basic outputs of size and maturation. As a result, we are unable to comment on the level of HIO functional maturity and recognize that functional assays of maturation play an important next step in the study of HIOs. Third, pilot work was initially performed to address the methodology of the model

and is not included. However, our reported findings are reproducible when the described methodology is followed.

Conclusion

Human intestinal organoids (HIOs) offer an exciting avenue to study human intestinal development, physiology, and pathogenesis. The mesentery of NSG mice is a suitable and viable location for the transplantation of HIOs, yielding grafts of reproducible size and quality.

We believe that this improved engraftment site serves to further aid in functional and translational studies of stem cell-based intestinal organoid development.

DISCUSSANT

DR. CARLOS CHAN (Iowa City, IA): The study of organoids has a broad range of applications, from organ development to cancer research. There have been quite a lot of studies on those recently. We have been doing these kinds of studies for many years in our lab, back in the ‘90s, when we didn’t know too much about stem cells. But now, with the growing knowledge of stem cells, I do think a better biologically relevant model is needed. I congratulate Dr. Cortez and his colleagues for moving this forward.

My specific questions about this study are both on a technical and theoretical level. One of the things that you were trying to say is your model seems to be better. It looks better as compared to the kidney capsule model because of mobilization and exposure using manipulation. That sounds really interesting in that sense.

You’re saying that the next step may be doing anastomosis with the bowel. In the immunocompromised animal model, would opening the bowel have a health impact? What would be the anticipated survival rate? The other question would be your mice are immunocompromised and living in a pathogen-free environment. Some of the studies that you may want to do would be to look at microbiota in the gut development. My second question is how would you do that study in such model.

My last question is, did you see a difference in terms of cell differentiation between the kidney capsule model versus your mesenteric model?

You have really great work. Enjoyed reading your paper.

CLOSING DISCUSSANT

DR. ALEXANDER CORTEZ: The first question related to doing bowel surgery on immunosuppressed mice and how they tolerate that surgery. In our lab, previously we performed in different studies ileocecal resections on immunosuppressed and not immunosuppressed animals, and they tolerated the surgery well. It just requires careful surgical technique to avoid spilling of the intestinal contents, and they are typically placed on a liquid diet beforehand.

In the pilot work that we have done with some anastomosis to the small bowel, the animals tolerate the surgery well from a postoperative standpoint. So it seems to be feasible doing this intestinal reconstructive work with the organoids.

The second question, yes, you're, in fact, correct. We do these in immunosuppressed animals, and these animals are housed in a pathogen-free environment. From a clinical standpoint, how this would translate. The reason we used immunosuppressed animals is from a graft-rejection standpoint. So if we have projects where we're using humanized animal models -- one of my colleagues in the lab is taking humanized animal and then putting these HIOs in -- those animals don't need to be immunosuppressed. She houses those in conventional housing as well.

For the third question, we looked at the basic markers, which I showed here for our mesenteric-grown organoids. From the standpoint of those basic markers, they grew and had the same differentiation as the kidney capsule, but we haven't gone into further detail on whether or not the mesentery is a better location in terms of maturity or functionality.

DISCUSSANT

DR. MATTHEW HERNANDEZ (Rochester, MN): Modeling with 3D formats in mice is very labor intensive, but I have questions about the scalability of this model. You only have 17 mice that essentially you were successful in. Based on that, have you considered using the chorioallantoic membrane as another source for injecting the organoids and having the ability to transplant these and visualize them more easily?

CLOSING DISCUSSANT

DR. ALEXANDER CORTEZ: That is a great point in terms of the efficiency of these models. We have done various experiments. We have not looked at the chorioallantoic membrane, but we have tried using the kidney mesentery, and previously attempted the epididymal fat pad. We have recently started to scale towards larger animal models and specifically in a rat. The advantage there is they are not necessarily larger from our preliminary work, but you're able to put four or five organoids within the mesentery of the rat, which it tolerates; whereas, in a mouse, the area is too small. We are looking at ways to have higher throughput of the system.

DISCUSSANT

DR. JUAN A. ASENSIO (Omaha, NE): You demonstrated you were able to at least test or stain for the four basic lines of the mucosa. Since you already are able to stain for the immunological layer, have you harvested any of the lymphocytes? If you have, have you tested function? Will you be able to isolate T-killer cells? I think this is something to be considered in the future, and I congratulate you on such fine work.

CLOSING DISCUSSANT

DR. ALEXANDER CORTEZ: Thank you very much. One of the things our lab has done previously is be able to grow these organoids with neural crest cells and develop a functional enteric nervous system. As we move forward, in terms of the complexity of these organoids

grown in vitro, we're looking at how to have a functional immune system because these organoids are void of that currently. Finding ways to grow a functional immune system within the organoid is ongoing work in our lab right now.

DISCUSSANT

DR. MARK MALANGONI (Philadelphia, PA): Your engraftment rate was just over 80%. I wonder if you could speculate as to why some of these organoids did not engraft properly and how that might affect what you're going to do in the future.

CLOSING DISCUSSANT

DR. ALEXANDER CORTEZ: That's a great question. When we do the kidney transplant, it's interesting. There's typically some bleeding from the kidney bed. So you would speculate that there's some angiogenesis occurring and ingrowth of the vessels into the organoid. It's been interesting because in the mesenteric model, in some preliminary work, we did a mesenteric pocket and placed it in. Here we used glue. There's really no disruption of the blood vessel, so how these vessels are growing in is still something we're looking into. We speculate that it might be an irritation from the glue, or some stimulation of the blood vessels in the mesentery that grow in. However, it is unclear exactly why some of them aren't growing.

The other thing that we are lucky to have in our lab is a very standardized protocol, and really there's one person that grows these organoids. But if there is a patterning issue with the stem cells in vitro, that can also impact how they grow in vivo.

DISCUSSANT

DR. RONALD WEIGEL (Iowa City, IA): Have you advanced this to the point that you have tried harvesting and creating organoids from syngeneic animals? Instead of using the single HIO, developing them from the syngeneic animals would allow use of the model in immune competent animals and other large animal models, such as pigs, which would be closer to the human model.

CLOSING DISCUSSANT

DR. ALEXANDER CORTEZ: Definitely. For your first question, we specifically use human organoids. If you grew an organoid from the stem cells of the mouse, you wouldn't need immunosuppressed. But since we specifically used human organoids, that's something we have to do. But, theoretically, if you were growing an iPS line-derived HIO and putting it back into that patient, from a clinical standpoint, you wouldn't need immunosuppression.

From a large animal standpoint, we're headed to Virginia Tech this week to transplant these into an immunosuppressed pig model through a collaborative project. We're intrigued to see how, not only implanting these into a large animal, but placing them in at the time of birth to determine the critical time of growth, and seeing how they grow in the large animal for more clinical relevance.

DISCUSSANT

DR. SCOTT GRUBER (Detroit, MI): The small bowel is the most prone to rejection of all of our transplanted organs. It has the lymph nodes, obviously, within the mesentery and all of that. So I think it is key that you pointed out that you use immunosuppressed animals to prove the model. But after that is done, you will not be in a transplant-type situation from that standpoint. So I think that's really important to emphasize because, in other words, you're creating additional hardships for yourself to prove this model by choosing the most antigenic of organs to place these organoids. I think there's a balance that you have to reach.

CLOSING DISCUSSANT

DR. ALEXANDER CORTEZ: That is a great point, and we appreciate that. Thank you.

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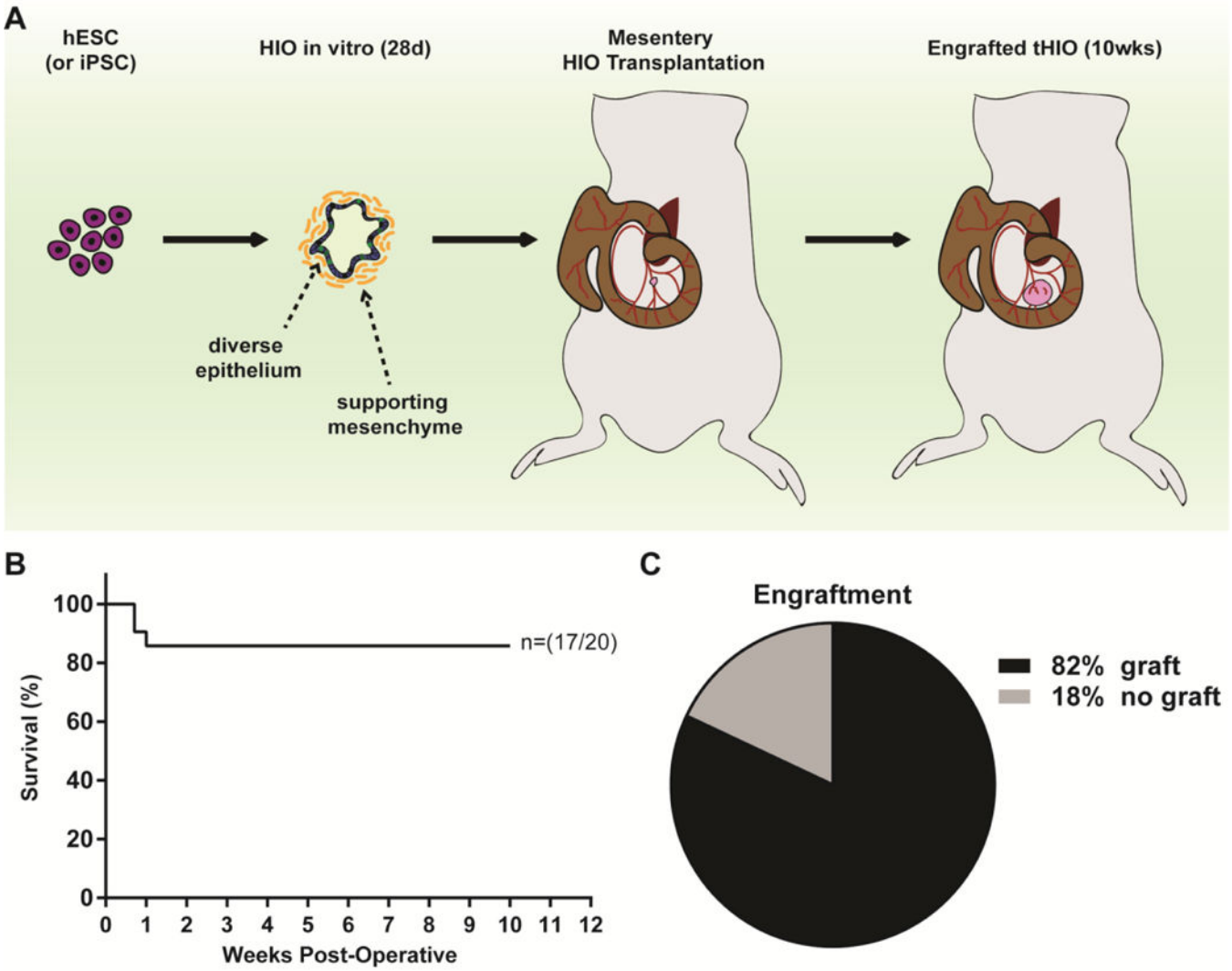

Figure 1.

Transplantation into the mouse mesentery is feasible with successful engraftment. (**A**) Schematic representing the development of HIOs *in vitro* followed by transplantation into the mesentery of mice with graft harvest at 10 weeks. (**B**) Kaplan-Meier curve associated with the mesentery transplant model. (**C**) Pie chart of HIO engraftment.

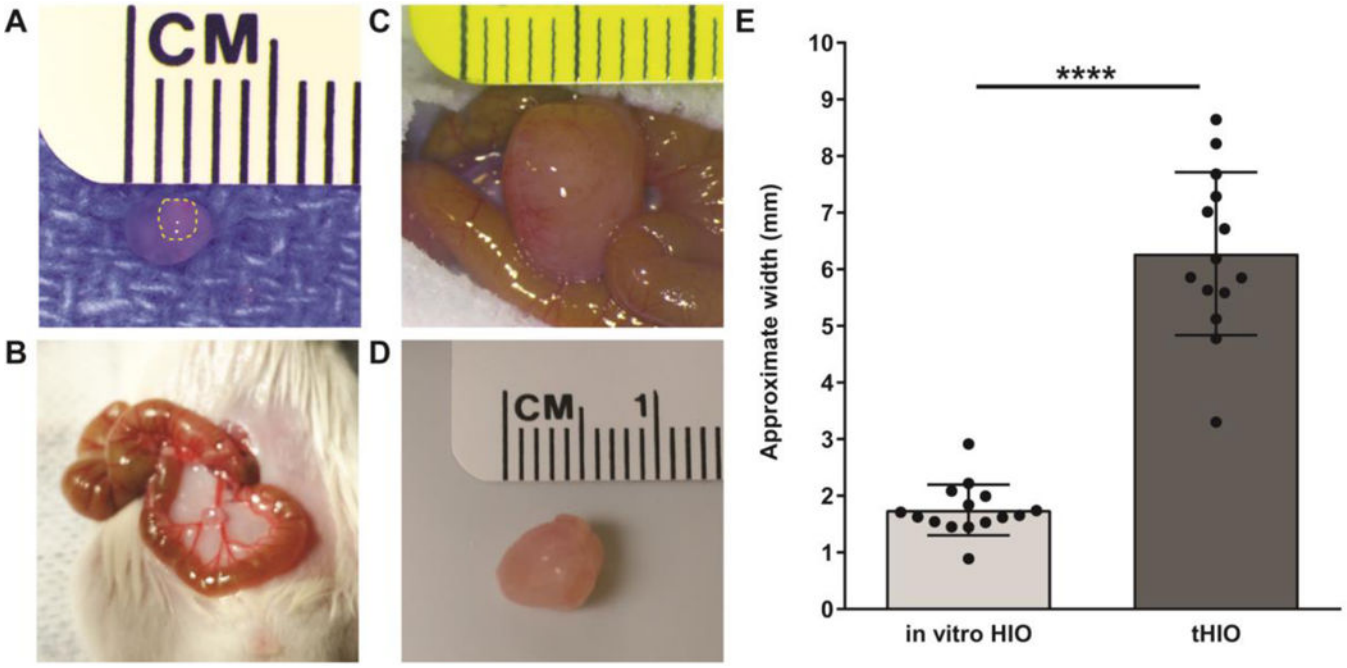

Figure 2.

HIOs grow significantly following *in vivo* growth. (**A**) Image of an HIO (outlined) in Matrigel being prepared for transplantation and (**B**) after placement into the mesentery. (**C**) Following 10 weeks of *in vivo* growth, the tHIO has engrafted into the mesentery and (**D**) is notably larger at harvest. (**E**) tHIOs were significantly larger than *in vitro* HIOs at the time of harvest (diameter = 6.27 mm ±1.43 vs 1.75 mm ±0.44, $p<0.0001$).

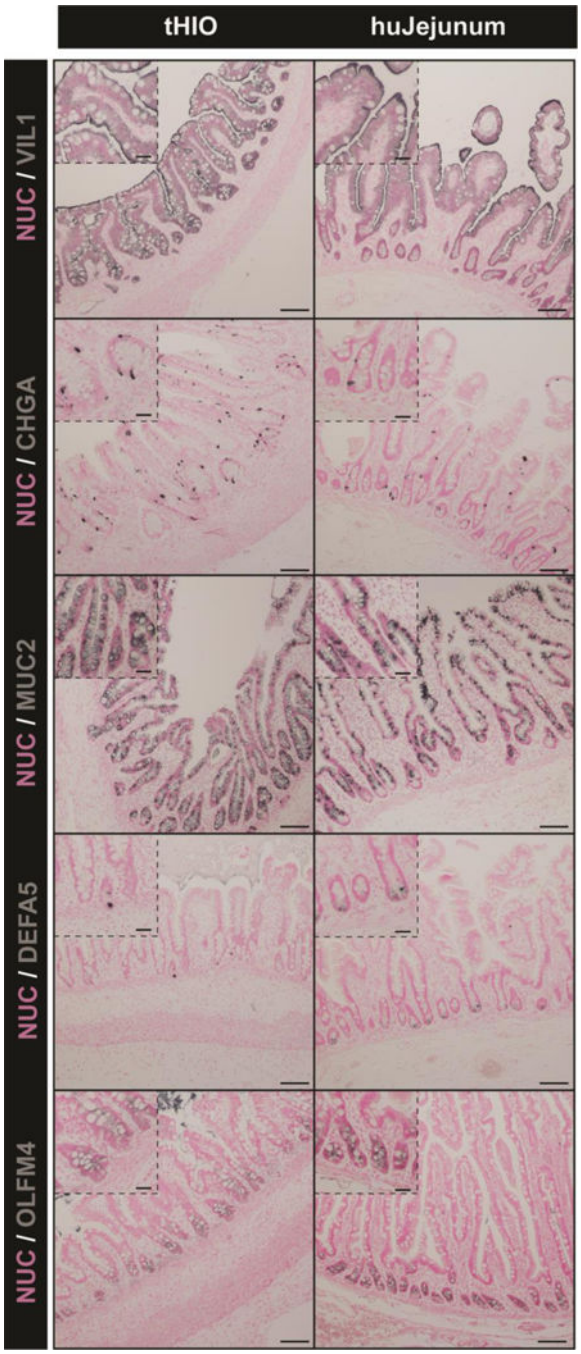

Figure 3.

Transplanted HIOs resemble human intestine. Analysis of tHIOs closely resemble human intestine with the presence of major cell lineages including enterocytes (VIL1), enteroendocrine cells (CHGA), goblet cells (MUC2), and Paneth cells (DEFA5). In addition, they stain positive for a marker of stem cell activity (OLFM4). Histology images are low-power field, insets are high-power field (scale bars = 100 μm).

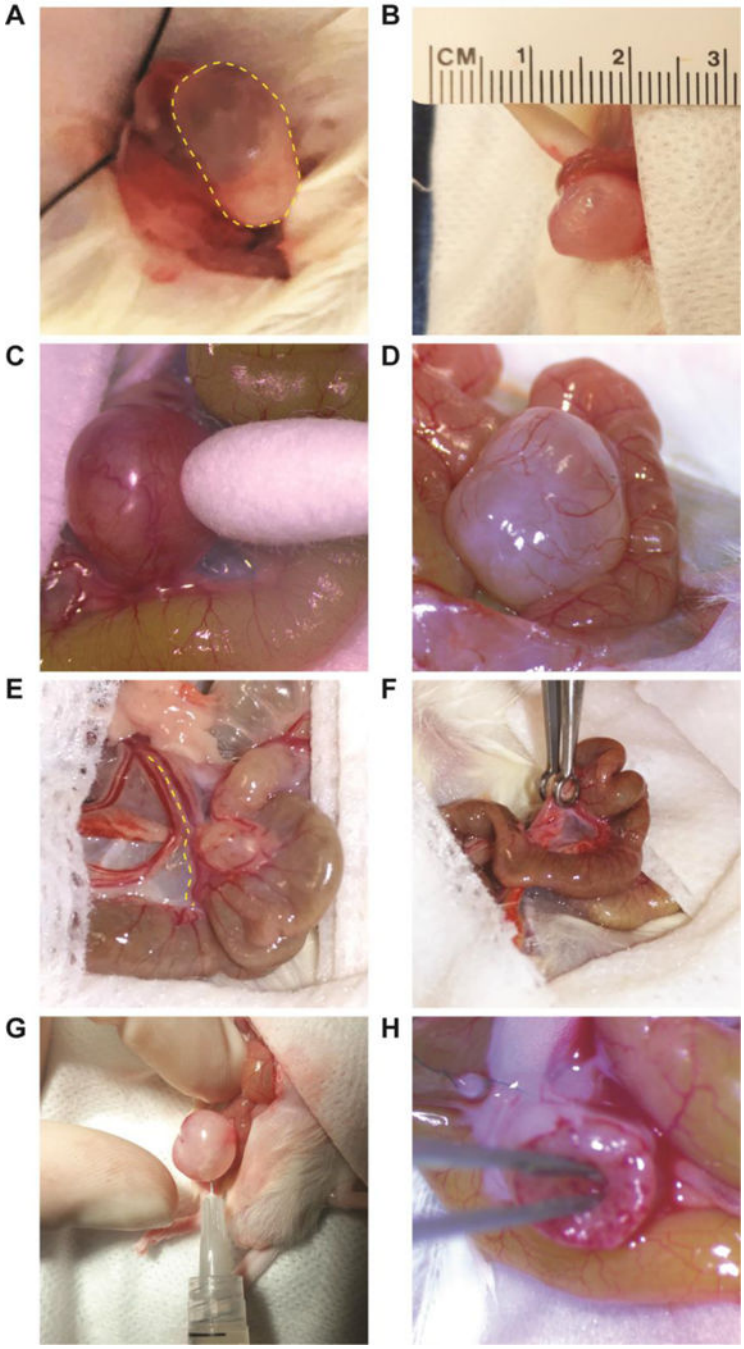

Figure 4.

The mesentery transplantation model has multiple advantages for *in vivo* study. (**A**) Following *in vivo* growth in the kidney model, the tHIO (outlined) is markedly larger but constrained to the retroperitoneum such that manipulation is difficult. (**B-D**) In contrast, tHIOs following *in vivo* growth in the mesentery are distinct from adjacent bowel and easily accessed and manipulated. (**E**) tHIOs that grow in the mesentery receive vascular supply from the mesenteric vessels and (**F**) can be manipulated and mobilized with its vascular

pedicle. (**G,H**) Subsequently, the tHIO can accessed for further interventions such as injections or direct placement of contents intraluminally.

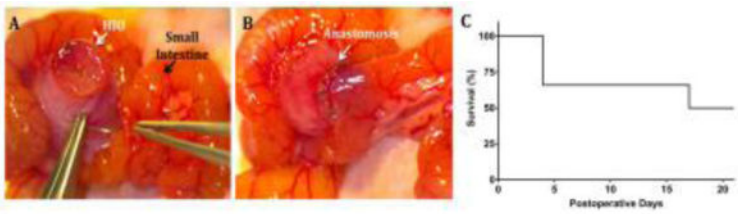

Figure 5.

Organoid-to-intestine anastomosis is feasible with the mesentery transplantation model. (**A**) The tHIO lumen is easily accessed and (**B**) an anastomosis was able to be performed in all mice (n=6). (C) 50% of these mice survived to 21 days at the time of harvest.