Cellular Localization, Invasion, and Turnover Are Differently Influenced by Healthy and Tumor-Derived Extracellular Matrix

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The interplay between tumor cells and the microenvironment has been recognized as one of the hallmarks of cancer biology. To assess the role of extracellular matrix (ECM) in the modulation of tissue homeostasis and tumorigenesis, we developed a protocol for the purification of tissue-derived ECM using mucosae from healthy human colon, perilesional area, and colorectal carcinoma (CRC). Matched specimens were collected from the left colon of patients undergoing CRC resection surgery. ECMs were obtained from tissues that were decellularized with hypotonic solutions containing ionic and nonionic detergents, hypertonic solution, and endonuclease in the absence of denaturing agents. Mucosae-derived ECMs maintained distribution and localization of proteins and glycoproteins typical of the original tissues, and showed different three-dimensional (3D) structures among normal versus perilesional and tumor-derived stroma. The three types of ECM differentially regulated the localization and organization of seeded monocytes and cancer cells that were located and organized as in the original tissue. Specifically, healthy, perilesional, and CRC-derived ECMs sustained differentiation and polarization of cancer epithelial cells. In addition, healthy, but not perilesional and CRC-derived ECM constrained invasion of cancer cells. All three ECMs sustained turnover between cell proliferation and death up to 40 days of culture, although each ECM showed different ability in supporting cell proliferation, with tumor > perilesional > healthy-derived ECMs. Healthy-, perilesional- and CRC-derived ECM differently modulated cell homeostasis, spreading in the stroma and turnover between proliferation and death, and equally supported differentiation and polarization of cancer epithelial cells, thus highlighting the contribution of different ECMs modulating some features of tissue homeostasis and tumorigenesis. Moreover, these ECMs provide competent scaffolds useful to assess efficacy of antitumor drugs in a 3D setting that more closely recapitulates the native microenvironment. Further, ECM-based scaffolds may also be beneficial for future studies seeking prognostic and diagnostic stromal markers and targets for antineoplastic drugs.

Introduction

EXTRACELLULAR MATRIX (ECM) is responsible for the three-dimensional (3D) structure of tissues. ECM is composed of a network of proteins and proteoglycans interacting with each other, and participating in supramolecular assemblies where their biological properties are modified. ECM regulates tissue functions by sequestering and storing growth factors, thus localizing their activity in specific area of the tissue and protecting them from degradation.¹ In particular, the glycosylated proteins of the ECM are reported to have the

capacity to bind to soluble extracellular factors present in the extracellular environment such as cytokines, chemokines, and growth factors.² Moreover, intrinsic domains of the stromal proteins have growth factor-like structure acting as ligands for canonical growth factor receptors. Therefore, ECM has been described as solid-phase organized ensemble of ligands.³ Consequently, the extracellular microenvironment of different tissues and organs influences cell recruitment from blood, in addition to cellular migration, growth, death, differentiation and polarization, as clearly highlighted by the ability of stem cells or monocytes to differentiate in a variety

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of cellular subpopulations in different tissues and organs. Indeed, one of the most important properties of ECM, and thus of the extracellular microenvironment, is its functional diversity.⁴ Nonetheless, extracellular microenvironment from pathological tissue differently modulates the outcome of the disease. For instance, changes in glycosylation and in the repertoire of glycans promote tumor cell invasion and distribution throughout the organism.⁵ Processing leading to the modification and accumulation of ECM components in the lamina propria surrounding tumors (desmoplastic reaction) have been associated with both containment of tumor growth and invasion,^{6,7} in addition to tumor progression and poor prognosis of colorectal carcinoma (CRC).^{8,9} A unifying hypothesis might be that the desmoplastic reaction represents the initial defensive response of the host that then turn out in favor of neoplastic cells.

Further, the success rate of many drugs evaluated *in vitro* or *in vivo* in animal models and tested in clinical trials is very low,¹⁰ and the spatial organization of cells, accumulation of ECM components, or altered branching patterns of certain glycans have been proposed to be responsible for the failure of conventional system.⁵

The study of ECM regulating cellular functions has greatly benefitted from the commercially available synthetic and murine matrices (e.g., fibroblast-derived ECM).^{11,12} On the other hand, those matrices show limitations: (i) synthetic matrices do not recapitulate the composition of tissue-derived ECM, lacking the presence of cytokines/growth factors and proper 3D structure, (ii) murine matrices are derived from mouse tumors and thus are not representative of human tissues, and (iii) ECM from tissue-purified fibroblasts does not represent the entire complexity of ECM observed *in vivo*. Moreover, ECMs purified from human tissues, which would recapitulate better the tumor microenvironment of healthy or pathological tissues are not commercially available.

Aim of this study was to assess the role of human ECM in the modulation of tissue homeostasis and tumorigenesis. In particular, we have focused our attention on ECM from human colon mucosa and CRC because this neoplasia is one of the leading tumors in the world and surgery still represents the primary treatment for this set of patients.¹³ To address this issue we have developed a protocol that allows decellularization of human colon mucosa and obtaining an ECM that maintains the proper structure and composition suitable as scaffold for cell culture experiments. We provide evidence that proper distribution of stromal proteins, glycoproteins, and 3D structure in healthy, perilesional area, and CRC-derived ECM supported specific cellular localization, organization, proliferation, and death of seeded monocytes and cancer cells. This novel methodology may find broad utility in (i) establishing the influence of healthy and pathological 3D microenvironment on tissue homeostasis; (ii) assessing the outcome of tumor progression; and (iii) as medical device for antitumor drugs.

Materials and Methods

Reagents

Chemicals and cell culture reagents are reported in the Supplementary Data (Supplementary Data are available online at www.liebertpub.com/tea).

Tissue specimens

Matched specimens were collected from the left colon of patients undergoing CRC resection surgery and obtained through the Unit of Surgical Pathology (Ospedale San Raffaele, Milan, Italy). Formal written waiver from the Institutional Review Board (Ethic Committee Ospedale San Raffaele, protocol 279/DG), and signed written informed consent was obtained. All mucosa specimens encompassed the luminal surface, mucosa and, upper submucosa. Neoplastic tissue was obtained at the edge of infiltrating neoplasia; perilesional tissue was 1 cm far from CRC, free of lesions and of macroscopically necrotic areas; healthy colon mucosa was from the resection margins (10 cm far from the CRC).

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats of healthy blood donors by Ficoll-Hypaque and monocytes following centrifugation of PBMC on iso-osmotic Percoll gradient.¹⁴

Preparation of ECM from human colon mucosa

All specimens were kept in cold and sterile phosphate buffered saline (PBS) for no longer than 1 h before processing. Each specimen was split in two fragments for assessing features of tissue-derived ECM versus the unprocessed tissue. All steps for decellularization were performed with sterile solutions and under tissue culture hood, and the below described protocol was developed from previous reports.^{15–17}

Wet tissue was washed with cold PBS, placed on Petri dish, and dissected with scalpel at 60–100 mg weight, then placed in 10 mL of cold solution A and kept on rotating wheel for 8 h at 4°C. Tissue was then transferred into a new falcon tube and washed thrice with 4 mL of cold 0.1× PBS, placed in 10 mL of cold solution B, and kept on rotating wheel overnight at 4°C. Tissue was then washed thrice with 4 mL of cold 0.1× PBS, placed in 10 mL of cold solution C, and kept on rotating wheel for 8 h at 4°C. Tissue was then washed thrice with 4 mL of cold 0.1× PBS, placed in 10 mL of solution D, and kept on rotating wheel overnight at room temperature. Tissue was then washed thrice with 4 mL of 0.1× PBS, and washed twice with solution E on rotating wheel for 15 min at room temperature. Tissue was then incubated twice with 5 mL of solution F kept shaking at 37°C for 4 h. After a final three washes with PBS ECM is ready to use. Alternatively, ECM was stored at 4°C in PBS containing 1% pen/strep, or at –80°C in 10% dimethyl sulfoxide (DMSO)/Dulbecco's modified Eagle's medium (DMEM) culture medium. Composition and mechanism of action of solutions A–F are reported in Table 1. All steps on the rotating wheel were at 30 rpm (slower rotation resulted in incomplete decellularization, according to previous reports showing incomplete removal of nucleic acids¹⁸).

The same protocol used for the preparation of ECM from fresh specimens has also been tested and validated using vital-frozen tissues (data not shown). Mucosa was frozen according to standard procedures, such as washed with cold PBS, transferred into chilled freezing solution (10% DMSO, 90% culture medium DMEM), and frozen at –80°C for 24 h followed by storage in liquid nitrogen.

Fluorescein isothiocyanate and AMAC staining of ECM and 3D reconstruction

Stromal proteins and carbohydrates were labeled with fluorescein isothiocyanate (FITC) and 2-aminoacridone (AMAC),

TABLE 1. LIST OF SOLUTIONS USED FOR THE PREPARATION OF EXTRACELLULAR MATRIX

<i>Solution</i>	<i>Composition</i>	<i>Mode of action</i>
Solution A	86% of 0.14× PBS, 5 mM EDTA, 10% DMSO, 1% protease inhibitor cocktail, 1% pen/strep, 1% fungizone, 50 µg/mL gentamicin,	Cell dissociation from the stroma and cell lysis
Solution B	96% of 0.1× PBS, 1% TrytonX-100, 1% protease inhibitor cocktail, 1% pen/strep, 1% fungizone, 50 µg/mL gentamicin	Removal of membrane lipids and cell residues
Solution C	0.5 M NaCl in 0.1× PBS	Dissociation of proteins from DNA
Solution D	10 mM sodium cholate hydrate in 0.1× PBS	Solubilization of nucleic membranes
Solution E	50 mM Tris-HCl pH 8.0, 2 mM MgCl ₂	Conditioning with Tris buffer
Solution F	Solution E containing 100 U/mL Benzonase	Degradation of DNA and RNA

Hypotonic (0.1× and 0.14×) PBS were prepared by diluting 1× PBS with ddH₂O and sterilized by filtering on 0.2 µm filter. All solutions were prepared in sterile conditions. All reagents were bought as for cell culture and at the highest grade of purification.

DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; PBS, phosphate buffered saline.

respectively, according to the manufacturer's instructions. Detailed methods are reported in the Supplementary Data (Supplementary Data are available online at www.liebertpub.com/tea). Laser scanning confocal microscope (Leica TCS SP2) and Leica Confocal Software were used to acquire images and perform 3D reconstruction (an average of 150–180 frames spanning 100–120 µm of ECM).

DNA extraction and quantification

DNA was extracted from 20 mg of colon mucosa or ECM by NucleoSpin Tissue Kit (Macherey-Nagel), according to the manufacturer's instructions. DNA was quantified by Nanodrop 1000 (Thermo Fisher Scientific) at 260/280 nm ratio. Agarose gel (1%, containing 0.01% SYBR) was used to separate DNA and bands were visualized by exposing the gel to UV light and images acquired by Gel Doc (Biorad). Five and 10 µL of mucosa or ECM lysates were loaded into the gel, and 1 kb ladder and 100/200/400/800 ng of lambda DNA were used as controls.

Western blot for actin

Sixty milligrams of mucosa and ECM or 10⁷ PBMC were suspended in 200 µL of buffer C (20 mM Hepes, 10% glycerol, 0.4 M NaCl, 0.2 M ethylenediaminetetraacetic acid (EDTA), 1.5 mM MgCl₂, 0.5% NP40, and 1 µL of proteases inhibitor cocktail each mL). After 5 h at 4°C on horizontal shaker, and vortex every 30 min, lysates were clarified by centrifugation (12,000 rpm at 4°C for 5 min). Protein content in the supernatants was quantified by Bradford assay. Because of low levels in the lysate from ECM, a volume equal to that used for the lysate from colon mucosa was used for western blot. Thirty-six micrograms of protein from mucosa lysate, 20 µg from PBMC (corresponding to 400,000 cells) lysate, and lysate from ECM were denatured by addition of an equal volume of 2× sample buffer (0.125 M Tris-HCl, pH 6, 8; 4% SDS; 10% 2-2-ME; and 20% glycerol) and heated for 5 min at 100°C before SDS-PAGE on a 14% gel and subsequently transferred to a nitrocellulose membrane (Hybond ECL; Amersham) by electroblotting. Membranes were blocked in 5% nonfat milk, 20 mM Tris, pH 7.6, 137 mM NaCl, and 0.2% Tween 20 overnight at 4°C and further incubated with goat-anti human actin antibody (1/1000 in 5% milk/TTBS). Ab binding was visualized by

using rabbit-anti goat antibody-HRP (1/5000 dilution in 5% milk/TTBS) and the signal was revealed by ECL (Amersham) according to the manufacturer's instructions.

Seeding of primary monocytes

Two-million monocytes were suspended in 1 mL of complete medium (DMEM culture medium containing 10% fetal calf serum (FCS), 1% penicillin/streptomycin sulfate, and 1% glutamine) and seeded onto 50 mg ECM (dipped in 1 mL of DMEM for 1 h at room temperature before cell seeding to replace PBS with DMEM) placed in 25 cm² sterile flask with ventilated cap. Two hours later 4 mL of complete medium was added. Twenty hours later ECM containing the seeded cells was transferred in a new culture flask and cultivated in 5 mL of complete medium: half of the conditioned medium was replaced by fresh complete medium after 3 days.

Presence and localization of CD68⁺ cells (i) seeded in the different ECMs and (ii) in the corresponding native surgical samples from which ECMs were prepared were evaluated by means of immunohistochemistry.

Seeding of HT-29, LoVo, and SW480 cancer cells

Five million cancer cells were suspended in 5 mL of culture medium (DMEM containing 10% heat-inactivated FBS, 1% penicillin/streptomycin sulfate, 1% glutamine, and 1% non-essential aminoacids) and added to 50 mg ECM (dipped in 1 mL of DMEM for 1 h at room temperature before cell seeding) placed in 25 cm² sterile flask with ventilated cap. After 2 h in cell incubator 5 mL of culture medium was added. Twenty-two hours later ECM containing the seeded cells was transferred in a new culture flask and cultivated in 10 mL of complete medium: half of the conditioned medium was replaced by fresh complete medium every 3–4 days.

Histological and immunohistochemistry analysis

Tissues and ECM were fixed in 10% buffered formalin for 24–48 h at room temperature and then embedded in paraffin. Morphological examination and antigen expression were performed as reported.¹⁹ Briefly, 3 µm paraffin sections from each sample were used. Hematoxylin-eosin stained sections were histologically evaluated. For immunohistochemistry, sections were pretreated in a microwave oven, two cycles

×5 min each, at 780W, in 0.25 mM EDTA buffer, and then incubated for 2 h with specific antibodies against human antigens (Supplementary Table S1). The reactions were revealed by nonbiotin peroxidase detection system with 3,3'-diaminobenzidine free base (DAB) as chromogen.

Mucus secreting cells were estimated by means of Alcian blue/periodic acid–Schiff (AB/PAS) staining, as reported.²⁰

Scanning electron microscopy

Paraffin section on coverslip was dewaxed, dehydrated with absolute ethyl alcohol, and dried overnight in hexamethyldisilazane. Section was coated with gold-palladium after evaporation of hexamethyldisilazane and examined in a Leica S420 scanning electron microscopy.

Apoptosis and proliferation rate of cells seeded into the ECM

Apoptotic cells were detected on tissue sections prepared and stained by using commercial kit (TdT-FragEL DNA fragmentation Kit) according to manufacturer's instructions (Calbiochem, Merck Chemicals Ltd.).

Proliferating cells were detected on tissue sections stained by Ki67 monoclonal antibody (Supplementary Table S1) and counterstained with hematoxylin; proliferation rate was measured as the percentage of Ki67⁺ cells on the total number of cells present in regions free of dead cells. Five high-magnification (40×) fields per each experiment were used for counting cells, by means of ImageJ software.²¹

FIG. 1. ECM maintains stromal proteins and hyaluronic acid distributed as in the healthy and neoplastic mucosa. Healthy mucosa from the left colon and CRC were analyzed before and after decellularization. (A) Mucosae and ECM were fixed by 4% formalin and embedded in paraffin. Three micrometer slices of mucosa and ECM were analyzed by histochemistry (hematoxylin-eosin [HE], staining) and immunohistochemistry for the presence of cellular proteins (cytokeratin and vimentin) and stromal components (collagen IV, fibrinogen, and hyaluronic acid). Magnification 20×. (B) Content of actin in colon mucosa, ECM, and PBMC was visualized by means of western blot analysis. (C) Colon mucosa and ECM were assessed for the content of DNA, visualized on SYBR agarose gel. (D) Mucosa from left colon was decellularized and visualized by white light transilluminator Gel Doc. One representative experiment out of seven is shown. CRC, colorectal carcinoma; ECM, extracellular matrix; PBMC, peripheral blood mononuclear cells. Color images available online at www.liebertpub.com/tea

Results

ECM lacks cell-associated antigens but maintains stromal-derived proteins and hyaluronic acid

Mucosa was removed during surgery for colon-rectal carcinoma. Healthy colon mucosa (from the resection margins) and CRC underwent treatments for the removal of cells, nuclei, and nucleic acids as described in the Materials and Methods section. Histological examination of hematoxylin-eosin stained sections from untreated and treated samples revealed that the decellularized healthy mucosa and carcinoma fully maintained the architectural structure as the corresponding untreated samples, as evidenced by the presence of colon crypts with a regular and homogeneous shape in healthy samples and ramified glands with heterogeneous shape and size in neoplastic tissues (Fig. 1A).

Immunohistochemistry was performed to assess the distribution of cellular and stromal components. ECM from both healthy colon mucosa and CRC lack cytokeratin and vimentin (Fig. 1A), indicating the absence of epithelial and mesenchymal cells, respectively. All ECMs maintained distribution of stromal components such as collagen IV, fibrinogen, and hyaluronic acid that are also observed in the untreated mucosae (Fig. 1A).

Lack of cellular antigens and nucleic acids in the ECM was confirmed by means of western blot analysis (Fig. 1B) and quantification of DNA content by Nanodrop 1000 (data not shown): SYBR-agarose gel confirmed lack of DNA fragments from ECM and a homogeneous band of DNA from colon mucosa (Fig. 1C). Moreover, the decellularized mucosa maintained a solid state (Fig. 1D).

ECM 3D structure is different among healthy, perilesional, and neoplastic tissue

To further assess the 3D structure of ECMs we took advantage of FITC that labels proteins and stained matrices from (i) healthy colon mucosa (ii) perilesional area, and (iii) CRC.

FITC staining of the ECM from the healthy area showed homogeneous distribution of (i) crypts size, and (ii) proteins

in between and around crypts (Fig. 2A, showing one single frame). Three-dimensional reconstructions showed that crypts maintain an intact structure and a round shape morphology with ECM proteins uniformly distributed throughout the decellularized mucosa (Fig. 2B).

FITC staining of the ECM from the perilesional area revealed that this tissue was characterized by heterogeneous distribution of (i) crypts size and (ii) proteins in between and around crypts (Fig. 2C). The 3D reconstructions showed that this ECM structure is maintained throughout the tissue (Fig. 2D). FITC staining of the ECM from the tumor mass revealed a dense matrix and glands with irregular shape and asymmetric profile (Fig. 2E, F).

Therefore, this protocol of colon decellularization allowed preservation of 3D structures that appeared different among healthy, perilesional, and neoplastic tissues. Those structures recapitulated features observed in the hematoxylin-eosin staining reported in Figure 1A, such as regular shape morphology of crypts in the colon mucosa and glands with irregular shape and asymmetric profile in the CRC, as previously reported.^{22,23}

Moreover, the protocol for decellularization was optimized and standardized, with the above data showing that complete removal of cells, cellular debris, and nucleic acid was obtained when 60–100 mg of tissue was used with 10 mL of each reported solution. FITC staining of ECM derived from increased amount of mucosa revealed incomplete removal of cells both in the stroma and crypts (Supplementary Fig. S1).

Carbohydrates distribution in the ECM is different among healthy, perilesional, and neoplastic tissue

To assess distribution of glycans in the different ECMs, carbohydrate moieties were labeled with AMAC.

Healthy colon mucosa derived-ECM was characterized by regular and homogeneous distribution of glycoproteins (Fig. 3A, one single frame; Fig. 3B, 3D reconstruction). In the ECM from perilesional area glycoproteins were also normally distributed, although with irregular pattern (Fig. 3C, one single frame; Fig. 3D, 3D reconstruction). Conversely, in

FIG. 2. Healthy colon mucosa, perilesional tumor area, and CRC ECM showed different protein 3D organization. ECM was prepared using mucosa collected from healthy colon (A, B), perilesional tumor area (C, D), and CRC (E, F). ECMs were stained with FITC and pictures acquired with confocal microscope. Single frame from Z-stack (A, C, E) and 3D reconstruction (B, D, F) of FITC-stained ECMs are shown. Magnification 20 $\times$. One representative experiment out of three is shown. FITC, fluorescein isothiocyanate. Color images available online at www.liebertpub.com/tea

FIG. 3. Healthy colon mucosa, perilesional tumor area, and CRC ECM showed different carbohydrate 3D organization. ECM was prepared using mucosa collected from healthy colon (A, B), perilesional tumor area (C, D), and CRC (E, F). ECMs were stained with 2-AMAC and pictures acquired with confocal microscope. Single frame from Z-stack (A, C, E) and 3D reconstruction (B, D, F) of FITC-stained ECMs are shown. Magnification $20\times$. One representative experiment out of three is shown. AMAC, aminoacridone. Color images available online at www.liebertpub.com/tea

the ECM from CRC, glycoproteins showed extremely irregular and heterogeneous distribution (Fig. 3E, one single frame; Fig. 3F, 3D reconstruction).

Healthy, perilesional, and CRC-derived ECMs supported organized localization of primary monocytes

Given the important role of the inflammatory infiltrate in cancer-related inflammation,²⁴ we assessed the ability of ECM to sustain infiltration and localization of leukocytes. We seeded peripheral blood-derived monocytes onto matrices derived from healthy, perilesional area, and tumor-bearing colon mucosa, and we performed immunohistochemical staining with anti-CD68 antibody to identify monocytes in the healthy colon mucosa, perilesional area, and CRC. Native tissues from healthy colon mucosa, perilesional area and, CRC were used as control for CD68 distribution (Fig. 4A).

Four (Fig. 4B) and 7 days (Supplementary Fig. S2) after seeding morphological examination showed that monocytes penetrated ECMs; in the healthy ECM CD68⁺ cells were homogeneously distributed below the luminal area and around crypts. Increased number of CD68⁺ cells was found in the perilesional and CRC-derived ECMs, where cells clustered around dysplastic and neoplastic glands (Fig. 4B), as observed in the native healthy colon mucosa, perilesional area, and CRC (Fig. 4A).

Healthy, perilesional, and CRC-derived ECMs supported organized localization, differentiation, and morphology, but not infiltration, of HT29 cells

Next we assessed the influence of ECM on localization and differentiation of transformed epithelial cells HT29. This cell line was isolated from a grade II primary colorectal adenocarcinoma, and it was reported to have a modest invasive capacity and ability to form tumors in xenograft models.²⁵

Four days after seeding, HT29 cells were excluded from the ECM but instead located inside the crypts and neoplastic glands, and on top of the luminal surface in all the samples (Fig. 5A). Moreover, longitudinal cross sections revealed that 4 days after seeding HT29 cells did repopulate the entire lumen of the crypt (Supplementary Fig. S3), indicating accessibility of the crypts/glands, likely as consequent to the noncollapsing 3D structure.

The same localization of HT29 cells was observed at later time points (day 9, 21, and 40 after seeding, Fig. 5A), with no signs of infiltration in all three ECMs at all time points.

Niches of dead cells formed both in the crypts and in the mass formed on top of the luminal area of all ECMs. Interestingly, cell death in healthy-derived and perilesional-derived ECM started at day 9 postseeding, while it occurred at later time points (day 21 postseeding) (Fig. 5A) when cells were seeded on CRC-derived ECM. Dead cells did not accumulate over time since crypts, tumor glands, and luminal area were still colonized by live cells after 40 days of culture (Fig. 5A). This set of data indicated that the ECM sustains cellular turnover, with the CRC-derived ECM delaying occurrence of apoptosis.

We then assessed differentiation of HT29 cells by means of mucopolysaccharides detection using AB/PAS staining. Mucus-secreting cells were absent at day 4 but appeared 9 days after seeding (Supplementary Fig. S4). Cells secreting acidic mucus were evident 21 days after seeding, irrespective of the ECM on which they were seeded (Alcian blue staining, Fig. 5B). Further, HT29 cells expressed abundant and short microvilli irrespective of the ECM on which the cells were seeded (Fig. 5C).

Postconfluent HT29 cells grown in two-dimensional (2D) system for 21 days did not produce mucus but expressed a brush border composed by short and irregular microvilli (Supplementary Fig. S5A).^{26,27}

FIG. 4. Healthy, perilesional, and CRC-derived ECMs supported organized localization of primary monocytes. (A) Native surgical samples were analyzed by IHC for CD68 expression. (B) An aliquot of the native tissue was used for preparation of the ECM, on which peripheral blood-derived monocytes were seeded. Four days later seeded cells were analyzed by IHC for CD68 expression: 100 $\times$ magnification is showing the characteristic staining pattern of CD68 antigen. Pictures are from one experiment performed with ECMs derived from surgical samples of the same patient. One representative experiment out of two is shown. Color images available online at www.liebertpub.com/tea

These results are in line with previous findings indicating that HT29 cells acquire a differentiated status when grown in the presence of differentiating agents or xenograft model, whereas they have shown an undifferentiated status (i.e., lack of goblet cells) in 2D system.^{28–31}

ECMs supported infiltration of LoVo and SW480 cells

We then tested the availability of the different ECMs to support infiltration of different transformed epithelial cells. The epithelial cell line LoVo (isolated from a metastatic nodule of an individual bearing Dukes' type C, grade IV, colorectal adenocarcinoma³²) were seeded onto the healthy-, perilesional- and CRC-derived ECM and analyzed for their distribution and differentiation status.

Four days after seeding in the healthy-derived ECM, LoVo cells were localized in the crypts, on top of the luminal area and only few cells localized into the lamina propria; the same distribution was observed at 9 and 21 days postseeding, although after 40 days no cells were in the stroma (Fig. 6A). On the contrary, in the perilesional- and CRC-derived ECMs LoVo cells were also distributed throughout the ECM 4 days postseeding, with an evident spreading occurring at the later time points (Fig. 6A).

Niches of apoptotic LoVo cells appeared at day 9 postseeding in the crypts, inside the mass formed on top of the luminal area and in the ECM (Fig. 6A). Indeed, areas of dead cells continued to accumulate over time (day 21 and 40

postseeding) in the healthy-, but not perilesional- and CRC-derived ECM (Fig. 6A).

LoVo cells secreted neutral mucins (PAS staining positive) at day 4, 9 (Supplementary Fig. S6), and 21 postseeding (Fig. 6B), and expressed a brush border (Fig. 6C), irrespective of the ECM on which the cells were seeded.

Postconfluent LoVo cells grown in 2D system for 21 days also produced mucus and expressed a brush border (Supplementary Fig. S5B).

These findings are consistent with previous reports showing LoVo cells expressing PAS⁺ mucus and microvilli in 2D system^{32,33} and xenograft models,³⁴ and we provide evidence that healthy, perilesional, and tumor-derived ECM did not influence differentiation of LoVo cells.

As observed for LoVo cells, SW480 cells (established from a primary colorectal adenocarcinoma, Dukes' type B) also infiltrated all three ECMs, both at day 4 and 9 postseeding, with apoptotic niches occurring at day 9 postseeding (Supplementary Fig. S7A).

Scant PAS staining was observed in SW480 cells seeded onto the ECMs both at day 4 and 9 postseeding (Supplementary Fig. S7B), as previously reported in the 2D system and xenograft models.³⁴

Different capacities of ECMs in supporting the proliferation of transformed epithelial cells

The appearance of apoptotic niches and the spreading of cancer cells suggested that the different ECMs might

FIG. 5. Healthy, perilesional, and CRC-derived ECMs supported organized localization, differentiation, and morphology, but not infiltration, of HT29 cells. **(A)** Hematoxylin-eosin staining was used to analyze the distribution of HT29 cells onto the different matrices at the indicated time points postseeding (magnification $20\times$). **(B)** Mucus secreting cells were analyzed by Alcian blue/PAS staining (magnification $40\times$). **(C)** Ultrastructural morphology of HT29 seeded onto healthy- and tumor-derived ECM was analyzed by scanning electron microscopy 21 days postseeding, searching for presence of microvilli (magnification $29630\times$). Symbols are representative of niches of apoptotic (asterisk) and mucus secreting (red arrow-head) cells. Pictures are from one experiment performed with ECMs derived from surgical samples of the same patient. One representative experiment out of four is shown. PAS, periodic acid–Schiff. Color images available online at www.liebertpub.com/tea

modulate turnover between proliferating and nonproliferating cells. Proliferation rate of live cells was estimated by means of Ki67 staining, thus excluding the areas of apoptotic niches.

Four days after seeding into the ECMs $70\% \pm 4\%$ (median $\pm$ SD, $n=3$) of the HT29 cells were in the proliferative phase, irrespective of the healthy, perilesional, and neoplastic origin of ECM. Percentage of HT29 cells in the proliferative

phase decreased 9 and 21 days postseeding, although the different ECMs showed different abilities to sustain cell proliferation until 40 days postseeding (Fig. 7A, B).

Four days after seeding into the different ECMs $94\% \pm 2\%$ (median $\pm$ SD, $n=3$) of LoVo cells were in the proliferative phase, irrespective of the origin of the stroma (Fig. 7C and Supplementary Fig. S8). At later time points

FIG. 6. ECMs supported infiltration of LoVo cells in the stroma. **(A)** Hematoxylin-eosin staining was used to analyze the distribution of HT29 cells onto the different matrices at the indicated time points postseeding (magnification 20×). **(B)** Mucus secreting cells were analyzed by Alcian blue/PAS staining (magnification 40×). **(C)** Ultrastructural morphology of LoVo cells seeded onto healthy and tumor-derived ECM was analyzed by scanning electron microscopy 21 days postseeding, searching for presence of microvilli (magnification 29630×). Symbols are representative of niches of apoptotic cells (asterisk), mucus-secreting cells (red arrow-head), and cells infiltrating the stroma (arrow). Pictures are from one experiment performed with ECMs derived from surgical samples of the same patient. One representative experiment out of three is shown. Color images available online at www.liebertpub.com/tea

proliferation rate of LoVo cells was influenced by the ECM on which cells were seeded. Proliferation rate decreased when cells were seeded onto healthy-derived ECM, whereas it remained at higher levels in cells seeded in the perilesional- and CRC-derived ECM (Fig. 7C and Supplementary Fig. S8). Superimposable findings were observed with SW480 cells (Supplementary Fig. S7C).

These findings are in agreement with previous report showing 50% decreased cell viability of oral squamous cell carcinoma cultivated for 10 days in a synthetic scaffold.³⁵ Indeed, with HT29, LoVo, and SW480 cells we observed that ECMs showed different capacity in supporting the proliferation of transformed epithelial cells, with tumor > perilesional > healthy-derived ECM (Fig. 7B, C), thus

FIG. 7. Different potency of ECMs in supporting the proliferation of transformed epithelial cells. Proliferating HT29 cells (**A**) were assessed by Ki67 staining (brown spot) at different time points after seeding into ECMs, with slides counterstained with hematoxylin (20x magnification). Proliferation rate of HT29 (**B**) and LoVo (**C**) cells was measured as the percentage of Ki67⁺ cells on the total number of cells present in regions free of dead cells. Five high-magnification (40x) fields per each experiment were used for counting cells, and data are presented as median $\pm$ SD of Ki67⁺ cells seeded into ECMs derived from surgical specimens of three different individuals. Differences were tested by one-way ANOVA (p -values are indicated by asterisk *0.01–0.05; **0.001–0.01; *** $\leq$ 0.001). Pictures are from one experiment performed with ECMs derived from surgical samples of the same patient. Color images available online at www.liebertpub.com/tea

extending relevance to the different contribution of healthy, perilesional, and CRC-derived ECM.

Conclusions and Discussion

The described methodology for purification of 3D ECM have been tested and validated on healthy human colon mucosa and CRC. In agreement with the criteria required to

satisfy the intent of decellularization (<50 ng DNA/mg ECM weight, <200 bp DNA fragment length, lack of visible nuclear material in ECM by hematoxylin-eosin staining),¹⁸ the ECM purified with this methodology has shown complete removal of cells, cellular debris, and nucleic acid. In addition, the protocol was further improved to preserve the complex composition of the different ECMs, including presence of glycans that are known to facilitate invasion of

tumor cells in colon neoplasia.⁵ In fact, a common problem with many agents and techniques for decellularizing tissue is the loss of glycosaminoglycans and partial disruption of ECM ultrastructure.¹⁸ Conventional scaffolds such as synthetic matrices, murine sarcoma-derived matrix, and matrix from fibroblasts do not recapitulate native ECM. Consistent with the above findings, our methodology allowed to obtain tissue-derived scaffolds that maintained the different characteristics of the ECM from different tissues, thus extending the purification of the matrix from healthy tissue to solid tumors, characterized by increased cellularity and desmoplasia.

We provide evidence that this protocol allows maintaining proper distribution of (i) ECM proteins, (ii) glycans, and (iii) 3D structure. Of particular note is the maintenance of hyaluronic acid, the main glycosaminoglycan modulating tissue homeostasis, epithelial mesenchymal transition, and tumor outcome.^{36,37} Moreover, despite visualization of carbohydrates by AMAC does not allow discriminating for the type of glycans present in the different ECMs, we have clearly shown different pattern and profile of glycoproteins distribution among ECMs from healthy colon, perilesional area, and CRC. The ability to maintain the presence of glycosaminoglycans is mandatory for the proper function of ECM, although many protocols of decellularization lack this important component due to the use of harsh detergents and aspecific enzymes.^{18,38,39} Indeed, all the above reported features of tissue-derived ECMs are relevant for assessing the role of microenvironment on tissue homeostasis, as (i) intrinsic domains within stromal proteins might act as ligands for canonical growth factor receptors, (ii) many growth factors bind to glycosaminoglycan chains,³ and (iii) the proper 3D structure allow for the specific and organized localization of ligands⁴⁰ and associated proteins, such as mucins, complement, metallo-proteases, metallo-protease inhibitors, latent TGF- β , growth factors, and chemokines.^{41,42}

To establish the availability of tissue-derived ECMs to be employed as scaffolds for the study of tumorigenesis we used monocytes and tumor epithelial cell lines.

We used primary monocytes because this cellular population is known to localize within the ECM and represents a fundamental component of inflammatory infiltrate modulating tumorigenesis.²⁴ ECMs sustained specific and organized localization of seeded monocytes, with CD68⁺ cells that were within the stroma of all ECMs, indicating accessibility of the stroma purified with this protocol. Second, infiltrated monocytes localized and distributed around dysplastic and neoplastic glands in the perilesional and CRC-derived ECMs while they homogeneously distributed around the crypts of the healthy-derived ECM and this was dependent on the unique properties of the different ECMs. The same localization and organization of CD68⁺ cells was observed in healthy and neoplastic tissues, thus indicating that ECMs obtained with this protocol recapitulated properties and components modulating cell localization and clusterization typical of the original tissue.

To assess the ability of ECMs to sustain homeostasis and localization of cancer cells, we used transformed epithelial cell lines isolated from colon tumor at grade II, III, and IV.

HT29 cells (CRC grade II) were located inside the crypts and on top of the luminal area, in which they remained localized up to 40 days postseeding. Moreover, HT29 cells re-

mained localized also inside the tumor glands and never spread throughout the ECM despite that the breakdown of the basal membrane is a feature of the CRC.⁴³ HT29 cells have been reported to have modest invasive capacity in Matrigel.⁴⁴ *In vivo*, depending on the xenograft model HT29 cells have been reported to remain confined in the tumor mass⁴⁴ or invade the surrounding tissue.^{44,45} All these findings indicate that the ability of HT29 cells to spread out from the primary site depend on the microenvironment in which they were seeded.

SW480 (CRC grade III) and LoVo cells (CRC grade IV) were found in the crypts and stroma of healthy-derived ECM, and over time few cells remained in the stroma. On the contrary, these cells spread in the perilesional- and CRC-derived ECM over time. These findings are in agreement with previous reports indicating an invasive phenotype of these cells in Matrigel⁴⁴ and xenograft models.^{34,46}

Our findings highlight a relevant role of the healthy ECM in constraining the spread of tumor cells with invasive capacity, as LoVo and SW480 cells. Second, CRC-derived ECM sustained the invasion of these cells, indicating that the original tissue was rearranged by tumor cells and tumor-associated cells to support the invasion of the transformed cells. Of note invasion was also observed in the perilesional-derived ECM purified from an area free of tumor cells, thus indicating a role of the tumor-associated cells in the remodeling of stroma surrounding the tumor mass. On the other hand, HT29 cells did not infiltrate healthy, perilesional, and tumor-derived ECM.

The ability of LoVo and SW480 cells, and the failure of HT29 cells to invade the tumor ECM likely reflect the different armamentarium of these epithelial cell lines, such as expression of integrins, MMPs, and proteases (i.e., the prometastatic protease uPA,⁴⁷ absent in HT29⁴⁸ cells but expressed by LoVo⁴⁹ and SW480⁵⁰ cells). On the other side, the ability of the healthy-derived ECM to constrain the spreading of cells with invasive ability highlighted the contribution of host factors versus the intrinsic capacity of neoplastic cells to invade the matrix.

Mucus and brush border expression are markers of functional and structural differentiation of epithelial cells.^{30,34} In our system the different matrices sustained cell differentiation of epithelial cells, such as the formation of glandular-like structures, expression of mucus and brush border, irrespective of the ECM on which cells were seeded. However, HT29 cells expressing mucus were evident starting from 9 days after cultivation on the ECM, but not in the 2D system. These findings highlight the capacity of ECM components sustaining the differentiation phenotype of HT29 cells. On the contrary, expression of mucus by LoVo cells was not different between the 2D system and ECM. All together these findings indicate the ability of ECM to sustain the differentiated phenotype of epithelial tumor cells, and that this feature is bypassed by cells acquiring metastatic potential.

On the other side, expression of the brush border was superimposable among the different ECMs and the 2D system, according to a previous report showing that the ECM did not play a major role in the formation of microvilli.³⁰ We here provide evidence that the ECM modulated mucus production in nonmetastatic cells, but not microvilli expression.

Most of the HT29, LoVo, and SW480 cells were in the proliferative phase after 4 days of seeding, irrespectively of the ECM to which they were seeded, similar to what was

reported for HT29 cells cultivated in 2D system.⁵¹ Decreased proliferation of seeded cells was observed at later time points, indicating the influence of the ECM on the cell cycle. Indeed, in our system the CRC-derived ECM induces high proliferation of transformed epithelial cells, and the healthy-derived ECM show a constraining effect on cell proliferation, indicating that the different ECMs induced different selective pressures on transformed cells. In parallel, we reported that the appearance of niches of apoptotic cells was also modulated by the stroma, as CRC-derived ECM (i) delayed presence of dead HT29 cells or (ii) constrained appearance of niches of dead LoVo cells.

In the last years it has become evident that transformed cells require the generation and recruitment of a microenvironment permissive for tumor growth,⁵² spreading of neoplastic cells into the vasculature and/or lymphatic system, and seeding in distant organs. All these tumorigenic steps are fine-tuned by tumor cells and host factors.⁵³ Tumor-associated cells appear to be nonmalignant in the sense of genetic mutations, although they exhibit epigenetic changes, which affect their behavior and protein expression, also modulating the composition of ECM and embedded growth factors.^{53–55}

Collectively, our system highlighted the different ability of healthy-, perilesional- and CRC-derived ECM to modulate cell homeostasis, spreading in the stroma, turnover between proliferation and death, and the functional differentiation phenotype of cancer epithelial cells.

Further studies are needed to unveil differential composition of structural and ECM-associated components^{41,42} responsible for the different cell behavior in the healthy and tumor ECM.

This article reports methodology that might also be useful to address biological questions giving the opportunity to dissect the contribution of different players of the microenvironment, by combining different cell types (e.g., tumor cells and fibroblast, with or without soluble factors) and ECM isolated from different tissues.

Nonetheless, colon-derived ECMs may likely represent the ideal scaffold for assessing the influence of microenvironment in the generation of colorectal cancer from cancer stem cells.⁵⁶

On the other hand, from the preclinical point of view this system is suitable for assessing prognostic and diagnostic marker and for drug screening. The success rate for many drugs evaluated in animal models and tested in clinical trials is very low.¹⁰ Tissue-derived ECM could contribute to reproduce human microenvironments to be used for evaluating (i) efficacy of drugs in the tumor microenvironment and (ii) side effects on cells seeded in the healthy-derived ECM. Finally, this methodology is likely to reduce use of animals, by providing a suitable 3D microenvironment that closely recapitulates the native tissues thus avoiding the excessive use of laboratory animals.

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Disclosure Statement

All authors have declared that no competing interests exist.

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