ORIGINAL ARTICLE

Extracellular Matrix Degradation Products Downregulate Neoplastic Esophageal Cell Phenotype

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Extracellular matrix (ECsM) bioscaffolds have been successfully used to treat five esophageal adenocarcinoma (EAC) patients following resection of neoplastic mucosal tissue. The present study evaluated the *in vitro* effect of ECM harvested from nonmalignant, decellularized tissue on EAC cell phenotype to understand the molecular mechanisms underlying the clinical findings. Nonmalignant (Het-1A), metaplastic (CP-A), and neoplastic (SK-GT-4, OE33) esophageal epithelial cells were exposed to ECM degradation products (250 µg/mL) prepared from heterologous urinary bladder tissue or homologous esophageal mucosa tissue, and evaluated for cell morphology, cell function, and EAC signaling pathways. Both the ECM sources downregulated neoplastic cell phenotype, but had distinctive tissue-specific effects. Urinary bladder ECM decreased OE33 and SK-GT-4 metabolism and increased CP-A apoptosis. Esophageal ECM decreased SK-GT-4, CP-A, and Het-1A proliferation; robustly downregulated PI3K-Akt-mTOR, cell cycle/DNA replication signaling, and upregulated autophagy signaling in OE33 cells; and increased cell cycle/DNA replication signaling in Het-1A cells. Both ECM sources decreased OE33 proliferation and phosphorylated AKT in OE33 cells, and in contrast, increased phosphorylated AKT in Het-1A cells. The results support the concept that the biochemical signals in non-malignant ECM can downregulate neoplastic cell phenotype with minimal, and sometimes opposite, effects on normal cells. PI3K-Akt signaling has been implicated in EAC progression and these ECM-mediated effects may be favorable for an esophageal therapy following cancer resection.

Keywords: extracellular matrix bioscaffold, esophageal cancer, cell/matrix interaction, dynamic reciprocity, gene expression

Impact Statement

Extracellular matrix (ECM) biomaterials were used to treat esophageal cancer patients after cancer resection and promoted regrowth of normal mucosa without recurrence of cancer. The present study investigates the mechanisms by which these materials were successful to prevent the cancerous phenotype. ECM downregulated neoplastic esophageal cell function (proliferation, metabolism), but normal esophageal epithelial cells were unaffected *in vitro*, and suggests a molecular basis (downregulation of PI3K-Akt, cell cycle) for the promising clinical results. The therapeutic effect appeared to be enhanced using homologous esophageal ECM. This study suggests that ECM can be further investigated to treat cancer patients after resection or in combination with targeted therapy.

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Introduction

BIOLOGIC MATERIALS COMPOSED of extracellular matrix (ECM)^{1,2} or individual components of ECM such as collagen^{3,4} or hyaluronan⁵ have been successfully used in every body system for the repair and reconstruction of injured tissues. However, such materials have been used only sparingly in the gastrointestinal tract. The ECM is composed of the naturally secreted products of the resident cells of each tissue and organ and constitutes the solid component of every cell's microenvironmental niche. The ECM is a potent regulator of cell homeostasis, proliferation, migration, differentiation, and spatial organization or patterning.

Abnormal or dysregulated cell phenotype can be mitigated by exposure to ECM or components of ECM harvested from normal tissues.^{6–13} The pathogenesis of gastroesophageal reflux disease with progression to metaplastic Barrett's esophagus (BE) and esophageal adenocarcinoma (EAC) is reasonably well established.¹⁴ Microenvironmental changes induce progressively more severe changes in cell phenotype and culminate in EAC,¹⁵ a disease with a 20% 5-year survival regardless of therapy.¹⁶

Preclinical studies^{17–23} and a cohort study of five patients⁶ show that placement of a xenogeneic ECM bioscaffold at the site of long-segment, full circumferential resection of the esophageal mucosa and submucosa facilitated the restoration of a near normal esophageal mucosa, with preservation of esophageal structure and function, and without clinically significant stricture. The five-patient cohort had T1A EAC before resection and placement of the ECM bioscaffold,⁶ and now 7–8 years cancer free. The ECM bioscaffold completely degraded within 2 weeks, suggesting that the matrix was not required to be physically present long term for effective results, but rather the bioinductive cues of the native matrix or matrix degradation products influenced cell phenotype in the post-tumor resection niche.

The results of the five-patient cohort study are consistent with the theory of “dynamic reciprocity”: the bidirectional cross talk between a cell and its surrounding matrix to influence cell behavior.²⁴ The molecular mechanisms by which the embedded signaling molecules of the ECM influence cell behavior are only partially understood. A more complete understanding of these mechanisms may allow more targeted therapy with ECM-based materials.

The objective of the present study was to determine the response of normal, metaplastic, and neoplastic esophageal epithelial cells to the degradation products of ECM harvested from porcine heterologous and homologous tissues; specifically, urinary bladder matrix (UBM-ECM) and esophageal mucosa ECM (eECM), respectively. Biologic scaffolds composed of allogeneic and xenogeneic ECM have been successfully used in a variety of clinical applications and have involved both homologous and heterologous tissues.²⁵ These materials have been manufactured by a variety of methods and all consist of a large number of structural and functional molecules such as collagen, laminin, fibronectin, glycosaminoglycans, and various cytokines and chemokines.²⁵ The heterologous UBM-ECM and homologous eECM materials used in the present study comprised similar molecular species, although the protein compositions would likely be distinct between tissue sources.²³

It was hypothesized that pepsin-digested degradation products of UBM-ECM and eECM would have the potential

to normalize neoplastic cell behavior and cancer signaling pathways, and that the homologous eECM would provide a more pronounced downregulation of neoplastic cell behavior compared with the heterologous UBM-ECM. Outcome measures included cell phenotype; cell function, including proliferation, metabolism, and apoptosis; and key cancer signaling pathways involved in EAC progression.

Materials and Methods

ECM degradation products

UBM-ECM and eECM were decellularized from porcine tissues using tissue-specific protocols to meet criteria of decellularization as previously described.^{26,27} ECM (10 mg/mL) was solubilized using pepsin (1 mg/mL) in 0.01 M HCl for 48 h²⁶ as previously described. Pepsin control (1 mg/mL) was similarly prepared without the addition of ECM.

Sodium dodecyl sulfate/polyacrylamide gel electrophoresis with silver stain

Pepsin-digested UBM-ECM, eECM, and pepsin control proteins were separated by gel electrophoresis and stained using silver stain as detailed in Supplementary Methods.

Cell culture and ECM treatment

Het-1A is an immortalized nonmalignant “normal” esophageal epithelial cell line and CP-A is an immortalized nondysplastic, metaplastic Barrett's esophageal epithelial cell line, both obtained from American Type Culture Collection (ATCC). Cells were cultured according to the manufacturer's guidelines and the media supplemented with 1% penicillin/streptomycin. OE33 and SK-GT-4 are immortalized EAC epithelial cell lines (Sigma-Aldrich) and were cultured according to the manufacturer's guidelines.

Cells were seeded at 70% confluency for experiments: Het-1A (400,000 cells/well), CP-A (250,000 cells/well), SK-GT-4 (200,000 cells/well), or OE33 (250,000 cells/well) in six-well plates; or scaled appropriately for 96-well plates.

Cells were treated with media supplemented with UBM-ECM (250 µg/mL), eECM (250 µg/mL), or pepsin control (25 µg/mL) for 24 h for cellular assays, unless otherwise noted.

Cell morphology

Brightfield micrographs were taken of six-well plates after 6, 24, and 48 h of treatment. Live-cell time-lapse microscopy was used to visualize the cell bridges over a period of 18 h after treatment, with a picture taken every 20 min. Immunolabeling was performed on the fixed OE33 cancer cell bridges for actin, and immunolabeling was performed for tubulin overlaid with differential interference contrast (DIC) microscopy after 24 h of culture to determine the bridge biochemical content as described in Supplementary Methods.

Proliferation

Cell proliferation was quantified with a 5-bromo-2-deoxyuridine (BrdU) colorimetric immunoassay based on

the manufacturer's guidelines (Roche) in 96-well plates. Each assay was performed in technical quadruplicates, and representative graphs of the biological replicates ($n=3$) are shown.

Metabolism

Cell metabolic activity was assayed with the Vybrant MTT colorimetric assay according to the manufacturer's instruction (Thermo Fisher) for the MTT Quick Protocol Option in 96-well plates. Each assay was performed in technical quadruplicates, and representative graphs of the biological replicates ($n=3$) are shown.

Apoptosis

Flow cytometry was performed to determine the percentage of early and late apoptotic cells after ECM treatment using Annexin V/propidium iodide (PI) staining in six-well plates ($n=3$), as detailed in Supplementary Methods.

Gene expression

Het-1A and OE33 cells were seeded in six-well plates and 24 h after treatment, cells were collected in TRIzol (Invitrogen), with technical duplicates ($n=3$). RNA was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. RNA concentration and purity were measured with the NanoDrop 1000. Gene expression was determined by whole-transcriptome analysis and qPCR as described in the following sections.

Whole-transcriptome analysis. RNA was prepared for whole-transcriptome analysis using the Human Clariom S Pico array as detailed in Supplementary Methods. Differentially expressed genes (>2 or <-2 -fold change, $p<0.05$) were analyzed with the Affymetrix Transcriptome Analysis Console Pathway software. Six pathways were selected for further analysis based on the following criteria: (1) within the top 15 signaling pathways of OE33 cancer cells treated with UBM-ECM or eECM and (2) related to the functional changes tested (i.e., proliferation, metabolism, apoptosis) and/or EAC tumorigenesis. TreeView was used to generate heat maps for the differentially expressed genes grouped by top signaling pathways.

Selection of candidate genes and validation by qPCR. The biological function of the differentially expressed genes associated with the selected signaling pathways is shown in Supplementary Table S1. Thirty candidate genes across the nine signaling pathways consistent with the hypothesis were selected for validation by qPCR. Genes were excluded if ECM treatment changed the normal and neoplastic cell in the same direction, unless the relative fold change between the normal and neoplastic cell was >2 or <-2 . Primer sequences are shown in Supplementary Table S2, and primers were optimized as detailed in Supplementary Methods. RNA was transcribed to cDNA (Invitrogen cDNA RT Kit), and qPCR was performed using SYBR Green Master Mix (Bio-RAD) in technical duplicates ($n=3$). Results were analyzed by the $\Delta\Delta C_t$ method and normalized to the housekeeping gene *ACTB*. Fold changes were calculated for each treatment compared with pepsin control.

Western immunoblotting

Detailed methods are provided in Supplementary Methods for the collection of OE33 and Het-1A cell lysates at 0, 6, and 24 h and Western immunoblotting for pAKT, AKT, and ACTB. The experiment was performed for $n=2$ independent lysates and technical duplicates. Representative images are shown.

Statistical analysis

Functional data sets were analyzed with a one-way analysis of variance (ANOVA) with *post hoc* Tukey test using GraphPad Prism 7.0 statistical software (GraphPad software, Inc., San Diego, CA). Data are reported as mean $\pm$ standard deviation. Whole-transcriptome statistics was performed by the Affymetrix Transcriptome Analysis Console software using one-way between-subject ANOVA (unpaired) based on gene-level intensities [biweight average signal ($\log_2$)]. qPCR gene expression data were $\log_2$ transformed and one-way analysis of variance was performed, with Sidak's multiple comparisons test. Significance was determined using the 95% confidence interval and p -values were adjusted for multiple comparisons.

Results

UBM-ECM and eECM show similar but distinctive protein compositions

The protein profiles of the soluble fraction of UBM-ECM and eECM were largely similar, but also showed distinctive bands such as those present at ~ 60 kDa for eECM and at ~ 65 kDa for UBM-ECM (Supplementary Fig. S1).

ECM degradation products promote morphologic changes in neoplastic cells

Cell morphology did not change after 6 h of treatment (Supplementary Fig. S2A), but OE33 cells treated with UBM-ECM and eECM showed markedly reduced confluency after 24 h of treatment compared with pepsin control (Fig. 1A). On closer inspection, the OE33 and SK-GT-4 cells showed a striking phenotypic change after ECM treatment with the appearance of "cell bridges" at 24 h (Fig. 1B), which were absent in the pepsin control and Het-1A and CP-A cells (Supplementary Fig. S2B).

The cell bridges had a membranous appearance and served as a physical connection between cells, as shown by brightfield and DIC microscopy. These bridges typically had a length of several cell diameters (Fig. 1B). The cellular bridges did not stain positive for actin (Supplementary Fig. S2D) or tubulin (Fig. 1B). The cell bridges were transient, forming and reforming between groups of cells, and lasting on the order of minutes-hours, as shown by the time-lapse microscopy video of the cell bridge formation in OE33 cells treated with UBM-ECM (Supplementary Movie S1).

OE33 cells treated with UBM-ECM and eECM for 48 h showed a reduced confluency compared with pepsin control, similarly to 24 h, with little change in the Het-1A or CP-A cells. SK-GT-4 cells also showed a slightly reduced confluency with UBM-ECM and eECM treatment compared with pepsin at 48 h (Supplementary Fig. S2C).

FIG. 1. Cell Morphology. OE33 cells showed reduced confluency with UBM-ECM and eECM treatment compared with pepsin control, while Het-1A, CP-A, and SK-GT-4 cells showed no change after 24 h. Scale bar = 250 μ m (A). OE33 cells form bridges with UBM-ECM and eECM treatment and SK-GT-4 cells form bridges with UBM-ECM treatment (B), visible with brightfield and DIC microscopy at 24 h. Bridges are outlined with white dotted lines. The bridges do not stain positively for cytoskeletal marker tubulin. Pictures shown are representative brightfield micrographs after 24 h of treatment. Scale bar = 50 μ m. DIC, differential interference contrast; UBM, urinary bladder matrix; ECM, extracellular matrix. Color images are available online.

UBM-ECM decreases OE33 cell proliferation and eECM decreases normal, metaplastic, and neoplastic cell proliferation

UBM-ECM decreased the proliferation of OE33 cancer cells compared with pepsin control ($p=0.0008$), but showed no change in proliferation compared with pepsin control for Het-1A, CP-A, or SK-GT-4 cells. eECM decreased proliferation of all cell types compared with pepsin control: Het-1A ($p<0.0001$), CP-A ($p<0.0001$), OE33 ($p=0.0003$), and SK-GT-4 ($p<0.0001$) (Fig. 2A).

Comparing ECM tissue types, eECM significantly decreased proliferation of all cell types compared with UBM-ECM for Het-1A ($p<0.0001$), CP-A ($p<0.0001$), and SK-GT-4 ($p<0.0001$). There was no difference between eECM and UBM-ECM in decreasing OE33 proliferation (Fig. 2A).

UBM-ECM decreases neoplastic cell metabolism

UBM-ECM and eECM did not affect the metabolic activity of Het-1A or CP-A cells compared with pepsin control. UBM-ECM decreased the metabolic activity of the OE33 ($p=0.0001$) and SK-GT-4 ($p=0.0035$) cells compared with pepsin control. UBM-ECM also decreased the metabolic activity of OE33 ($p<0.0001$) and SK-GT-4 ($p=0.0024$) cells compared with eECM (Fig. 2B).

UBM-ECM increases metaplastic cell apoptosis

The fold change of early and late apoptotic cells with UBM-ECM or eECM treatment compared with pepsin is shown for Het-1A, CP-A, OE33, and SK-GT-4 (Fig. 2C). For CP-A cells, UBM-ECM treatment increased late apoptosis ($p=0.0095$) compared with pepsin control. UBM-ECM and eECM did not show differences for early or late apoptosis in Het-1A, OE33, or SK-GT-4 cells compared with pepsin control.

eECM can downregulate PI3K-Akt, cell cycle, and DNA replication pathways, and can increase autophagy signaling in OE33 cells; eECM can upregulate cell cycle and DNA replication signaling in Het-1A cells

The gene expression workflow is shown (Fig. 3A). Genes that were differentially expressed (defined as greater or less than twofold change, with significance by one-way ANOVA $p<0.05$) were identified (Supplementary Fig. S3A). Notably, eECM had 1445 differentially expressed genes in OE33 cells and 2274 differentially expressed genes in Het-1A cells. In contrast, UBM-ECM had 149 differentially expressed genes in OE33 cells and 24 differentially expressed genes in Het-1A cells.

The top differentially expressed pathways are shown (Supplementary Fig. S3B). The six differentially expressed pathways in OE33 cells with UBM-ECM and eECM treatment selected for further analysis included the focal adhesion-PI3K-Akt-mTOR, cell cycle, DNA replication, autophagy (Fig. 3B), G1 to S phase transition, and Wnt (Supplementary Fig. S4). Signaling pathway networks for other cancers, that is, gastric cancer, breast cancer, and glioblastoma signaling pathways, were also shown to be downregulated in OE33 cells with eECM treatment (Supplementary Fig. S5), but not further validated.

FIG. 2. Cell Function. Het-1A, CP-A, OE33, and SK-GT-4 cells were cultured with media supplemented with pepsin (25 $\mu\text{g/mL}$), UBM-ECM (250 $\mu\text{g/mL}$), or eECM (250 $\mu\text{g/mL}$) for 24 h. Cell proliferation was measured by BrdU (A), cell metabolic activity was measured by MTT assay (B), and cell apoptosis was measured by flow cytometry using AnnexinV/propidium iodide (PI) stain (C). Representative graphs are shown for $n=3$, technical quadruplicates for the BrdU and MTT assays. Cell count fold change is shown normalized to pepsin for apoptosis (early and late apoptosis) ($n=3$). Bar represents mean $\pm$ standard deviation. Summary tables show positive (+) or negative (-) regulation for UBM-ECM or eECM treatment compared with pepsin control for each cell line (ns - not significant $p>0.05$; $*p<0.01$; $***p<0.001$; $****p<0.0001$). eECM, esophageal mucosa ECM. Color images are available online.

A Gene Expression Analysis Overview

B Pathway Analysis and qPCR Validation

FIG. 3. Gene Expression. (A) Overview of gene expression analysis. (B) Differentially expressed genes (>2 or <-2 -fold change, with $p < 0.05$) grouped by signaling pathway for Het-1A and OE33 cells treated with UBM-ECM (250 $\mu\text{g/mL}$) or eECM (250 $\mu\text{g/mL}$) for 24 h, normalized to pepsin control treatment (25 $\mu\text{g/mL}$) ($n = 3$). Heat maps show the whole-transcriptome analysis with corresponding qPCR for each signaling pathway: focal adhesion-PI3K-Akt-mTOR, autophagy, cell cycle, and DNA replication. Purple bars represent downregulated genes, and yellow bars represent upregulated genes compared with pepsin control. Genes are labeled as positive regulators (red) or negative regulators (green) and candidate genes that were further validated by qPCR are marked (*). (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.) Color images are available online.

FIG. 4. Phosphorylated AKT Expression. PI3K-Akt is a signaling pathway that regulates proliferation and metabolism, and shown to be upregulated in EAC cells. OE33 and Het-1A cells were treated with pepsin (25 μ g/mL), UBM-ECM (250 μ g/mL), or eECM (250 μ g/mL) for 0, 6, or 24 h. All time points were collected at the same time ($t=0$). Immunoblot for pAKT, AKT, and ACTB expression is shown for OE33 (**A**) and Het-1A (**B**) cells. Representative blots from a single experiment, $n=2$, technical duplicates. Independent blots were loaded with equal protein concentrations and confirmed for equal protein loading by ACTB. The initial/baseline levels of pAKT were much lower in OE33 compared with Het-1A and $\sim 6\times$ as much protein was needed to detect pAKT levels in OE33 cells.

qPCR validation of 30 candidate genes selected from the top signaling pathways largely agreed with the whole-transcriptome analysis (Fig. 3B), in which eECM had a more pronounced effect compared with UBM-ECM at 24 h, and Het-1A and OE33 cells responded differently to the same ECM treatment. eECM showed a net effect of decreasing PI3K-Akt-mTOR signaling and increasing autophagy signaling in OE33 cells. eECM robustly downregulated cell cycle, DNA replication, and G1 to S phase signaling in OE33 cells, and in contrast, upregulated these signaling pathways in Het-1A cells.

UBM-ECM and eECM decrease phosphorylated AKT protein expression in OE33 cells and increase phosphorylated AKT in Het-1A cells

Western immunoblot was performed on the OE33 and Het-1A lysates for pAKT, a key upstream regulator of proliferation, cell cycle, and metabolism. OE33 cells and Het-1A cells showed no change in pAKT in pepsin control. OE33 cells show a downregulation in pAKT expression with UBM-ECM treatment at 24 h, and a downregulation in pAKT expression with eECM treatment at 6 and 24 h (Fig. 4A). In contrast, Het-1A cells showed an upregulation in pAKT expression with UBM-ECM and eECM treatment at 6 and 24 h (Fig. 4B). However, it is important to note that the initial/baseline levels of pAKT were much lower in OE33 compared with Het-1A and $\sim 6\times$ as much protein was needed to detect pAKT levels in OE33 cells.

Discussion

The results of the present study show that signaling molecules present in solubilized ECM derived from non-malignant “normal” tissue can downregulate the neoplastic phenotype of EAC epithelial cells. The same ECM had minimal, and sometimes opposite, effects on nonmalignant “normal” esophageal epithelial cells. The rationale for the two types of xenogeneic (porcine) ECM used in the present study is based on the results of two previous studies.^{6,23}

UBM-ECM is commercially available (MatriStem, ACell) and one of the ECM types used to treat 14 EAC patients (stage T1A) to date after aggressive, long-segment

mucosal resection. The patients, ineligible for esophagectomy due to comorbidities, have survived for 1–8 years to date and returned to a normal diet. The bioscaffolds degraded completely within 2 weeks, suggesting the importance of ECM degradation products as an inductive cue for resident cells in the post-tumor resection niche.⁶ The work is currently in a Phase I clinical trial (ClinicalTrials.gov Identifier: NCT02396745).

eECM is a more recently developed biomaterial²⁷ and the logical tissue source for esophageal reconstruction. eECM enhanced esophageal stem cell migration and organoid formation compared with UBM-ECM and small intestinal submucosa (SIS-ECM).²³ In the present study, the heterologous UBM-ECM and homologous eECM showed similar but distinctive effects on the normal, metaplastic, and neoplastic cells for phenotype, cell function, and gene expression, as summarized in Figure 5.

Homologous and heterologous ECM: similar and distinctive effects

The similar effects of both UBM-ECM and eECM include decreased proliferation in OE33 cells, decreased focal adhesion-PI3K-Akt-mTOR signaling pathway in OE33 cells, and downregulated phosphorylated AKT at 24 h in OE33 cells, and upregulated phosphorylated AKT in Het-1A cells at 6 and 24 h. These similar responses elicited by the two ECM sources suggest the presence of common signaling molecules derived from nonmalignant tissue with the ability to downregulate certain neoplastic cell behaviors.

UBM-ECM and eECM treatment also showed reduced confluency and a striking morphologic change with the formation of cell bridges in OE33 and SK-GT-4 cells at 24 h, which were absent in pepsin control and Het-1A and CP-A cells. Interestingly, the cell bridges in the present study did not positively label for actin or tubulin, as characteristic of other cell bridges, commonly referred to as “tunneling nanotubes.”^{28–30}

Tunneling nanotubes are a recently described form of intercellular communication found in a variety of cell types, including nonmalignant and neoplastic cells *in vitro* and *in vivo*.²⁸ The function of the tunneling nanotubes in neoplastic cells range from “enabling” (e.g., escape mechanism,³¹

FIG. 5. Summary Graphic. Summary of significant findings divided by treatment (UBM-ECM vs. eECM) and by cell line. *Arrows* represent directionality of change with ECM treatment compared with pepsin control. **↓**: similar response across ECM treatment types, **↓**: specific response to UBM-ECM, **↓**: specific response to eECM, **↓**: validated by qPCR, *Blank box*: no change, *black box*: not tested.

		UBM-ECM				eECM			
		Het1A	CP-A	OE33	SK-GT-4	Het1A	CP-A	OE33	SK-GT-4
Phenotype	Cell bridges			↑	↑			↑	
Cell Function	Proliferation (BrdU)			↓		↓	↓	↓	↓
	Metabolism (MTT)			↓	↓				
	Apoptosis (Flow Cytometry)		↑						
Cell Signaling Pathways	PI3K-Akt-mTOR pathway			↓				↓	↓
	Autophagy signaling							↑	↑
	Cell Cycle					↑		↓	↓
	DNA replication					↑		↓	↓
Protein	pAKT expression	↑ (6,24h)		↓ (24h)		↑ (6,24h)		↓ (6,24h)	

Legend

- ↓** UBM and eECM shared response
- ↓** eECM only
- ↓** UBM only
- ↓** Validated by qPCR

increased invasiveness,³² chemoresistance, and metastasis,³³ to “reactionary” (e.g., stress response^{31,32}) to “anti-tumorigenic” (e.g., reduction in proliferation³⁴). Tunneling nanotubes have been shown to transfer healthy organelles and energy between cells in response to metabolic stress, mediated by PI3K-Akt activation, or before apoptosis.³⁵ It is plausible the cell bridges in the present study are also related to a PI3K-Akt-mediated stress response to the ECM treatment, but future work is needed to further understand this observation.

The distinctive effects of the two ECM materials included decreased metabolic activity of OE33 and SK-GT-4 cells, and increased late apoptosis of CP-A cells caused by UBM-ECM but not eECM. Previous studies have shown the effect of a heterologous ECM, specifically SIS-ECM, to suppress the malignant growth of neoplastic cells, including pancreatic,⁷ sinonasal,⁸ bladder,^{9–12} melanoma,¹³ and esophageal⁶ cancer cells in small-animal studies^{7,12,13} and human patients.^{6,8} Hurst *et al.*¹² showed that SIS-ECM hydrogel delayed and reduced incidence of tumor growth when bladder cancer cells were directly mixed and injected into the flank of mice compared with Matrigel. The present study corroborates Hurst *et al.*¹² by showing that heterologous UBM-ECM degradation products can specifically reduce proliferation and metabolic activity of neoplastic esophageal epithelial cells *in vitro* compared with pepsin control.

eECM showed distinctive effects compared with UBM-ECM by decreasing the proliferation of SK-GT-4 neoplastic

and CP-A metaplastic cells, as well as Het-1A nonmalignant esophageal epithelial cells. Only eECM strikingly down-regulated the focal adhesion-PI3K-Akt-mTOR and cell cycle/DNA replication pathways, and upregulated autophagy signaling at 24 h in OE33 cells by qPCR. It is important to note that while Het-1A proliferation was decreased with eECM treatment, Het-1A metabolic activity showed no change and Het-1A mitogenic signaling pathways, including cell cycle, DNA replication, and pAKT signaling, increased with eECM treatment at 24 h. Explanations for the discrepancy can include the time point selected for the proliferation assay, or other downstream functions of the upregulated signaling pathways that remain to be determined.

Despite the tissue-specific differences in neoplastic cell function and gene expression results shown between UBM-ECM and eECM, the protein signature of UBM-ECM and eECM using sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) showed that the banding patterns between the UBM-ECM and eECM were largely similar, except for a difference at ~60 kDa for eECM and at ~65 kDa for UBM-ECM. However, SDS-PAGE is not sensitive enough to detect if there are distinct protein isoforms present for the protein bands. The specific factors in normal ECM that actively suppress the neoplastic phenotype remain to be determined.

Hurst *et al.*¹² showed that collagen control did not provide the same suppressive effect on bladder cancer cell

phenotype as the SIS hydrogel provided in an *in vitro* invasion assay and in a flank xenograft coinjection model. These results suggest that collagen is not the primary suppressive bioactive component; however, future experiments are required to confirm this finding for esophageal cancer cells. The suppressive factor could be a soluble protein,³⁶ cryptic peptide,^{37,38} recently discovered matrix-bound nanovesicle,³⁹ combination of the aforementioned, or other factors.

Signaling pathways modulated by ECM degradation products: PI3K-Akt-mTOR, autophagy, cell cycle/DNA replication

eECM showed a downregulation of the PI3K-Akt-mTOR, cell cycle/DNA replication pathways and upregulation of autophagy pathways in OE33 cells by whole-transcriptome analysis and qPCR. PI3K-Akt-mTOR has been shown to be upregulated in many cancers,⁴⁰ including the progression from BE to EAC.^{41,42} Notably, PI3K-Akt-mTOR signaling regulates key cancer functions evaluated in the present study, including metabolism, proliferation, and apoptosis.⁴⁰ The identification of PI3K-Akt-mTOR pathways, and more specifically, upregulation of negative regulators of the pathway *PIK3PI*, *ULK1*, and *TSC2* and downregulation of positive regulator *HRAS* in OE33 cells treated with eECM, was shown by whole-transcriptome analysis and qPCR.

Other studies investigating the effect of (acellular) ECM on neoplastic cells have implicated the Akt pathway.^{11,12} Hurst *et al.*¹² showed that the PI3K-Akt pathway was found to be the most significantly downregulated pathway at the protein level of bladder cancer cells grown on top of SIS gel, in comparison with those grown on top of Matrigel or collagen. The identification of PI3K-Akt signaling in the present study could suggest a common pathway that is altered in neoplastic cells with ECM treatment, although the responsible signaling molecules would likely be distinct between cancer types and ECM tissue sources.

PI3K-Akt-mTOR negatively regulates autophagy signaling, and consistent with the present study results, autophagy was shown to be increased in OE33 cells treated with eECM by whole-transcriptome analysis and qPCR. The role of autophagy in EAC progression is not fully understood. However, a decrease in autophagy was proposed to be related to EAC cancer progression.⁴³ The net increase in autophagy, as a controlled form of cellular death, may further support the use of ECM to treat EAC.

UBM-ECM regulated fewer genes in the PI3K-Akt-mTOR pathway in OE33 cells by qPCR, despite the whole-transcriptome predictions. In OE33 cells, UBM-ECM decreased a negative regulator of the PI3K-Akt-mTOR pathway (*DDIT4*) and decreased a positive regulator of PI3K-Akt-mTOR (*SLC2A3*). *SLC2A3* is a gene that facilitates uptake of glucose needed to support proliferation and metabolic activity, and could be related to the downregulation of metabolic activity and proliferation shown in OE33 cells with UBM-ECM treatment at 24 h.

Cell cycle and DNA replication genes were strikingly regulated in opposite ways in Het-1A and OE33 cells treated with eECM, specifically the marked decrease of positive regulators in OE33 cells and marked increase of positive regulators in Het-1A cells. Similar to the present study,

Barkan *et al.*⁴⁴ showed that basement membrane ECM could arrest the cell cycle of breast cancer cells cultured in 3D, specifically with an increase in cell cycle inhibitors CDKN2A (p16) and CDKN1A (p27). Adding a specific component of the basement membrane matrix, $\alpha 2$ non-collagenous type IV collagen (canstatin), was shown by recombinant protein experiments to directly increase CDKN2A (p16) expression in cultured melanoma cells and promote cellular quiescence.⁴⁵ It remains unknown if the same basement membrane proteins are coordinating the changes in the cell cycle/DNA replication pathways in the present study, or if the effects are initiated by other proteins within the complex ECM.

At the protein level, UBM-ECM and eECM both downregulated phosphorylated AKT in OE33 cells at 24 h, and eECM also downregulated phosphorylated AKT in OE33 cells as early as 6 h. In contrast, UBM-ECM and eECM both upregulated phosphorylated AKT in Het-1A cells at 6 and 24 h. PI3K-Akt is an EAC signaling pathway, but its activation is also shown to be vital in wound healing processes in nonmalignant cells.⁴⁶ The opposite regulation of phosphorylated AKT in Het-1A and OE33 cells in response to the same ECM bioscaffold degradation products could be favorable for a therapy promoting tissue reconstruction in a setting of previous cancer.

Why would a normal and neoplastic cell show divergent intracellular signaling pathway responses to a normal ECM? Although the ability of the microenvironment to “normalize” neoplastic cell signaling pathways is not fully understood, the neoplastic cell may overexpress or downregulate cell surface receptors (e.g., integrins, growth factor receptors) that integrate ECM signaling molecules,^{47,48} or show differences in downstream histone modifications and epigenetic remodeling.⁴⁹ A normal ECM likely causes changes in multiple signaling pathways to downregulate the neoplastic phenotype.⁵⁰

A corollary question is: why is understanding these intracellular signaling pathways important? Liu *et al.*⁵⁰ used specific PI3K pathway inhibitors on human mammary epithelial cancer cells and showed phenotypic reversion to normal phenotype with reduced proliferation, reduced size, and corrected basal polarity. Stated differently, even though the neoplastic breast cells retained their genetic mutations, the cells were biochemically normalized by manipulating a pathway that had previously shown to be initiated by deregulated cell/matrix contact. As the cell becomes normalized biochemically and phenotypically, the ECM that the cell secretes will change. Many ECM proteins, including thrombospondin,^{51,52} tenascin X, and tropoelastin, among others,⁵³ have p53 binding sites, showing the coordination between tumor suppressor gene activation and the synthesis of new ECM. In this manner, the ECM can function as a tumor suppressing unit in response to dynamic intracellular signaling changes.

Even a transient application of normal ECM can reprogram the PI3K-Akt signaling pathway in OE33 cells, as early as 6 h with eECM treatment or at 24 h with UBM-ECM treatment, as shown in the present study. The multifactorial signaling pathways (PI3K-Akt, cell cycle, DNA replication, autophagy, among others) that are targeted with ECM treatment could be advantageous for EAC with documented genetic heterogeneity.⁵⁴

Limitations and future work

There were limitations to the present study. First, immortalized cell lines were used and are by definition transformed; however, other studies have shown similar effects with ECM on cancer cell lines.^{7,9–12} Second, the progression series were cultured on tissue culture plastic with ECM spiked in the media. The limitation of culturing cells on tissue culture plastic is well understood,^{55,56} and future work can validate the functional differences and signaling pathways of normal and EAC cells in 3D models and *in vivo*. Spiking the cell culture media with ECM means that only the biochemical cues of the ECM were evaluated, and not the potential effect of the structural/mechanical cues. Third, only one time point (24 h) was investigated for functional tests. This study, along with other studies investigating the effect of solubilized ECM on cell behavior that used short time points (1–2 days),^{27,57–60} demonstrates the rapid effect of ECM degradation products on cell phenotype. However, future work could investigate later time points. Finally, the isolated role of ECM on epithelial cells alone was investigated without the associated effects on supporting stromal cells (e.g., fibroblasts, immune cells) in the tumor niche that also influence the final remodeling outcome.

Despite the limitations, the role of ECM bioscaffold degradation products in modulating neoplastic phenotype is strongly supported. Similar and distinctive effects between a heterologous UBM-ECM and homologous eECM to modulate neoplastic cell proliferation and metabolism were shown, and the PI3K-Akt-mTOR, autophagy, and cell cycle/DNA replication pathways were identified as possible regulating pathways. While both heterologous UBM and homologous eECM showed the potential to decrease neoplastic phenotype, it appears as if the homologous eECM may show a more pronounced therapeutic effect. Interestingly, the suppressive effects of ECM are primarily shown with neoplastic cells, while normal cells were minimally affected. Normal cells have repeatedly been shown to not be adversely affected by normal ECM degradation products,⁶¹ and ECM biomaterials have been safely implanted in millions of patients to date. ECM bioscaffolds can also form a hydrogel with pepsin solubilization, which is liquid at room temperature, and gels *in vivo*, expanding the potential clinical use for minimally invasive or oral delivery to treat the cancer precursor diseases, BE and high-grade dysplasia. ECM could be used in combination with targeted therapy or immunotherapy, or as an adjunct therapy, to treat established esophageal cancer. As the mechanisms by which a normal ECM downregulates neoplastic phenotype are better understood, ECM-based therapies can be tailored for patients of this devastating disease.

Acknowledgments

This work was supported by the National Cancer Institute of the National Institutes of Health under Award Number F31CA210694 (L.S.). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

The authors thank Lynda Guzik from the McGowan Institute Flow Cytometry core at the University of Pittsburgh for access and assistance in the design and performance of flow cytometry experiments. The project used the University

of Pittsburgh HSCRF Genomics Research Core Whole Transcriptome Array and gratefully acknowledges the technical assistance of Liane Fairfull for processing the samples and gene expression data; and Deborah Hollingshead and Dr. Janette Lamb for helpful discussions in whole-transcriptome experimental design.

Authors' Contributions

L.S., S.P., L.H., and N.T. were responsible for study design, data collection, data analysis, and article writing. L.Z. and G.H. were responsible for study design, data collection, and data analysis. D.N., L.Q., X.L., D.R., A.B., N.S., and C.C. were responsible for data collection and data analysis. A.O., J.K., and B.J. were responsible for data analysis. A.Z. was responsible for study design and data analysis. S.B. was responsible for study design, data analysis, and article writing.

Disclosure Statements

L.S. reports grants from the National Institutes of Health National Cancer Institute during the conduct of the study. L.S., S.P., L.Z., L.H., G.H., D.N., L.Q., X.L., D.R., A.B., N.S., C.C., N.T., and S.B. report “sponsored research in the Badylak laboratory” from CR BARD, during the conduct of the study. S.B. reports “sponsored research in the Badylak laboratory” from ACell and CR BARD outside the submitted work. L.H. reports and is currently employed by and owns stock in ACell, Inc., which commercializes UBM as Gentrix™, MatriStem®, Cytal™, and MicroMatrix®. L.S., S.B., and G.H. have a patent “Extracellular Matrix (ECM) Hydrogel and Soluble Fraction Thereof for the Treatment of Cancer” pending. L.S. and S.B. have a patent “ECM Hydrogel for Treating Esophageal Inflammation” pending. The remaining authors have nothing to disclose.

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Received: April 12, 2018

Accepted: August 23, 2018

Online Publication Date: February 26, 2019