Tissue-engineered esophagus: Experimental substitution by onlay patch or interposition

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Objectives: We proposed to fabricate a tissue-engineered esophagus and to use it for replacement of the abdominal esophagus.

Methods: Esophagus organoid units, mesenchymal cores surrounded by epithelial cells, were isolated from neonatal or adult rats and paratopically transplanted on biodegradable polymer tubes, which were implanted in syngeneic hosts. Four weeks later, the tissue-engineered esophagus was either harvested or anastomosed as an onlay patch or total interposition graft. Green Fluorescent Protein labeling by means of viral infection of the organoid units was performed before implantation. Histology and immunohistochemical detection of the antigen α-actin smooth muscle were performed.

Results: Tissue-engineered esophagus grows in sufficient quantity for interposition grafting. Histology reveals a complete esophageal wall, including mucosa, submucosa, and muscularis propria, which was confirmed by means of immunohistochemical staining for α-actin smooth muscle. Tissue-engineered esophagus architecture was maintained after interposition or use as a patch, and animals gained weight on a normal diet. Green Fluorescent Protein–labeled tissue-engineered esophagus preserved its fluorescent label, proving the donor origin of the tissue-engineered esophagus.

Conclusions: Tissue-engineered esophagus resembles the native esophagus and maintains normal histology in anastomosis, with implications for therapy of long-segment esophageal tissue loss caused by congenital absence, surgical excision, or trauma.

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Our laboratory first reported making a tissue-engineered small intestine by means of the transplantation of organoid units (OUs) on a polymer scaffold into the omentum of the Lewis rat.[1] OUs are multicellular units derived from neonatal or adult tissue or other tissue-engineered constructs, containing a mesenchymal core surrounded by a polarized intestinal epithelium and all of the cells of a full-thickness intestinal section.[2] This protocol has been transformed to yield greater numbers of OUs more efficiently from an additional area of the gastrointestinal tract, the sigmoid colon. The protocol changes lead to increased

viability: production of tissue-engineered colon (TEC) occurs 100% of the time.[3] In addition, there is initial evidence of in vivo physiologic replication of major functions by TEC, which produces short-chain fatty acids and absorbs moisture and sodium when compared with animals that lack a TEC.[3] We hypothesized that these techniques could be translated to the production of a functional, living, tissue-engineered esophagus (TEE) that could be used for replacement.

A major advantage of tissue engineering is exact replacement rather than replacement with prosthetic materials or by using another tissue type by proxy. The creation of living replacement organs and tissues has the advantages of self-propagation and self-repair without compounding tissue loss to perform surgical replacement.[4]

Multiple strategies to reconstruct pharyngogastric transit currently exist. Most commonly, the colon or the denervated stomach replace the esophagus.[5-8] Free jejunal grafts can also be used.[9-12] Although these conduits have each served as acceptable substitutes, the choice of conduit still remains open to debate,[8,9] and none exactly recapitulate esophageal architecture and function. In addition, they require an exchange in which some native tissue and its in situ function are lost to replicate a more important function. Options for esophageal replacement might be limited in some patients by previous resection or intestinal disorders. TEE might enable reconstruction, even when the usual surgical alternatives are unavailable.

Materials and Methods

Generation of TEE

Animals were cared for in compliance with the "Institute of Laboratory Animal Resources Guide." Esophagus OUs were produced by dissecting the abdominal esophagus without mesentery from 6-day-old Lewis rat pups (n = 30) or adults (n = 3), which was cut into full-thickness 2 mm × 2 mm sections after lengthwise opening along the antimesenteric border. Because in the rat the margo plicatus, the epithelial transition from the esophagus to the stomach, occurs within the anatomic stomach, the lower margin of resection was just above the cardia. The upper margin was the diaphragm. The resected specimens were washed 2 times in 4°C Hanks balanced salt solution, sedimenting between washes, and digested with 0.25 mg/mL dispase (Boehringer Ingelheim) and 800 U/mL collagenase type I (Worthington) on an orbital shaker at 37°C for 20 minutes. The digestion was immediately stopped with three 4°C washes of a solution of high-glucose Dulbecco's modified Eagle's medium, 4% heat-inactivated fetal bovine serum, and 4% sorbitol. The OUs were centrifuged between washes at 150*g* for 5 minutes, and the supernatant was removed. OUs were reconstituted in high-glucose Dulbecco's modified Eagle's medium with 10% heat-inactivated fetal bovine serum, counted with a hemocytometer, loaded at 100,000 units per polymer at 4°C, and maintained at that temperature until implantation, which occurred in less than 1.5 hours.

Green Fluorescent Protein Methods

Green Fluorescent Protein (GFP)–labeled OUs underwent an additional step of incubation with the Green Fluorescent Protein–vesicular stomatitis virus (GFP-VSV) at a multiplicity of infection of 10^3 at 37°C on an orbital shaker for 1.5 hours before undergoing 3 washes of the reconstitution solution and treatment as above. Two hundred thousand OUs were maintained in a 12-well plate to measure GFP production in vitro in 24 hours, and the remaining OUs were implanted. Production of GFP was performed as follows. A Moloney murine leukemia virus retroviral vector with GFP expressed from an endogenous cytomegalovirus promoter was used. The virus was pseudotyped with the VSV glycoprotein to allow efficient infection of the rat organoids. Virus was produced after transient transfection in 293-GPG cells. This retroviral producer cell line provides the retroviral packaging functions in trans and allows expression of the VSV-G protein in a tetracycline-regulated fashion.[13] Seventy-five dishes were transfected with the retroviral vector DNA (10 μg) in the absence of tetracycline by lipofectamine (Invitrogen Corporation, Carlsbad, Calif). The medium was changed every 24 hours. Tissue-culture supernatants containing virus were harvested every 24 hours at the 72-, 96-, 120-, and 144-hour time points. Harvested supernatants were clarified by means of low-speed centrifugation. The viral supernatants were then filtered through a 0.45-μm filter and immediately concentrated up to 1000-fold by means of centrifugation at 17K rpm and 4°C. The resulting pellets containing concentrated virus were resuspended in buffer containing 8 μg/mL polybrene (Sigma H 9268; Sigma Chemical Co, St Louis, Mo) at 4°C overnight and pooled. Titers were estimated by using the GFP signal after infection of 293 cells. GFP detection 2 weeks after anastomosis was performed on 10-μm cryosections with native tissue controls.

Polymer Design

Scaffold polymers were constructed of 2-mm-thick nonwoven polyglycolic acid (fiber diameter, 15 μm; mesh thickness, 2 mm; bulk density, 60 mg/cm^3; porosity, >95%; Smith & Nephew, Andover, Mass) formed into 1-cm tubes (outer diameter = 0.5 cm, inner diameter = 0.2 cm) and sealed with 5% poly-L-lactic acid (Sigma-Aldrich Corp, St Louis, Mo) in chloroform. Polymer tubes were sterilized in 100% ethanol at room temperature for 20 minutes, washed with 500 mL of phosphate-buffered saline solution-(PBS) coated with 1:100 collagen type 1 (Vitrogen)/PBS solution for 20 minutes at 4°C, and washed again with 500 mL of PBS.

TEE Surgical Groups

During pentobarbital-induced anesthesia, eight 150-g male Lewis rats were implanted with neonatal-derived OUs, and 3 were implanted with adult-derived OUs. Implantation was achieved through a 1.5-cm upper abdominal incision, through which the greater omentum was externalized and wrapped in a specific manner around the TEE construct, secured with a 6-0 Prolene suture (Ethicon, Inc, Somerville, NJ), and returned to the peritoneum before closing the abdomen in layers. At 4 weeks, 2 groups of 3 neonatal-derived TEE animals each underwent an additional operation. All remaining animals were harvested for histology and immunohistochemistry. In the TEE patch group (TEEP) a 2.5 cm × 1 cm portion of the abdominal esophagus was removed, and the TEE was opened and trimmed to the size of the native esophagus

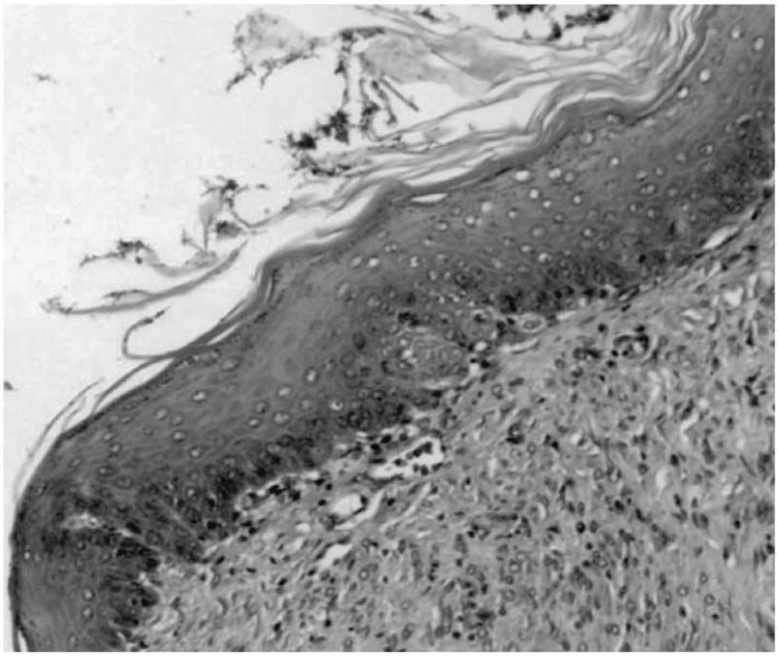

Figure 1. TEE derived from a neonatal rat pup. (Hematoxylin and eosin; original magnification 10×.)

deficit. By using a 6-0 Prolene suture in running fashion, the TEE patch was applied. In the TEE interposition group (TEEI) a 2-cm-long circumferential segment of native esophagus down to the esophageal junction with the stomach was removed, and 2 end-to-end anastomoses were performed after creating two 1.5-cm incisions on either end of the TEE to completely interpose the TEE between the remaining abdominal esophagus and the native stomach. Both groups of animals were given nothing by mouth after these operations for 3 days, given free access to water, and then given free access to normal rat chow on postoperative day 4. Weights were measured every other day. Animals were filmed and observed by both the investigators and a blinded viewer to assess for respiratory or gastrointestinal symptoms or difficulty with deglutition. Both the TEEI and TEEP animals were killed at day 42, and tissue was collected for histology. Sections of TEEs were fixed in 10% formalin and routinely processed. Four-micrometer sections were analyzed by a pathologist. Immunohistochemical detection of the antigens α-smooth muscle actin and S100 was performed. Fluoroscopy was performed on day 42 in both groups, as well as in a control animal, by using radiocontrast administered during inhaled sedation before harvest.

Results

TEE formed in all implantations from neonatal animals and in 2 of 3 implantations from adult-derived tissue. When successful, TEE from neonates and adults were histologically indistinguishable (Figures 1 and 2). The TEE appeared as a single-lumen, mobile cyst in the omentum and ranged from dimensions of 3 cm $\times$ 2 cm $\times$ 2 cm to 4 cm $\times$ 3 cm $\times$ 2 cm. The tissue was pliable and held sutures in a manner that was similar to native tissue. The lumen of the TEE was filled with slightly viscous clear mucus, with no evidence of purulence confirmed by means of histologic examination. The TEE resembled native rat esophagus with a keratinized stratified squamous epithelial lining. The lining could be stripped with vigorous swabbing away from the muscularis but with proper care was continuous with the muscularis propria. There were some areas of deep glandular invaginations after anastomosis that were more pronounced adjacent to the stomach (Figure 3). Immunohistochemical staining for α-actin smooth muscle was positive in the muscularis; however, it was less developed than in the native esophagus, with separate muscle slips rather than a continuous layer (Figure 4). Immunohistochemical staining for S100 did not detect antigen.

One animal in each surgical group did not survive the operation. In both cases animals did not awaken after the initial operation. Of the surviving 2 animals in each group, there was an initial weight loss, followed by a subsequent weight gain. By day 42, all animals had regained weight, ranging from 105% to 100% of their preoperative weight (Figure 5). On routine observation to assess for respiratory or gastrointestinal symptoms or difficulty with deglutition, no difference between the TEEI, TEEP, or control animals was noted.

TEE formed from OUs marked with the GFP virus (Figure 6) maintained GFP signal on frozen sections after 4 weeks of growth and 42 days of anastomosis (Figure 7). Native esophagus was not autofluorescent. Esophagus his-

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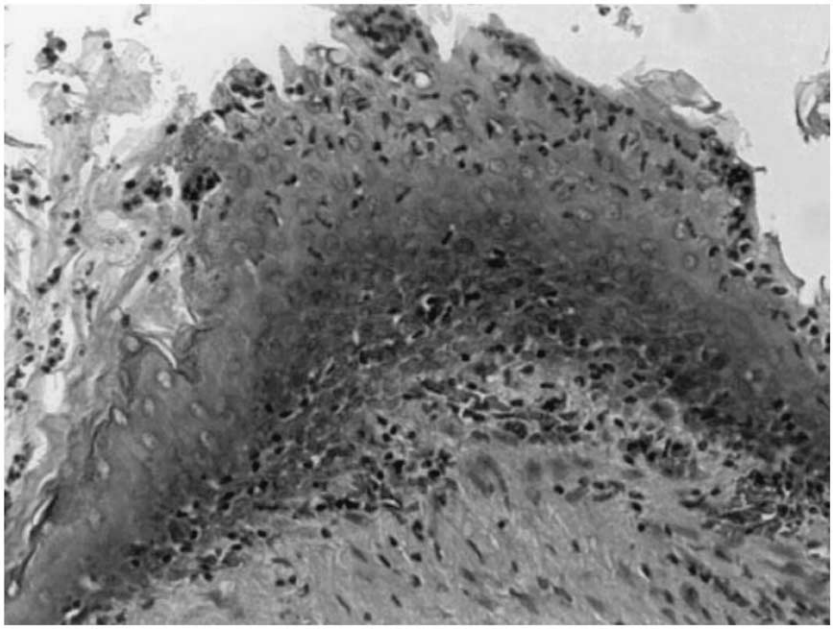

Figure 2. TEE derived from an adult rat. (Hematoxylin and eosin; original magnification 10×.)

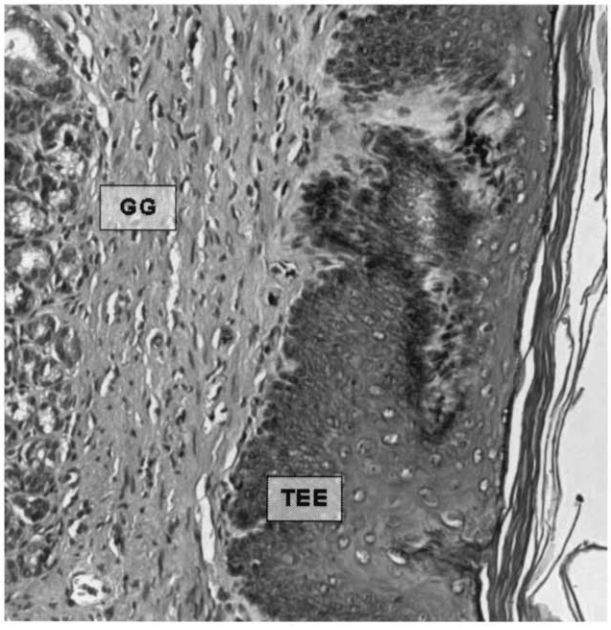

Figure 3. TEE from the TEEI group harvested at day 42, including the gastroesophageal junction. A segment of engineered esophageal epithelia *(TEE)* on the right is seen next to native stomach gastric glands *(GG)* on the right. (Hematoxylin and eosin; original magnification 10×.)

tology was maintained in the anastomosed animals in the TEE.

Fluoroscopy, performed on the same animals for which weight gain over 42 days was observed, as seen in Figure 5, revealed a wide unobstructed lumen in TEEP animals (Figure 8). In the TEEI group both animals had a stenosis at the upper anastomosis, whereas the lower anastomosis, contiguous with the native stomach, was widely dilated (Figure 9). Esophageal dilation above the stricture was not observed in either animal at autopsy. Dilation was induced at fluoroscopy to force radiocontrast throughout the upper gastrointestinal tract. Passive relaxation after radiocontrast filling was seen in each group. On gross examination of both groups at the time of death, the TEE remained strong yet pliable, with no gross signs of ulceration or tissue injury (Figure 10). The upper anastomosis stenosis of TEEI animals was grossly visible.

Discussion

The focus of tissue engineering is the successful replacement of tissue without resorting to using other tissue by proxy or manmade substitutes that might not be regenerative. In this novel method for the generation of an architecturally correct TEE, we show the broad application of an improved technique that now successfully produces both TEC and TEE from both neonatal and adult animals and its use as a replacement tissue. In addition, the successful labeling of TEE with GFP virus ascertains its provenance and has implications for the eventual inclusion of gene therapy in this versatile model.

A dominant question in using this new protocol to create tissue-engineered intestinal tissue has been whether the tissue-engineered neointestine is solely donor tissue or

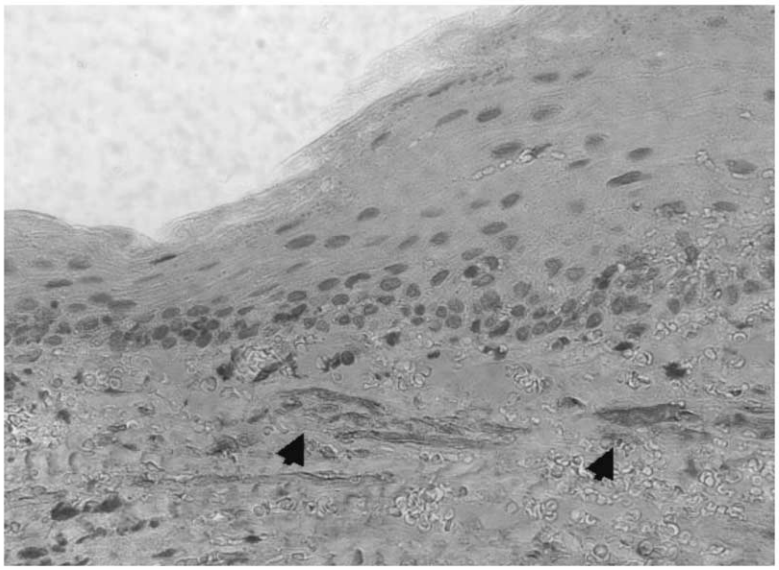

Figure 4. Immunohistochemical detection of the antigen α-smooth muscle actin in TEE shows appropriate staining in the muscularis, although it is more sparse than that seen in the native esophagus. (Original magnification 10×.)

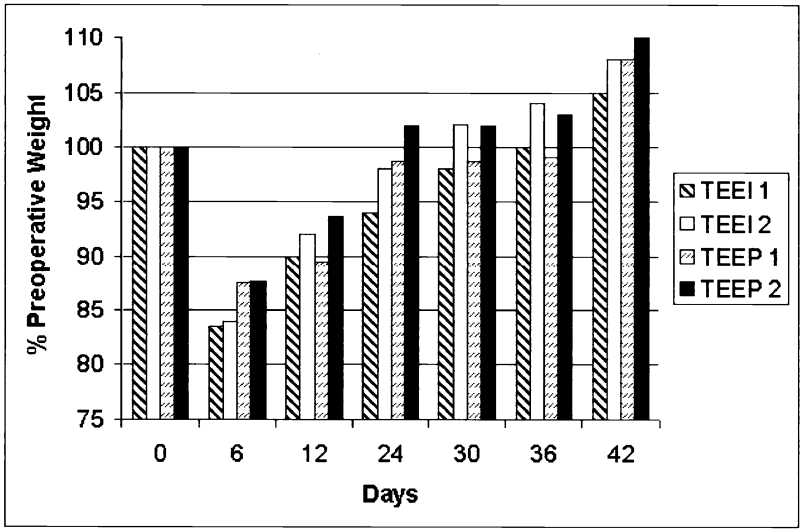

Figure 5. Percentage of preoperative weight per day of TEEP and TEEI animals. Animals either underwent onlay patching to the abdominal esophagus with TEE (TEEP) or complete interposition grafting in the abdominal esophagus (TEEI). After 3 days with nothing by mouth, rats resumed normal laboratory rat chow consumption for the subsequent 42 days. Weights were taken every other day.

whether the donor tissue is merely a good construct overgrown by host tissue. The GFP label that persists in anastomosis addresses this question by proving the origin of cells from OUs. Previous evidence only demonstrated continued donor histology in anastomosis to varied host sites.

The architecture of the TEE recapitulates the donor, the Lewis rat, with keratinized squamous epithelium and an actin-positive muscularis. The architecture formed in every successful experiment and did not deteriorate for 42 days in continuity with the gastrointestinal tract. Appropriate glandular invaginations occurred close to the anastomosed stomach, and the anastomosis itself healed to a smooth tissue transition. Although ganglion cells were not identified by means of histologic assessment or S100 immunohistochemical staining, we have seen that ongoing remodeling after anastomosis improves muscle appearance and leads to the

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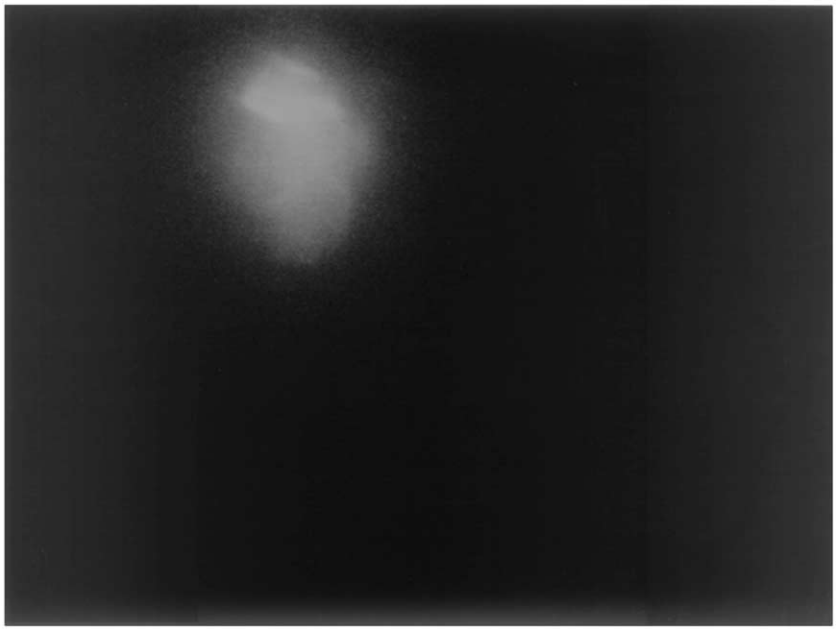

Figure 6. TEE OUs labeled with GFP 24 hours after isolation in culture. Resolution is limited by the bright signal, multicellularity, and culture condition. (Original magnification 20×.)

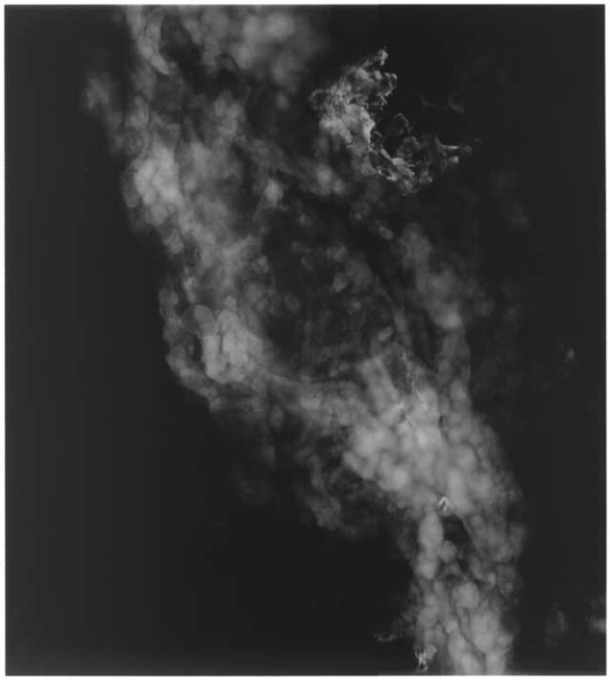

Figure 7. TEE labeled with GFP after 4 weeks of growth and 2 weeks of anastomosis. (Original magnification 10×.)

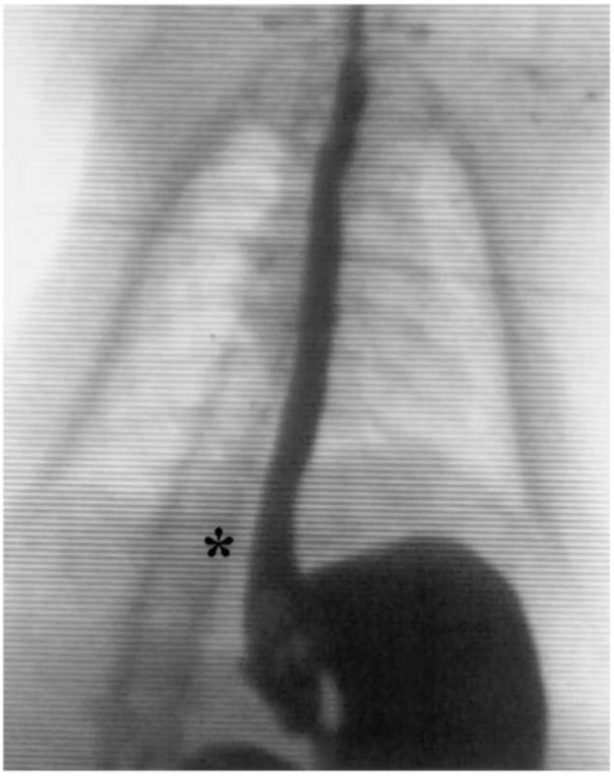

Figure 8. Fluoroscopy in TEEP animals reveals a wide unobstructed lumen. The *asterisk* indicates the upper margin of the onlay patch.

formation of Meissner and Auerbach plexi, including ganglion cells in other tissue-engineered intestinal projects.[3]

In addition to using neonatal rats as donors, the use of OUs isolated from adult rats was assessed and resulted in

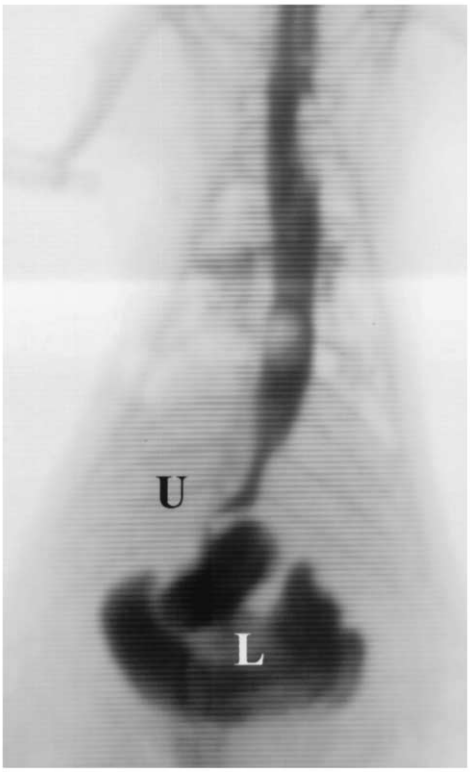

Figure 9. Fluoroscopy in TEEI animals reveals stenosis at the upper anastomosis *(U)* and a dilated TEE that flows into the stomach at the lower anastomosis *(L)*.

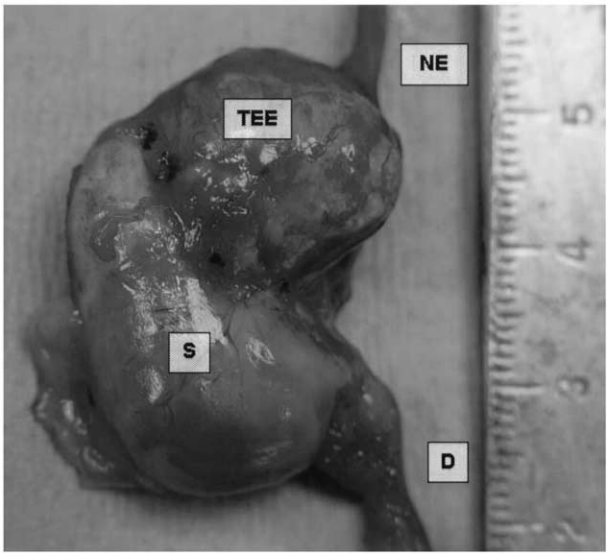

Figure 10. Gross examination of TEEI construct at harvest. Native esophagus *(NE)* is seen joining dilated TEE. Visible Prolene sutures that were part of the initial lower anastomosis have been marked with a black pen to show the margin between the TEE and the stomach *(S)*. The duodenum *(D)* exits the stomach.

the formation of TEEs with similar histology, an important step for the autologous therapy that would be a clinically acceptable distant target of this project.

Because animals were not pair-fed and sample size in both groups was only 2 animals, it is not possible to draw conclusions about the rates of weight gain for this novel tissue or to compare between the 2 groups. However, it can be concluded that animals successfully ate normal rat chow after postoperative day 3 and gained weight in all cases to greater than their weight preoperatively. This is definite proof of the TEE serving as an acceptable conduit. This was true even in the TEEI group, in which both animals had an upper anastomosis stricture, and no obstruction during deglutition was seen in these animals, even when filmed and replayed to a blinded viewer. Because stricture occurred, as is usually seen at the suture line, it is not likely a specific deficit of the TEE but rather a result of surgical technique. Fluoroscopy confirmed patent conduit with the stricture as noted.

In conclusion, the TEE formed from transplanted esophagus OUs is a complex tissue resembling native esophagus. TEE can be formed from adult autologous esophagus. Because the TEE grows orders of magnitude beyond the original implanted construct, this success could lead to an autologous approach to complicated pharyngogastric reconstruction problems when conventional approaches are not useful, perhaps with a laparoscopic implantation of the construct. The presence of an omentum might not be an absolute requirement because tissue-engineered constructs have been successfully vascularized in other locations, such as the mesentery, interscapular fat pad, or latissimus dorsi.[14] A tissue-engineered vascular bed is also possible, with silicone micromachining. MicroElectricalMechanical Systems, also used in inertial guidance and navigation,[15] can etch trench patterns on silicone and Pyrex templates with resolution to 10 μm. Endothelial cells are then cultured, remaining viable and forming a vascular bed.[16] With further study assessing longer time points and translation to larger animals, this technique could possibly be refined to a procedure of choice in cases in which native proxy substitution is unacceptable.

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