TISSUE ENGINEERING: Part A
Volume 21, Numbers 17 and 18, 2015

DOI: 10.1089/ten.tea.2015.0322

termis
Tissue Engineering
& Regenerative Medicine
International Society

2015 MARY ANN LIEBERT, INC. OUTSTANDING STUDENT AWARD OF TISSUE ENGINEERING AND REGENERATIVE MEDICINE INTERNATIONAL SOCIETY–AMERICAS

Tissue-Specific Effects of Esophageal Extracellular Matrix

Timothy J. Keane, BSc,[1,2,*] Aaron DeWard, PhD,[1,3*] Ricardo Londono, BSc,[1,4] Lindsey T. Saldin, BSc,[1,2] Arthur A. Castleton,[1] Lisa Carey, BSc,[1,2] Alejandro Nieponice, MD, PhD,[1,5] Eric Lagasse, PharmD, PhD,[1,3] and Stephen F. Badylak, DVM, PhD, MD[1,2,6]

Biologic scaffolds composed of extracellular matrix (ECM) have been used to facilitate repair or remodeling of numerous tissues, including the esophagus. The theoretically ideal scaffold for tissue repair is the ECM derived from the particular tissue to be treated, that is, site-specific or homologous ECM. The preference or potential advantage for the use of site-specific ECM remains unknown in the esophageal location. The objective of the present study was to characterize the *in vitro* cellular response and *in vivo* host response to a homologous esophageal ECM (eECM) versus nonhomologous ECMs derived from small intestinal submucosa and urinary bladder. The *in vitro* response of esophageal stem cells was characterized by migration, proliferation, and three-dimensional (3D) organoid formation assays. The *in vivo* remodeling response was evaluated in a rat model of esophageal mucosal resection. Results of the study showed that the eECM retains favorable tissue-specific characteristics that enhance the migration of esophageal stem cells and supports the formation of 3D organoids to a greater extent than heterologous ECMs. Implantation of eECM facilitates the remodeling of esophageal mucosa following mucosal resection, but no distinct advantage versus heterologous ECM could be identified.

Introduction

Scaffolds derived from extracellular matrix (ECM) have been investigated for their ability to support tissue remodeling in nearly every body system, including parts of the gastrointestinal tract. Similarly, ECM scaffolds have been isolated from tissues ranging from the urinary bladder and small intestine to the spinal cord and brain,[1–3] among others. Implantation and subsequent degradation of ECM scaffolds lead to the release or exposure of cryptic peptide fragments that affect cell behavior and tissue remodeling events. However, the necessity or preference for a homologous ECM remains unknown for many therapeutic applications.

The ECM represents the structural and functional molecules secreted by the resident cells of a tissue or organ. The biochemical composition and mechanical and ultrastructural characteristics of an ECM scaffold therefore vary according to the tissue source from which the ECM is isolated. Logically, the ideal substrate for cell survival, proliferation, differentiation, and functional tissue remodeling is the native ECM of the homologous tissue or organ. Recent work has described the potential benefits of ECM scaffold materials derived from homologous tissue versus heterologous tissue when used in selected anatomic locations.[2,4–11] The homologous ECM can preferentially maintain tissue-specific cell phenotypes,[4–7] promote cell proliferation,[6,8] induce tissue-specific differentiation,[9] and enhance the chemotaxis of stem cells.[2,10,12] However, the preference or necessity for tissue-specific ECM has not been shown for all therapeutic applications.[13–15]

The objective of the present study was to compare the cellular response to esophageal ECM (eECM) versus small intestinal submucosa (SIS)-ECM and urinary bladder matrix (UBM) *in vitro* and *in vivo*. The chemotaxis, proliferation, and capacity for eECM to support the formation of three-dimensional (3D) organoids of esophageal stem cells, which are a candidate cell population that may play a role in functional tissue remodeling of the esophagus, were investigated. The eECM scaffold was then implanted in a rat esophageal mucosal defect model to determine the ability of the scaffold to facilitate remodeling of esophageal mucosa.

[1]McGowan Institute for Regenerative Medicine, Pittsburgh, Pennsylvania.
Departments of [2]Bioengineering and [3]Pathology, University of Pittsburgh, Pittsburgh, Pennsylvania.
[4]School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania.
[5]Hospital Universitario, Fundación Favaloro, Buenos Aires, Argentina.
[6]Department of Surgery, University of Pittsburgh, Pittsburgh, Pennsylvania.
*Cofirst authors; these authors contributed equally.

Materials and Methods

Overview of experimental design

Porcine esophageal mucosa was decellularized to produce the eECM. Using a population of murine-derived esophageal stem cells,[16] the ability of the eECM to promote migration, proliferation, and 3D organoid formation was evaluated and compared to two benchmark ECM scaffolds, UBM and SIS. Subsequently, eECM and UBM scaffolds were implanted into a rat model of esophageal mucosa resection and the remodeling response was evaluated at 14 days postsurgery.

Mice and rats

Transgenic EGFP C57BL/6 mice were bred and housed in the Division of Laboratory Animal Resources facility at the University of Pittsburgh McGowan Institute for Regenerative Medicine. Female Sprague Dawley rats (350–400 g at implantation) were purchased from Harlan Laboratories and housed in the Division of Laboratory Animal Resources facility at the University of Pittsburgh McGowan Institute for Regenerative Medicine. Experimental protocols followed NIH guidelines for animal care and were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh.

Harvest and preparation of ECM scaffolds and hydrogels

The esophagus, urinary bladder, and small intestine were isolated from market weight pigs and frozen at −20°C until use. All ECM scaffolds were prepared according to established decellularization protocols (Fig. 1). Briefly, esophageal ECM (eECM) was prepared by mechanically separating the mucosa and submucosa from the muscularis externa and subjecting the mucosal layers to 1% trypsin/0.05% EDTA (Invitrogen, Carlsbad, CA) for 1 h at 37°C on a rocker plate, deionized water for 15 min, 1 M sucrose (Fisher Scientific, Pittsburgh, PA) for 30 min, deionized water for 30 min, 3.0% Triton X-100 (Sigma-Aldrich, St. Louis, MO) for 48 h, deionized water for 15 min, phosphate-buffered saline (PBS; Fisher Scientific) for 15 min, 10% deoxycholate (Sigma-Aldrich) for 4 h, deionized water for 30 min, 0.1% peracetic acid (Rochester Midland Corp., Rochester, NY) in 4.0% ethanol for 4 h, 100 U/mL DNAse (Invitrogen) for 2 h on a rocker plate, followed by 15-min washes with PBS, deionized water, PBS, and deionized water.[17] All washes were agitated at 300 rpm on a shaker plate, unless otherwise specified.

SIS was prepared by mechanically removing the superficial layers of the tunica mucosa, tunica serosa, and tunica muscularis externa from the intact small intestine, leaving the submucosa, muscularis mucosa, and basilar stratum compactum intact. UBM was prepared by mechanically removing the tunica serosa, tunica muscularis externa, the tunica submucosa, and majority of the tunica muscularis mucosa from the intact bladder, leaving the lamina propria and basement membrane intact. The SIS and UBM were then subjected to 0.1% peracetic acid in 4.0% ethanol for 4 h, followed by 15-min washes with PBS, deionized water, PBS, and deionized water as described above. All treatments were performed at room temperature with agitation on a shaker plate at 300 rpm, unless otherwise stated.

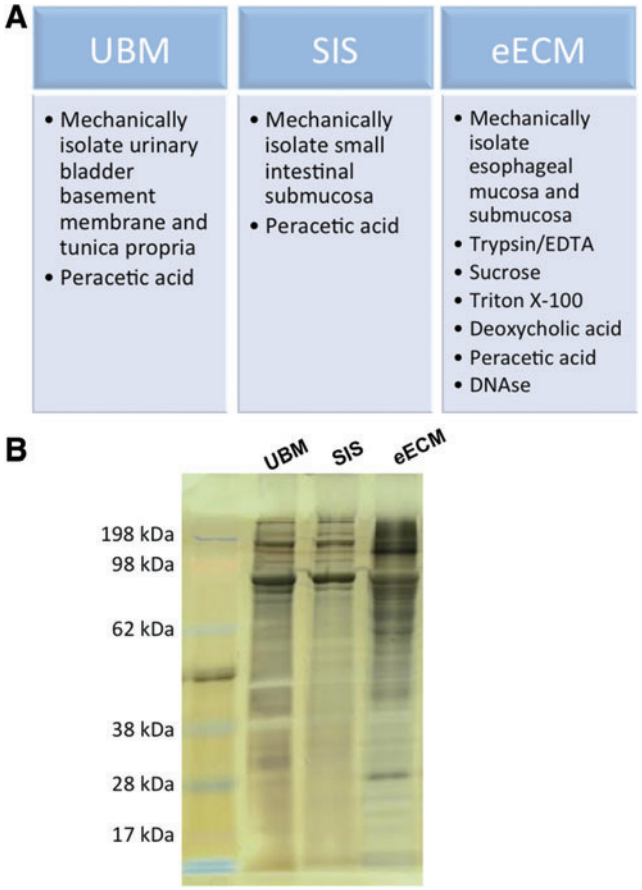

FIG. 1. Preparation and characteristics of extracellular matrix (ECM) scaffolds. **(A)** Overview of decellularization process for preparing urinary bladder matrix (UBM), small intestinal submucosa (SIS), and eECM. **(B)** Gel chromatography of ECM materials showing features in banding patterns of different ECM materials. Color images available online at www.liebertpub.com/tea

For implantation studies, the ECM scaffolds were lyophilized using an FTS Systems Bulk Freeze Dryer (Model 8-54) and sterilized with ethylene oxide. For studies using a hydrogel form of the ECM, the decellularized ECM sheets were lyophilized and comminuted to a particulate form using a Wiley Mini Mill. One gram of lyophilized ECM powder and 100 mg of pepsin (Sigma-Aldrich) were mixed in 100 mL of 0.01 M HCl and kept at a constant stir for 48 h at room temperature.

Sodium dodecyl sulfate gel chromatography

Pepsin solubilized (0.1% pepsin) forms of SIS, UBM, and eECM were diluted 1:1 in 2×Laemmli sample buffer (Bio-Rad, Hercules, CA) and boiled at 95°C for 8 min. Samples were diluted and protein concentrations were approximated using bovine serum albumin (BSA) with a Pierce BCA Protein Assay (Thermo Fisher, Waltham, MA) according to the manufacturer's instructions. Samples (5 μg) were run on a 10%, 12-well sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE; Bio-Rad, Hercules, CA) at 50 V until the tracking dye moved below the stacking gel. The gel was then run at 150 V for 50 min, until the dye reached

the end of the gel. After running, the gel was washed and fixed with 30% ethanol/10% acetic acid in a rocking staining dish. Silver stain was applied according to the manufacturer's instructions (Silver Stain Plus Kit; Bio-Rad) and developed for 15 min. The developed gel was imaged using a Nikon D7000 over a light table.

Isolation and culture of esophageal stem cells

The esophagus was removed from transgenic EGFP C57BL/6 mice (Jackson Laboratories) followed by physical separation of the mucosa using forceps. The mucosa was minced into small pieces and digested with 0.25% trypsin for 60 min. Cells and remaining tissue were passed through a 70-μm filter to obtain a single-cell suspension. Cells were then placed in tissue culture flasks that were previously seeded with irradiated LA7 feeder cells. LA7 cells have been shown to select for esophageal epithelial cells with stem cell properties.[16] Esophageal epithelial cells were expanded by passaging cells to flasks coated with irradiated LA7 cells.

Migration assay

Migration assays were performed using a 48-well chemotaxis chamber with polycarbonate filters containing 5 μm pores (Neuro Probe, Gaithersburg, MD). The filters were coated with 20 μg/mL laminin (Sigma-Aldrich) and dried before use. The bottom wells of the chamber were loaded with 30 μL containing 25 or 100 μg/mL of ECM digest and the top wells were loaded with $50\,\mu L$ containing 7×10^4 esophageal stem cells. Control wells consisted of serum-free media and media containing 10% fetal bovine serum (FBS). Following a 6-h incubation, the top filter surface (nonmigratory) was scraped and the bottom of the filter was fixed in 95% methanol for 5 min and then mounted on a glass slide with mounting media containing 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Inc., Burlingame, CA) and imaged. Migrated cells were quantified using ImageJ (NIH, Bethesda, MD) to set the threshold and count cells, with binning to resolve cell clusters of various counts. The same ImageJ macro was used to analyze all images. Experiments were performed using four technical replicates with four separate biologic replicates ($n = 4$).

Generation of 3D organoids

Esophageal cells were suspended in ECM hydrogels and were placed as a droplet in a tissue culture plate. Cells were incubated at 37°C for 30 min to allow for solidification of the hydrogels. A growth medium was added to the plate to initiate organoid formation. The growth medium consisted of advanced Dulbecco's modified Eagle's medium/F12, 1× N2, 1× B27 supplements, 1× Glutamax, 1× HEPES, 1× penicillin/streptomycin, 1 mM *N*-acetyl-L-cysteine, 100 μM gastrin, 10 mM nicotinamide, 10 μM SB202190, 50 ng/mL epidermal growth factor, 100 ng/mL Noggin, 100 ng/mL Wnt3A, 100 ng/mL R-Spondin 2, and 500 nM A8301. Media were changed every 2–3 days.

Proliferation assay

Esophageal organoids were generated followed by the addition of 5-ethynyl-2′-deoxyuridine[18] to the culture media. Cells were exposed to EdU for 2 h to allow incorporation of EdU into dividing cells. Organoids were fixed in 4% paraformaldehyde and embedded in gelatin. Organoids were sectioned at 10 μm and EdU was detected using the Click-iT Alexa Fluor 594 EdU labeling kit (Life Technologies, Carlsbad, CA). Cells were counterstained with Hoechst to detect cell nuclei. EdU-positive cells were counted to determine the number of proliferating cells per organoid.

Surgical procedure and ECM implantation

All surgical procedures were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh and the animal care complied with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Induction and surgical plane of anesthesia were achieved with 2% inhaled isoflurane, and the animals were placed on a warming pad in a supine position. The cervical skin was shaved and aseptically prepared with ethanol and Betadine. A midline cervical incision was performed above the clavicle and the esophagus exposed by means of blunt dissection. A transverse incision of the muscularis externa layer was performed, and a window of full-thickness mucosa extending 5–10 mm in length, including 70% of the circumference, was resected. Animals treated with the ECM received a single layer sheet that was placed over the denuded area and secured in place with interrupted 10.0 Prolene sutures (Ethicon, Somerville, NJ). The denuded mucosa was left exposed in nontreated control animals. Skin was closed with 4.0 VICRYL (Ethicon) and buprenorphine (0.5 mg/kg; Benckiser Healthcare (UK) Ltd., Hull, England) was administered intramuscularly immediately postoperatively and twice a day for 3 days. Animals were placed on a soft diet for 5 days postoperatively.

Histology and immunolabeling

Two histology and immunolabeling studies were performed as follows: (i) on the 3D organoids and (ii) on the explanted esophageal tissue sections. Organoids were collected upon digestion of the hydrogel using 0.2% dispase, 0.1% collagenase type II, and 20 μg/mL DNase I in 1× HBSS containing 1% HEPES. Organoids were embedded in 5% gelatin, fixed in 4% paraformaldehyde for 1 h, and embedded in paraffin. Explanted esophageal tissue sections were fixed in 10% neutral buffered formalin and paraffin embedded.

Serial sections (5 μm) of the organoids and tissue sections were stained with hematoxylin and eosin or immunolabeled.

Organoid and tissue sections were deparaffinized with xylene and rehydrated through a graded ethanol series. Antigen retrieval was performed by heating a citrate antigen retrieval buffer (10 mM citric acid with 0.05% Tween 20, pH 6) until boiling, and incubating the slides in the solution until returning to room temperature. Three washes in PBS for 5 min at room temperature were performed. Organoid and tissue sections were permeabilized with 1× Tris-buffered saline with Tween 20 (TBST) for 10 min at room temperature. The slides were incubated in a blocking solution (5% BSA in 1× PBS) at room temperature to prevent nonspecific binding. The slides were incubated in a primary antibody blocking solution at 4°C overnight. Five washes in PBS were performed for 5 min at room temperature. Slides were incubated in a secondary antibody blocking buffer at

room temperature for 1 h. Five washes in PBS were performed for 5 min at room temperature. The primary antibodies used for the immunolabeling studies were cytokeratin 13 (1:250, Ab92551; Abcam, Cambridge, United Kingdom) and cytokeratin 14 (1:500, Ab7800; Abcam) for the organoids, and cytokeratin 14 (1:200, NBP1-67606; Novus Biologicals, Littleton, CO) for tissue sections. The secondary antibodies used were Alexa Fluor 488 (1:200, A-11034; Invitrogen) and Alexa Fluor 594 (1:200, A21203; Invitrogen). Sections were counterstained with DAPI and mounted in a fluorescence mounting medium (Dako, Glostrup, Denmark). Stained sections were visualized on a Nikon E600 fluorescence microscope with the Cri Nuance FX Multispectral Imaging System.

Statistical analysis

Data sets were analyzed with a one-way analysis of variance using SPSS Statistical Analysis Software (SPSS, Inc., IBM, Chicago, IL). A Student's *t*-test was used to identify the differences between means when the observed *F* ratio was statistically significant ($p < 0.05$). Data are reported as mean ± standard error.

Results

ECM characteristics

The esophagus, small intestine, and urinary bladder were decellularized according to established methods of decellularization. The attributes (thickness, cellularity, density, etc.) of each tissue are unique and therefore require different protocols for decellularization (Fig. 1A). Effective decellularization, as defined by previously described metrics,[19] was achieved for each tissue. Each ECM scaffold had a unique protein banding pattern and the eECM had a prevalent band at ~30 kDa that was absent in both UBM and SIS (Fig. 1B).

Esophageal stem cell chemotaxis

The chemotaxis of esophageal stem cells was evaluated using a Boyden chamber assay. Two concentrations of ECM were chosen to determine whether a migration dose response existed. The stem cells did not migrate toward serum-free media or 10% FBS. Representative images of migratory cells after DAPI staining (Fig. 2A) showed an increased migration response of the esophageal stem cells toward eECM and SIS, but not UBM, versus the pepsin control. Quantification of migrating cells (Fig. 2B) showed that the eECM enhanced migration of esophageal stem cells at both 25 μg/mL ($p = 0.01$) and 100 μg/mL ($p = 0.04$). The SIS-ECM enhanced stem cell migration at 100 μg/mL ($p < 0.01$) but not at 25 μg/mL.

Organoid forming capacity of ECM hydrogels

The ability of ECM hydrogels to support the formation of esophageal organoids was tested using two concentrations of ECM: 2 and 6 mg/mL (Fig. 3A). The number of organoids formed in the eECM was greater than those formed in SIS and UBM at both 2 mg/mL ($p = 0.04$) and 6 mg/mL ($p = 0.04$). Interestingly, the lower concentration hydrogel (2 mg/mL) better supported the formation of organoids

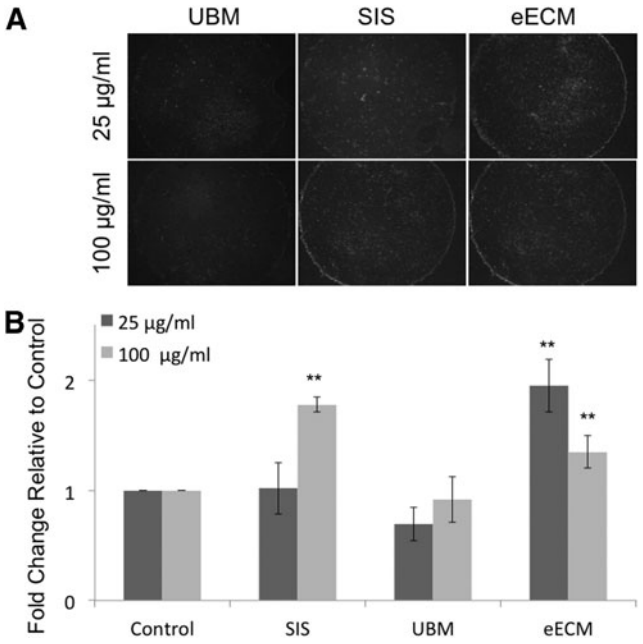

FIG. 2. Migration of esophageal stem cells. **(A)** Representative images of 4′,6-diamidino-2-phenylindole (DAPI)-stained migrating cells toward varying concentrations of ECM. **(B)** Quantification of migrated cells in response to ECM scaffolds. $^{**}p<0.01$.

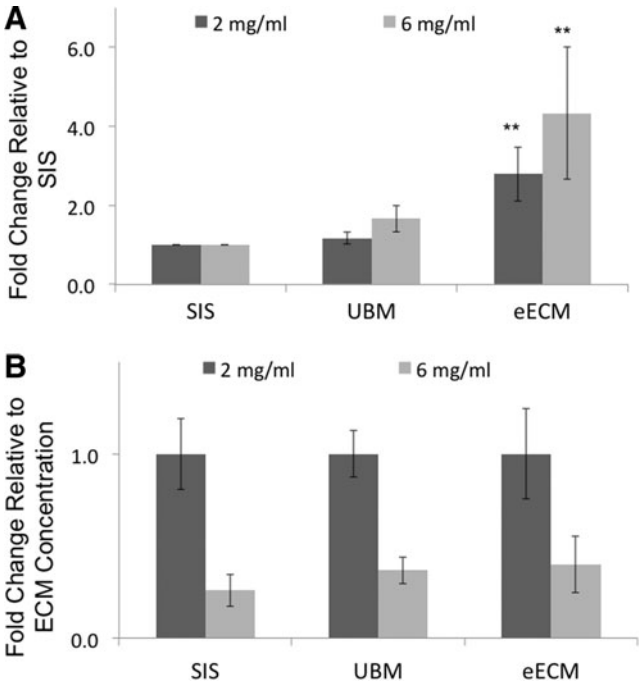

FIG. 3. Capacity of ECM hydrogels to support organoid formation. **(A)** Comparison number of organoids formed in different ECM types. Data are normalized to the number of organoids formed in SIS. **(B)** Comparison of number of organoids formed in ECM at 2 and 6 mg/mL. Data are normalized to the number of organoids present at 2 mg/mL concentration of ECM. $^{**}p<0.01$.

compared to the 6 mg/mL ECM hydrogel (Fig. 3B). Furthermore, SIS consistently performed the poorest among the ECM hydrogels in the ability to support organoid formation. Based upon these results, only the 2 mg/mL ECM hydrogels were used for subsequent proliferation studies and only eECM and UBM were used for *in vivo* studies.

Only eECM was able to support the growth of a substantial number of organoids necessary for histologic processing. Organoids that formed in the eECM were sectioned and immunolabeled for cytokeratin 14 (CK14), a marker of basal epithelium, and CK13, a marker of suprabasal differentiated epithelial cells (Fig. 4A).[16] Compared to native esophageal mucosa (Fig. 4B), the organoids largely maintained the $CK14^+$ phenotype at the periphery of the organoid, while the cells at the organoid center exhibited a more differentiated $CK13^+$ phenotype.

Proliferation of esophageal stem cells

Proliferating cells were identified by incorporation of EdU and immunolabeling (Fig. 5A). Proliferating cells within organoids were present in numbers similar to that in native tissue[16] and there was no difference among the values for proliferating cells in organoids formed eECM versus UBM (Fig. 5B), although there were substantially more organoids in eECM, as stated above. The sizes of organoids (i.e., number of cells per organoid) were equivalent in the UBM and eECM hydrogels.

Esophageal mucosal remodeling

The ability of ECM to mediate tissue repair in the esophageal mucosa was tested in a rat model of mucosal resection. Following resection of ~7 mm length of esophageal mucosa consisting of 70% of the circumference, a size-matched ECM scaffold was placed at the site of tissue resection. Rats weighed 228 ± 2.5 g before operation, and all animals lost weight following mucosal resection. The ECM-treated rats gradually gained weight over time. Eighty-three percent (5 out of 6) of the untreated control animals showed anorexia and complications secondary to anastomotic leaks and stricture formation that required removal from the study before the predetermined experimental endpoint. The remaining control rat showed no signs of mucosal coverage of the implant site (Fig. 6A). The eECM-treated rats lost −7.4% ± 1.3% versus −11.9% ± 2.5% for the UBM-treated rats by 3 days postsurgery compared to the UBM-treated rats, although the difference was not significant ($p = 0.243$). By 14 days postsurgery, both groups had recovered from the weight loss and exceeded their initial weight (eECM + 2.4% ± 2.7% vs. UBM + 1.4% ± 2.6%). All of the ECM-treated rats recovered from surgery and survived to the experimental endpoint (14 days) without complications. Representative images show that remodeling of the esophageal mucosa was indistinguishable in rats treated with UBM (Fig. 6B) versus eECM (Fig. 6C). Positive staining for CK14, a marker of basal esophageal epithelium, was absent in the control animals (Fig. 6D), but was shown in cells lining the basement membrane of the esophageal mucosa in rats treated with both UBM (Fig. 6E) and eECM (Fig. 6F).

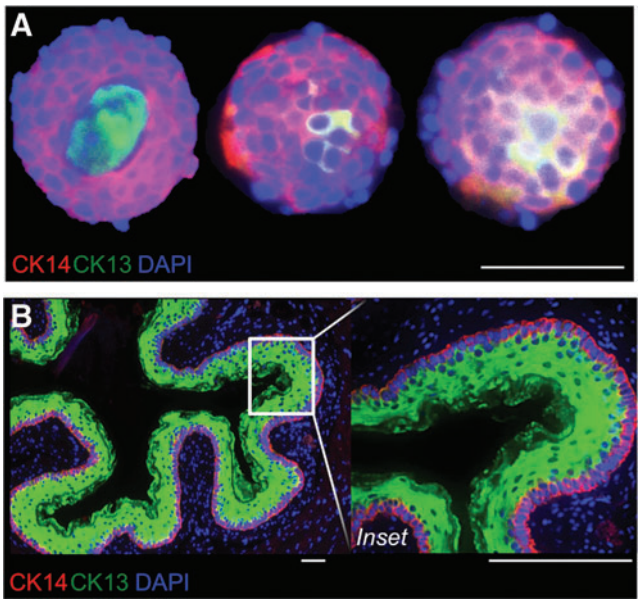

FIG. 4. Cytokeratin immunolabeling. **(A)** Representative images of organoids formed in eECM. **(B)** Representative image of normal esophageal mucosa. Cytokeratin 14, a basal epithelial cell marker, is stained *red*. Cytokeratin 13, a marker of suprabasal epithelial cells, is stained *green*. Nuclei (DAPI) is shown *blue*. Scale bars = 50 μm. Color images available online at www.liebertpub.com/tea

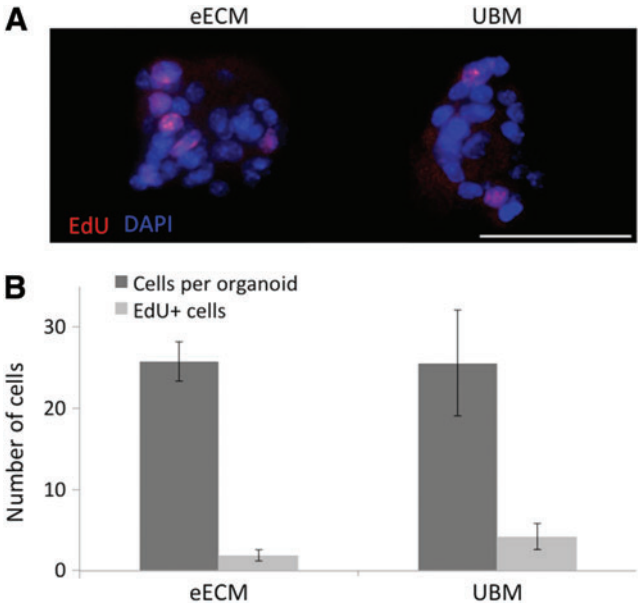

FIG. 5. Proliferation of organoid cells. **(A)** Representative images of EdU-stained organoids following 2-h EdU exposure. EdU^+ cells are shown in *red*. Nuclei are shown in *blue*. **(B)** Quantification of number of cells per organoid and number of EdU^+ cells per organoid. Scale bar = 50 μm. Color images available online at www.liebertpub.com/tea

Discussion

Results of the present study show that a homologous eECM preferentially enhances the migration of esophageal stem cells and supports the formation of 3D organoids in culture, but *in vivo* remodeling of the esophageal mucosa

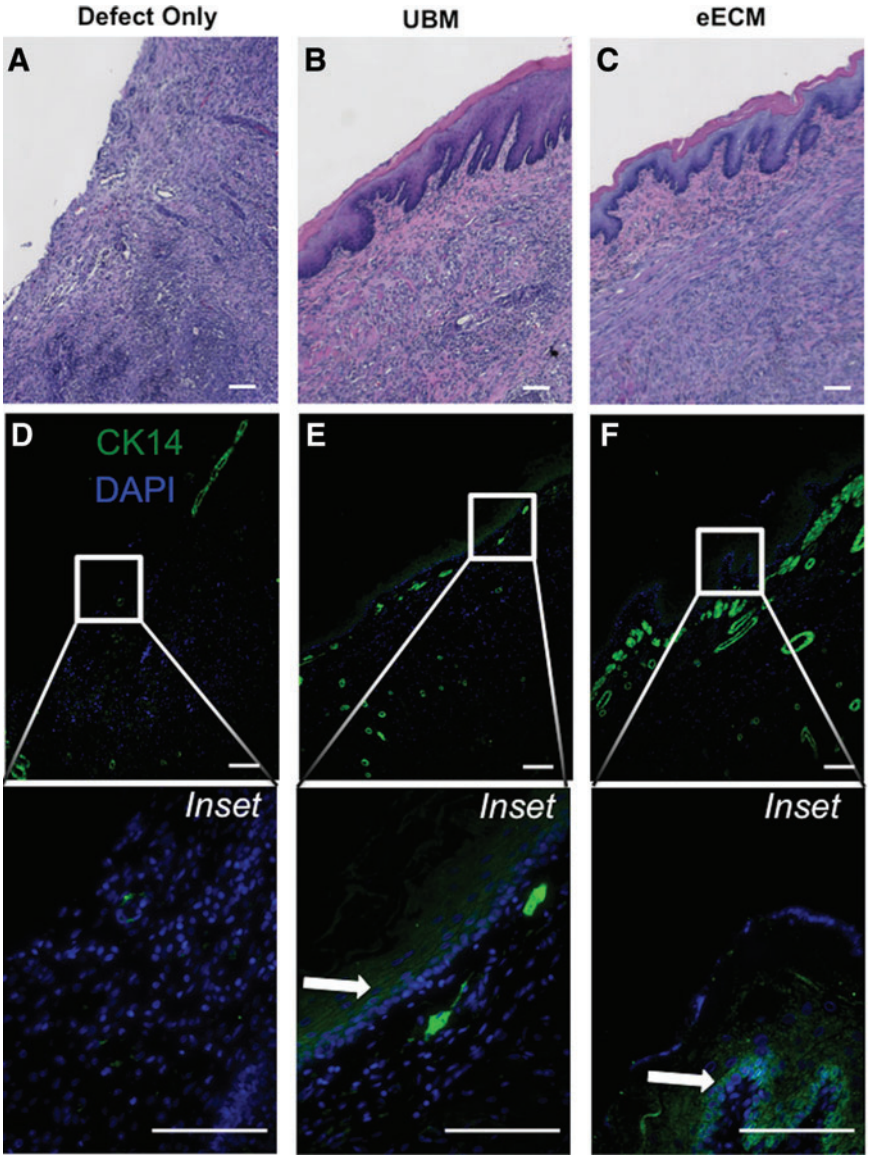

FIG. 6. Histology and immunolabeling of explants at 14 days postsurgery. The *in vivo* host response to no treatment **(A, D)**, UBM scaffold **(B, E)**, and eECM **(C, F)** was assessed histologically with hematoxylin and eosin (H&E) staining and by immunolabeling for stratified squamous epithelium (cytokeratin 14, *green*). Blood vessel endothelial cells stain positive for cytokeratin 14. *Arrows* indicate positive staining and scale bars = 100 μm. Color images available online at www.liebertpub.com/tea

was similar with the use of heterologous (UBM) versus homologous (eECM) scaffolds. These collective results suggest that the mucosa of the esophagus contains favorable tissue-specific properties that are retained following the decellularization process, but the contribution of these properties to the overall *in vivo* remodeling process is either not identifiable in this model or is not required for a constructive remodeling outcome.

While the types of cells that contribute to esophageal remodeling following mucosal resection in the present study are not completely known, the resident esophageal stem cell population represents a plausible and logical candidate.[20] These cells are present in the basal layer of the esophageal mucosa and following tissue resection must migrate the length of the mucosal resection to aid in tissue repair. Results of the present study show that the ECM constituents facilitate the migration and differentiation of esophageal stem cells and that the eECM, in particular, supports these processes. Interestingly, the present study showed an inverse dose response of migrating cells toward the esophageal ECM. It is known that chemoattractants often exhibit a bell-shaped dose–response curve.[21] It is plausible that tissue-specific chemoattractant molecule(s) are present in the eECM, just as similar findings appear to be true for other tissues and organs.[10,22] A unique gel chromatography protein-banding pattern in the eECM was identified in the present study, but the identities and biologic activity of the individual proteins have not yet been characterized.

A key determinant of the success or failure of an ECM scaffold to facilitate constructive and functional tissue repair is the host response to the material following implantation. While a distinct and tissue-specific ECM-dependent cellular response was observed *in vitro* in the present study, the *in vivo* remodeling outcome at 14 days postsurgery yielded an indistinguishable constructive outcome regardless of which ECM scaffold was used for repair. The poor outcome for the control animals clearly indicated that the mucosal defect was critically sized. Both ECM scaffolds promoted a constructive remodeling response compared to the healing response of the untreated control animals. Whether the

temporal remodeling response differed between the UBM and eECM is unknown since only a single postoperative time point was studied.

Previous studies in the esophagus have shown constructive outcomes with the use of UBM and SIS,[14,23,24] both heterologous forms of ECM. The heterologous ECMs were successful in reducing stricture formation, but the remodeled tissue did not fully reconstitute all components of normal esophageal tissue; for example, glandular tissue was absent. The present study showed that heterologous source ECM scaffolds were inferior to site-specific ECM *in vitro*, but *in vivo* differences in outcomes in eECM versus UBM at 14 days postmucosectomy were not identified. Species differences in the rat and human esophageal histology, namely the lack of submucosal glands in the rat esophagus, would require testing in a large animal model to determine whether eECM may have clinical benefits.

It should be noted that the ECM materials used in the present study were derived from xenogeneic tissues. However, this is quite representative of the clinical scenario, where a large majority of commercial scaffolds composed of ECM are from xenogeneic (typically porcine) source.[25] Practical considerations favor the use of xenogeneic tissues as they are in abundant supply through the agricultural supply chain. More importantly, the constituent molecules of ECM are some of the most highly evolutionarily conserved proteins across species.[26–28] The present study shows that porcine eECM regulates murine esophageal stem cell behavior and also mediates esophageal remodeling in the rat, consistent with known species homology of ECM constituents.

There were limitations to the present study. First, the response of only one cell type was evaluated. Esophageal stem cells are not the only cell population that may contribute to remodeling of the esophageal mucosa. Another potential contributing cell population is the multipotent perivascular stem cell.[22,29] Perivascular stem cells are found surrounding the endothelium of normal tissue and are likely to be present in the vasculature within the esophagus. A number of studies have reported the chemotactic and mitogenic potential of ECM for the perivascular stem cell population.[29,30] Another limitation of the present study was the use of different decellularization protocols for preparing the ECM scaffolds. Decellularization protocols are typically dictated by tissue-specific characteristics, which almost always differ to achieve effective decellularization. The use of a single decellularization protocol for all tissues in the present study would have resulted in ECM scaffolds with a different content of cell remnants and thus would have added a major variable.[31] The effect of the different decellularization protocols on the results in the present study is unknown, but protocols similar or identical to those in the present study would likely be used in the clinical setting and therefore have potential clinical relevance.

In conclusion, the present study showed a superior *in vitro* response of esophageal stem cells to homologous ECM versus heterologous ECM. Surgical placement of the scaffold into a rodent mucosal defect, however, showed no differences in the remodeling response for homologous versus heterologous ECM. A single time point limited conclusions from the *in vivo* portion of the present study. The preference of homologous ECM in the esophageal location is worthy of further investigation considering the unmet clinical need for therapeutic options for esophageal pathology.

Acknowledgments

The authors gratefully acknowledge Li Zhang for her assistance with Boyden chamber assays, Lori Whalton for histologic processing, and Victoria Messermidt for her assistance with imaging. T.J.K. is supported, in part, by the National Science Graduate Research Fellowship Program (DGE124782). Any opinion, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the National Science Foundation.

Disclosure Statement

No competing financial interests exist.

References

1. Badylak, S., Liang, A., Record, R., Tullius, R., and Hodde, J. Endothelial cell adherence to small intestinal submucosa: an acellular bioscaffold. Biomaterials **20,** 2257, 1999.
2. Crapo, P.M., Medberry, C.J., Reing, J.E., Tottey, S., van der Merwe, Y., Jones, K.E., *et al.* Biologic scaffolds composed of central nervous system extracellular matrix. Biomaterials **33,** 3539, 2012.
3. Freytes, D.O., Stoner, R.M., and Badylak, S.F. Uniaxial and biaxial properties of terminally sterilized porcine urinary bladder matrix scaffolds. J Biomed Mater Res Part B Appl Biomater **84,** 408, 2008.
4. Sellaro, T.L., Ravindra, A.K., Stolz, D.B., and Badylak, S.F. Maintenance of hepatic sinusoidal endothelial cell phenotype in vitro using organ-specific extracellular matrix scaffolds. Tissue Eng **13,** 2301, 2007.
5. Sellaro, T.L., Ranade, A., Faulk, D.M., McCabe, G.P., Dorko, K., Badylak, S.F., *et al.* Maintenance of human hepatocyte function in vitro by liver-derived extracellular matrix gels. Tissue Eng Part A **16,** 1075, 2010.
6. Allen, R.A., Seltz, L.M., Jiang, H., Kasick, R.T., Sellaro, T.L., Badylak, S.F., *et al.* Adrenal extracellular matrix scaffolds support adrenocortical cell proliferation and function in vitro. Tissue Eng Part A **16,** 3363, 2010.
7. French, K.M., Boopathy, A.V., Dequach, J.A., Chingozha, L., Lu, H., Christman, K.L., *et al.* A naturally derived cardiac extracellular matrix enhances cardiac progenitor cell behavior in vitro. Acta Biomater **8,** 4357, 2012.
8. Zhang, Y., He, Y., Bharadwaj, S., Hammam, N., Carnagey, K., Myers, R., *et al.* Tissue-specific extracellular matrix coatings for the promotion of cell proliferation and maintenance of cell phenotype. Biomaterials **30,** 4021, 2009.
9. Cortiella, J., Niles, J., Cantu, A., Brettler, A., Pham, A., Vargas, G., *et al.* Influence of acellular natural lung matrix on murine embryonic stem cell differentiation and tissue formation. Tissue Eng Part A **16,** 2565, 2010.
10. Brennan, E.P., Tang, X.H., Stewart-Akers, A.M., Gudas, L.J., and Badylak, S.F. Chemoattractant activity of degradation products of fetal and adult skin extracellular matrix for keratinocyte progenitor cells. J Tissue Eng Regen Med **2,** 491, 2008.
11. Medberry, C.J., Crapo, P.M., Siu, B.F., Carruthers, C.A., Wolf, M.T., Nagarkar, S.P., *et al.* Hydrogels derived from central nervous system extracellular matrix. Biomaterials **34,** 1033, 2013.

12. Beattie, A.J., Gilbert, T.W., Guyot, J.P., Yates, A.J., and Badylak, S.F. Chemoattraction of progenitor cells by remodeling extracellular matrix scaffolds. Tissue Eng Part A **15,** 1119, 2009
13. Wolf, M.T., Daly, K.A., Reing, J.E., and Badylak, S.F. Biologic scaffold composed of skeletal muscle extracellular matrix. Biomaterials **33,** 2916, 2012.
14. Badylak, S.F., Hoppo, T., Nieponice, A., Gilbert, T.W., Davison, J.M., and Jobe, B.A. Esophageal preservation in five male patients after endoscopic inner-layer circumferential resection in the setting of superficial cancer: a regenerative medicine approach with a biologic scaffold. Tissue Eng Part A **17,** 1643, 2011.
15. Badylak, S.F., Tullius, R., Kokini, K., Shelbourne, K.D., Klootwyk, T., Voytik, S.L., *et al.* The use of xenogeneic small-intestinal submucosa as a biomaterial for Achilles-tendon repair in a dog-model. J Biomed Mater Res **29,** 977, 1995.
16. DeWard, A.D., Cramer, J., and Lagasse, E. Cellular heterogeneity in the mouse esophagus implicates the presence of a nonquiescent epithelial stem cell population. Cell Rep **9,** 701, 2014.
17. Keane, T.J., Londono, R., Carey, R.M., Carruthers, C.A., Reing, J.E., Dearth, C.L., *et al.* Preparation and characterization of a biologic scaffold from esophageal mucosa. Biomaterials **34,** 6729, 2013.
18. Mendoza-Novelo, B., Avila, E.E., Cauich-Rodríguez, J.V., Jorge-Herrero, E., Rojo, F.J., Guinea, G.V., *et al.* Decellularization of pericardial tissue and its impact on tensile viscoelasticity and glycosaminoglycan content. Acta Biomater **7,** 1241, 2011.
19. Crapo, P.M., Gilbert, T.W., and Badylak, S.F. An overview of tissue and whole organ decellularization processes. Biomaterials **32,** 3233, 2011.
20. Doupe, D.P., Alcolea, M.P., Roshan, A., Zhang, G., Klein, A.M., Simons, B.D., *et al.* A single progenitor population switches behavior to maintain and repair esophageal epithelium. Science **337,** 1091, 2012.
21. Johnston, B., Kim, C.H., Soler, D., Emoto, M., and Butcher, E.C. Differential chemokine responses and homing patterns of murine TCR alpha beta NKT cell subsets. J Immunol **171,** 2960, 2003.
22. Crisan, M., Yap, S., Casteilla, L., Chen, C.W., Corselli, M., Park, T.S., *et al.* A perivascular origin for mesenchymal stem cells in multiple human organs. Cell Stem Cell **3,** 301, 2008.
23. Badylak, S.F., Vorp, D.A., Spievack, A.R., Simmons-Byrd, A., Hanke, J., Freytes, D.O., *et al.* Esophageal reconstruction with ECM and muscle tissue in a dog model. J Surg Res **128,** 87, 2005.
24. Nieponice, A., Gilbert, T.W., and Badylak, S.F. Reinforcement of esophageal anastomoses with an extracellular matrix scaffold in a canine model. Ann Thorac Surg **82,** 2050, 2006.
25. Keane, T.J., and Badylak, S.F. The host response to allogeneic and xenogeneic biological scaffold materials. J Tissue Eng Regen Med **9,** 504, 2015.
26. Hynes, R.O., and Zhao, Q. The evolution of cell adhesion. J Cell Biol **150,** F89, 2000.
27. Hutter, H., Vogel, B.E., Plenefisch, J.D., Norris, C.R., Proenca, R.B., Spieth, J., *et al.* Conservation and novelty in the evolution of cell adhesion and extracellular matrix genes. Science **287,** 989, 2000.
28. Kramer, J.M. Extracellular matrix. In: Riddle, D.L., Blumenthal, T., Meyer, B.J., and Priess, J.R., eds. *C elegans II.* 2nd ed., Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1997, Chapter 17.
29. Agrawal, V., Johnson, S.A., Reing, J., Zhang, L., Tottey, S., Wang, G., *et al.* Epimorphic regeneration approach to tissue replacement in adult mammals. Proc Natl Acad Sci U S A. **107,** 3351, 2010.
30. Tottey, S., Corselli, M., Jeffries, E.M., Londono, R., Peault, B., and Badylak, S.F. Extracellular matrix degradation products and low-oxygen conditions enhance the regenerative potential of perivascular stem cells. Tissue Eng Part A **17,** 37, 2011.
31. Keane, T.J., Londono, R., Turner, N.J., and Badylak, S.F. Consequences of ineffective decellularization of biologic scaffolds on the host response. Biomaterials **33,** 1771, 2012.

Address correspondence to:
Stephen F. Badylak, DVM, PhD, MD
McGowan Institute for Regenerative Medicine
450 Technology Drive
Suite 300
Pittsburgh, PA 15219

E-mail: badylaks@upmc.edu

Received: July 10, 2015
Accepted: July 13, 2015
Online Publication Date: August 12, 2015