J Mammary Gland Biol Neoplasia (2010) 15:353–364
DOI 10.1007/s10911-010-9185-x

Isolation of Mammary-Specific Extracellular Matrix to Assess Acute Cell-ECM Interactions in 3D Culture

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Received: 15 July 2010 / Accepted: 25 July 2010 / Published online: 3 August 2010
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Abstract Studies of mammary epithelial cells (MECs) cultured with reconstituted basement membrane proteins derived from EHS tumors have contributed greatly to the understanding of both normal physiology and transformation. Only when plated on such biologically relevant substratum are MECs able to form morphologically correct, differentiated structures, highlighting a critical role for extracellular matrix (ECM) proteins in MEC organization and function. Here, we describe methods modified from the original EHS matrix protocol for isolating tissue-specific ECM from rat mammary glands, and for subsequent use in short-term 3D cell culture models designed to assess acute cell-ECM interactions. Using this protocol, the final matrix is enriched up to 58-fold for ECM proteins such as fibronectin and laminin, while cellular proteins such as GAPDH are reduced 98-fold. We have previously shown that MECs plated in mammary-specific ECM form more elaborate duct-like and alveolar-like structures compared to MECs plated in Matrigel™, demonstrating the biological relevance of tissue-specific ECM. Use of mammary-specific ECM in 3D cell culture models will further our ability to study the intricate interplay between a cell and its microenvironment, and permit identification of modifying factors.

Financial Support Supported by Department of Defense Idea Award #BC051532 and the Komen Foundation Grant #KG090629 to PS and Department of Defense Predoctoral Grants #BC073482 to JO and #BC093130 to JF.

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Electronic supplementary material The online version of this article (doi:10.1007/s10911-010-9185-x) contains supplementary material, which is available to authorized users.

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Keywords Extracellular matrix · ECM · 3D culture · Mammary

Abbreviations

ECM	Extracellular matrix
LN	Laminin
FN	Fibronectin
TN	Tenascin
MECs	Mammary epithelial cells
TGF-β	Transforming growth factor beta
EHS	Engelbreth-Harm-Swarm
NEM	n-ethylmaleimide
PMSF	Phenylmethylsulfonyl fluoride

Introduction

Extensive manipulations of mammary epithelial cells in 3D model systems employing extracellular matrix (ECM) proteins have vastly increased our understanding of normal and transformed breast tissue. These studies have shown that ECM proteins are required for correct acinar modeling and expression of terminal differentiation genes such as milk proteins. In total, critical roles for ECM have been identified for mammary tissue structure, function and homeostasis [1–7]. Similarly, insight into cancer progression has been obtained by studying breast tumor cells in the context of ECM. Depending on ECM protein composition, both disruption of tissue organization similar to that observed during breast cancer progression, as well as reversion of the transformed state have been observed [8–10]. Further, mammary ECM composition and function differ by developmental stage and reproductive state [11, 12]. Cumulatively, these studies indicate that mammary ECM can be used as a functional readout of stromal activity, and further, that epithelial cell organization in 3D culture provides critical information regarding in vivo stromal-epithelial interactions. These observations raise numerous hypotheses that could be investigated with respect to developmental stage, reproductive status and disease specific mammary ECM composition and function. Since ECM-epithelial interactions are finely tuned to reflect tissue specific functions, it is anticipated that method development in mammary ECM isolation and 3D culture methods can be readily translated to the study of ECM-cell interactions for other organs and diseases. In this review we highlight advances made in the field of mammary ECM-epithelial cell interactions, provide protocols for isolating mammary ECM, describe 3D culture assays optimized for investigating tissue-specific ECM-cell interaction and discuss the salient and modifiable features of these protocols.

Extracellular Matrix-Cell Interactions

Embryonic mammary mesenchyme can direct skin-derived epithelium toward mammary-specific morphogenesis and lactogenesis [13, 14], identifying an instructive role for stroma in determining mammary epithelial cell fate. ECM is a dominant component of tissue stroma, suggesting a role for ECM in driving embryonic mammary development. Further, ECM components such as collagen IV, laminin (LN), entactin, collagen I, fibronectin (FN), and tenascin (TN) show differential expression in the mammary gland from birth through stages of development [12, 15], indicating that mammary stroma evolves to facilitate changes in epithelial cell function as the gland develops. Integrins, transmembrane receptors for ECM proteins, are critically important receptors for linking the external ECM milieu to internal cell signaling pathways [6, 16, 17]. In culture, mouse mammary epithelial cells (MECs) will only proliferate in response to ovarian hormone stimulation (R5020) when cultured on FN or collagen IV, but not when cultured on LN, collagen I, or TN [18]. This interaction is thought to be dependent on the type of integrin engaged by FN and/or collagen IV and the subsequent signaling pathway activated [19]. These studies indicate important links between mammotropic hormones, mammary epithelial cell function and ECM composition, interactions anticipated in a hormone responsive tissue. ECM composition can also influence both integrin expression and new ECM protein production by cells, providing evidence of reciprocal interactions between these tissue compartments. For example, when mouse MECs are plated on a LN-1 rich substratum they produce less β1-integrin, FN, LN and collagen IV mRNA and protein than when plated on plastic [20, 21]. Such experiments demonstrate that ECM can receive, impart and integrate the variety of signals that direct functional differentiation of the mammary gland [1–4, 6, 7, 22]. These observations led Bissell and colleagues to define the functional unit of the mammary gland as the epithelial cell plus it ECM, and to propose the model of 'Dynamic Reciprocity', where continual crosstalk between stroma and epithelium dictates epithelial form and function [3, 23].

In addition to protein composition, ECM function is further defined by the proteolytic state of the individual protein components, as these fragments are often bioactive. One famous example is endostatin, a fragment of collagen XVIII that exhibits anti-angiogenic activity [24]. As the activation state of ECM proteins can be changed by proteolysis, subsequent tissue functions may be modified if physiologic cleavage is altered. This is demonstrated by mammary-specific overexpression of matrix metalloproteinase 3, an enzyme responsible for ECM protein proteolysis. In these mice there is excess branching of the ductal network and precocious alveolar development, with mam-

mary glands in virgin female mice resembling those of mid-pregnancy [25, 26]. These studies identified targeted ECM proteolysis as a requisite for branching morphogenesis and alveologenesis in the mammary gland. ECM also functions as a storage depot for many signaling molecules [27, 28], with partial proteolysis of ECM controlling the activation or local concentration of these factors. For example, interaction between latent TGF-β and latent TGF-β binding protein-1 tethers TGF-β to the ECM by binding fibrillin, collagen and fibronectin. In this manner, the ECM serves as a critical regulator of TGF-β bioavailability [29]. Through these and other functions, ECM has been demonstrated to be essential for correct mammary structure and function throughout development and adulthood.

Biomatrices and 3D Culture

Due to the instructive requirement for ECM in organizing and maintaining mammary gland function, the use of ECM has been proven invaluable for in vitro studies of normal and disease function. Epithelial cells grown in 2D on tissue culture plastic simply do not represent their counterparts in vivo. Early experiments culturing hepatocytes on floating membranes of stromal collagen revealed the benefits of ECM proteins and 3D cultures for the study of cells in vitro [30]. The 3D culture techniques allowed recovery of primary liver cells that, for the first time, differentiated into morphologically correct, functioning hepatocytes [30]. Emerman and Pitelka adapted these techniques in 1977 in the first 3D culture of primary MECs [1]. The use of floating collagen gels resulted in alveolus-like structures with apical/basal polarization, including microvilli, tight junctions at the luminal surface, and a basal lamina layer deposited between the MECs and the collagen gel. In addition, these cells were lactogenic [1]. Early in the development of ECM isolation protocols, mammary-specific acellular matrix was extracted from rat mammary glands to use for 3D culture of rat MECs. Again, the MECs in these experiments were able to produce α-lactalbumin, a marker of mammary differentiation, and secrete the milk sugar lactose [2]. Of note, the mammary cells were not able to differentiate to this stage when plated in 3D on an ECM biomatrix isolated from rat liver, suggesting tissue specificity of stromal instruction. Around this time, isolation began of a more diverse biomatrix from Engelbreth-Harm-Swarm (EHS) chondrosarcoma mouse tumors [31]. EHS matrix (commercially available as Matrigel™, BD Biosciences) is a mixture of basement membrane proteins including LN, entactin, collagen IV, and heparan sulfate proteoglycans, as well as several growth factors including epithelial growth factor and insulin-like growth factor [27]. More recently, a proteomic analysis of Matrigel™ highlights the protein complexity of this commonly used 3D culture substratum [32]. An original paper on the isolation and structure of EHS matrix displayed the range of biological activity of cells grown on this biomatrix, including melanoma, endothelial and liver cells [33]. Since then, EHS matrix has been shown to support the differentiation of many cells types, and in particular has revolutionized the experimental capacity of the field of mammary gland biology.

Contributions of EHS Matrix to the Study of Mammary Gland Biology

Using EHS as a substratum, Bissell and colleagues have shown that 3D mammary structures form a specialized basal lamina, have apical junctions, and exhibit vectorial secretion of milk proteins [4]. Importantly, these 3D assays also served to distinguish the behavior of normal MECs from cancer cells. Normal MECs organize in EHS matrix into alveolar-like structures or ‘mammospheres’ with growth regulation, lumen formation, correct polarity and deposition of endogenous basement membrane, while cancer cells do not [4, 10, 34, 35]. The robustness of these phenotypic differences observed in 3D/EHS culture has permitted the study of genes that influence proliferation, apoptosis, polarity, invasion and other tumor cell phenotypes. Functional confirmation of an oncogene can be discerned through disrupted ability to form mammospheres after retroviral infection of normal epithelial cells with the gene of interest [9]. For example, when immortalized but non-tumorigenic MCF10A cells were transfected with the oncogene ErbB2, ascinar forming capabilities were lost as observed through lumen filling, increased cell proliferation and disrupted cell polarity [36]. Conversely, renewed ability to form ascinar-like structures was observed when a β1-integrin blocking antibody was added to the 3D culture of tumorigenic mammary HMT-3522-T4-2 cells, strongly implicating ECM signaling through β1-integrin in mammary tumor progression [8]. Further, in vivo xenograft assays have been improved with the use of EHS matrix. Both tumor growth and metastasis of mammary tumors increase when carcinoma cells are coinjected with EHS matrix into the mammary fatpad of athymic mice, compared to cells injected without matrix [37–39]. Clearly, the development of EHS biomatrix has expanded experimental opportunities available for biological research in ways comparable to that permitted by the original advent of cell culture technology.

Beyond EHS Matrix

Our lab has revised the protocols for isolating EHS matrix in order to isolate tissue and developmental stage specific

matrix from rat mammary glands for use in 3D culture [12, 32]. Utilizing these matrices, we have observed that the matrix is re-organized by mammary epithelial cells in the immediate areas surrounding the cells, as well as throughout the substratum, indicating the components of the matrix function as an interconnected meshwork rather than as individual proteins [40].The biological relevance of these matrices is evident by the observations that MECs plated on mammary matrix form more elaborate duct-like and alveolar-like structures in 3D culture than when plated under identical conditions in Matrigel™ alone [12, 32]. Further, we have demonstrated that mammary ECM function changes to facilitate epithelial expansion, differentiation, and regression associated with pregnancy, lactation and postpartum involution respectively, as well as with tamoxifen treatment [11, 12, 41, 42]. These studies highlight the dynamic and modifiable nature of ECM-epithelial interactions in the mammary gland. Recent proteomic analysis has confirmed that rat mammary matrix obtained from nulliparous animals is significantly distinct in both composition and relative abundance of specific ECM proteins in comparison to Matrigel™ [32], further supporting the relevance of tissue specific ECM preparations for the study of cellular function in culture.

In an attempt to optimize acute ECM-cell interactions, we have modified the commonly used 3D culture protocols originally utilized in the Bissell lab and subsequently standardized by the Brugge lab [9, 43]. In the classic 3D culture models, mammospheres/organoids develop in situ from single cells embedded in matrix over a course of 2-3 weeks. Importantly, interactions between the proliferating epithelial cells and their EHS matrix environment influences new ECM production by the cells, with the final organoid encapsulated in a thin layer of matrix of its own origin, whose overall structure is influenced by the underlying Matrigel scaffold [9, 43]. In our assays, cells are plated at a high density onto experimental matrix (Overlay assay) or within experimental matrix (Coating assay), and the ability of the experimental matrix to directly influence organoid organization is evaluated in relatively short term assays of 2–5 days [12, 42, 44]. Using these assays, we have reported that mammary ECM function changes with reproductive state, consistent with ECM facilitating the response of MECs to changes in hormone status [12]. In brief, immortalized but non-transformed MECs organize into duct-like structures on nulliparous ECM, alveolar-like structures on pregnant ECM, experience death on postpartum involution ECM, and have restricted ductal morphogenesis on ECM isolated from fully regressed glands; thus modeling the histology of the gland throughout these stages [12]. Further, mammary ECM isolated from these distinct reproductive stages differentially promotes metastasis in xenograft assays, providing insight into crosstalk between hormone stimulation, stromal composition and risk of cancer progression [45]. In summary, these 3D culture models can determine whether ECM contains information necessary for the cells to reorganize into duct-like, alveolar-like, or transformed structures.

Isolation of Extracellular Matrix from Rat Mammary Gland

Method Overview

The following ECM isolation protocol has been developed to obtain mammary ECM from adult Sprague Dawley rats, based on the pioneering work of Kleinman et al [33]. Briefly, mammary tissue lysates are enriched for high molecular weight, insoluble proteins through a series of centrifugations and high salt and urea extractions. The final matrix is enriched up to 58-fold for ECM proteins, such as FN and LN, when compared to the starting tissue, with a concurrent 98-fold reduction in cellular proteins, such as GAPDH (Fig. 1). Of note, fibrillar collagens, including collagen 1 are underrepresented in the final matrix in comparison to other high molecular weight ECM proteins due to their highly insoluble nature [32]. Starting with 3 g of mammary tissue from nulliparous rats, we routinely recover approximately 3.5–4.5 ml of ECM, ranging in concentration from 300–700 μg/ml. In addition to variations due to inter-animal and inter-prep differences, the final concentration of the matrix is highly dependent on the developmental or reproductive stage of the animal from which the mammary tissue is harvested [12].

Protocol 1. Mammary ECM Isolation

Before beginning, prepare and autoclave the appropriate volume of Buffers 1, 2 and 3. Buffers 1 and 2 are stored at room temperature and Buffer 3 at 4°C. Buffers 1, 2 and 3 can be prepared 1-2 weeks in advance. Also, prepare fresh stock solutions of 200 mM n-ethylmaleimide (NEM) and 15 mg/ml phenylmethylsulfonyl fluoride (PMSF). These can be stored throughout the 5 day isolation procedure at 4°C and −20°C, respectively. Between removal of mammary tissue from long term storage at −80°C and the first homogenization step, tissue must be kept frozen in liquid nitrogen to maintain integrity of ECM proteins. Perform tissue pulverization in liquid nitrogen, and all subsequent steps on ice. The homogenization protocol is written for a Polytron tissue homogenizer (PT 10–35, Kinematica) with a 7 mm diameter generator with serrated teeth (PT-DA 0721). The following mammary ECM isolation protocol is written for 3 g starting tissue. The amount of starting tissue

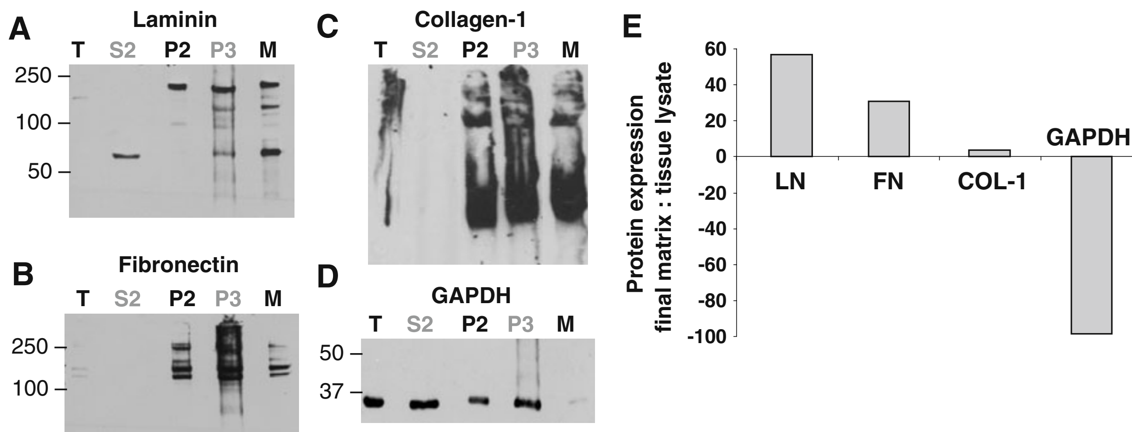

Figure 1 Enrichment for ECM proteins during matrix isolation. Western blot analyses of samples from sequential steps of the ECM isolation procedure show enrichment of high molecular weight ECM proteins laminin **a**, fibronectin **b**, and collagen-1 **c**, with concurrent reduction of soluble cellular contaminant GAPDH **d** in the final matrix, with densitometry analysis of fold changes in **e**. T=tissue lysate, S2=2nd supernatent, P2=2nd pellet, P3=3rd pellet, M=final matrix, *Gray*=discarded during isolation procedure, numerical values are in kilodaltons (kDa)

can be varied to increase or decrease the final matrix volume, however the protocol must be proportionally scaled with particular attention paid to the homogenization times and buffer volumes (Supplementary Worksheet 1).

Solutions

Buffer 1 (high salt buffer): 3.4 M NaCl, 50 mM Tris-HCl (pH 7.4), 4 mM EDTA-Na_2
Buffer 2 (urea extraction buffer): 0.2 M NaCl, 50 mM Tris-HCl (pH 7.4), 4 mM EDTA-Na_2, 2 M urea
Buffer 3 (tris dialysis buffer): 0.15 M NaCl, 50 mM Tris-HCl (pH 7.4), and 4 mM EDTA-Na_2

Day 1

1. Prior to use for ECM isolation, abdominal and inguinal rat mammary glands 4–6 with the lymph node region removed are harvested, snap-frozen in liquid nitrogen, and stored at −80°C.
2. Using a pre-chilled mortar and pestle, pulverize frozen mammary tissue to a fine powder in liquid nitrogen
3. Maintaining mammary tissue in a frozen state at all times, pool pulverized tissue from 3-4 animals to achieve a final mass of 3 g. At this point, some tissue may be removed for future biochemical analyses, and stored at −80°C.
4. Pre-chill Buffers 1 and 2 on ice.
5. Prepare solutions for high salt washes 1 and 2 by placing 2 ml of Buffer 1 per gram of tissue into two 50 ml polypropylene conicals. Add stock NEM, PMSF, and PI cocktail (Sigma, P8340) to each tube to obtain the following working concentrations: 2 mM NEM, 200 μg/ml PMSF, 2× PI cocktail. Prepare solution for urea extraction by placing 1.8 ml of Buffer 2 per gram of tissue into 50 ml polypropylene conical. Add stock NEM, PMSF, and PI cocktail to achieve the following working concentrations: 2 mM NEM, 100 μg/ml PMSF, 2× PI cocktail. Keep solutions on ice. These buffers will now be referred to as Buffer 1B and 2B, respectively.

High Salt Wash 1

6. Add approximately 1/3 of the starting tissue to the 50 ml conical containing Buffer 1B solution for high salt wash 1, as prepared in Step 5. Homogenize tissue on ice for 8 s on speed 3 using Polytron tissue homogenizer. Add ½ the remaining tissue, and homogenize for an additional 8 s on ice. Add remainder of starting tissue, and perform a final homogenization on ice of 2×8 s, for a total homogenization time of 4×8 s. Allow 5–10 s between homogenization steps to prevent tissue from warming.
7. Transfer homogenized tissue to polyallomer centrifuge tubes. Centrifuge at 100,000×*g* for 30 min at 4°C. Discard top layer of lipid and supernatant.

High Salt Wash 2

8. Add ½ the volume of Buffer 1B solution for high salt wash 2 (prepared in Step 5) to centrifuge tube containing ECM-enriched pellet. Homogenize tissue on ice for 5 s on speed 3 using Polytron. Transfer homogenized tissue back into conical containing the remainder of Buffer 1B.
9. Homogenize on ice for 5 s on speed 3.

10. Transfer homogenized tissue to polyallomer centrifugation tubes. Centrifuge at 100,000×*g* for 30 min at 4°C. Discard top layer of lipid and supernatant.

Urea Extraction

11. Add ½ the volume of Buffer 2B (prepared in Step 5) to the centrifuge tube containing the ECM-enriched pellet. Homogenize on ice for 5 s on speed 3. Transfer homogenized tissue into conical containing the remainder of the Buffer 2B.
12. Homogenize on ice for 5 s on speed 3.
13. Transfer homogenized tissue to a 75 cm^2 tissue culture-treated polystyrene flask with non-ventilated cap. Shake on orbital shaker overnight at 4°C.

Preparation of Dialysis Tubing

Note: Preparation of dialysis tubing and all subsequent steps should be carried out under sterile conditions in a laminar flow hood.

14. Using sterile scissors, cut regenerated cellulose dialysis tubing (MWCO 12–14 kDa, Spectrum Laboratories) based on manufacturer's recommended length per sample volume, plus 5–10 cm extra. Submerge in 100 ml Buffer 3 containing 25 μg/ml gentamicin. Cover and store at 4°C overnight (12–18 h).

Day 2

15. Remove ECM-enriched sample from orbital shaker and transfer to a sterile centrifuge tube. Use a cell scraper to collect as much sample from the flask as possible. Both the liquid and particulate component of the sample should be transferred to the centrifuge tube.
16. Centrifuge at 26,000×*g* for 1 h at 4°C. The supernatant from this step will be retained for subsequent dialysis, whereas the pellet will be discarded.

Dialysis 1

17. After centrifugation, prepare the solution for the first dialysis. Place 70 ml of Buffer 3 per gram of starting tissue into an Erlenmyer flask. Add NEM and PMSF to achieve the following working concentrations: 2 mM NEM, 100 μg/ml PMSF. This will now be referred to as Buffer 3B.
18. Remove prepared dialysis tubing from 4°C. Using sterile technique, tie a knot in one end of the dialysis tubing, as near the end as possible. Place a dialysis tubing clip inside of the knot.
19. Transfer the supernatant from Step 16 into the dialysis bag. Clamp the dialysis tubing closed using a second dialysis tubing clip. Tie a knot in the tubing outside of the second clip.
20. Place dialysis bag in the flask containing Buffer 3B solution prepared in Step 17. Add a sterile stir bar. Be sure dialysis bag does not touch the inside of the flask to assure adequate liquid and solute exchange. Cover flask tightly with Parafilm® (Pechiney Plastic Packaging Company). Dialyze at 4°C overnight (~24 h) with gentle stirring.

Day 3

Dialyses 2 and 3

21. After dialysis 1, discard Buffer 3B solution and replace with 70 ml of Buffer 3 per gram of starting tissue containing 100 μg/ml PMSF and 30 μg/ml gentamicin (Buffer 3C). Place at 4°C with gentle stirring for ~10 h.
22. After ~10 h, replace dialysis solution with 70 ml of Buffer 3 per gram of starting tissue containing 30 μg/ml gentamicin (Buffer 3D). Dialyze overnight (~14 h) at 4°C with gentle stirring.

Day 4

Dialysis 4

23. After 48 h of total dialysis against Buffer 3 solutions, replace Buffer 3 dialysis solution with 40 ml sera-free culture medium per gram of starting tissue containing 1 μg/ml gentamicin. Dialyze overnight (~24 h) at 4°C with gentle stirring. If sample is to be used in proteomic analysis, it must be removed prior to dialysis against culture medium.

Day 5

24. Following dialysis, transfer extracellular matrix preparation to a polypropylene tube, and store on ice at 4°C until use. Fresh matrix should be used within 2 weeks for cell culture work, and can be frozen at −20°C, without evidence of degradation, for biochemical analyses (data not shown). The consistency of the final matrix should be that of a viscous liquid, with no precipitate present. Note that unlike commercially available matrices, the final matrix does not solidify at 37°C. Matrigel™ may be used to facilitate gelation of mammary matrix. It is important to batch test Matrigel™

for this purpose, and reserve sufficient quantities of a particular Matrigel™ lot for subsequent 3D studies.

Short-Term Three-Dimensional Cell Culture Assays

Method Overview

We have developed two assays for studying acute ECM-cell interactions in culture. In the Overlay assay, cells are suspended in culture medium and plated (overlaid) at high density onto a substratum containing experimental rat mammary matrix co-polymerized with Matrigel™ (Fig. 2a, top). Matrigel™ is utilized to facilitate gel formation of mammary matrix, which solidifies poorly in its absence, as described above. Standard Matrigel™ is used in our studies; however, growth-factor reduced and high concentration options are also available. In the Coating assay, cells are first coated in experimental mammary matrix and then plated on a diluted Matrigel™ substratum (Fig. 2a, bottom). Stage-specific mammary ECM differences have been observed within both Overlay and Coating assays [[12] and data not shown]. Additionally, the structuring of cells within the same ECM preparation differs between assays. For example, mammary cells plated on ECM from nulliparous mammary glands in the Overlay assay form large, highly organized structures, indicative of cell clustering due to increased motility (Fig. 2b). In contrast, in the Coating assay, mammary cells plated in the same nulliparous ECM form smaller structures indicative of decreased motility (Fig. 2b). These differences between assays may result from increased 2-dimensional cell movement on tracts of long ECM fibers present in nulliparous ECM in Overlay assays verses impediment of 3-dimensional cell movement when the cells are surrounded by long ECM fibers in the Coating assay (Fig. 2c).

Protocol 2-1. Short-Term Overlay Method

The optimal cell number to use in these assays is cell- and assay-specific, and will need to be determined for each cell type studied. For example, using 96-well plates, the appropriate number of non-transformed human mammary MCF12A cells has been determined to be 45,000 cells/well for the Overlay assay, and 22,500 cells/well for the Coating assay. When more motile Ras-transformed MCF12A cells are used, less cells are required for optimal organization, and 30,000 cells/well and 15,000 cells/well are plated in the Overlay and Coating assays, respectively. We routinely use our experimental matrices at 200 µg/ml, however, the concentration of matrix used may be empirically determined based on the experimental question and design.

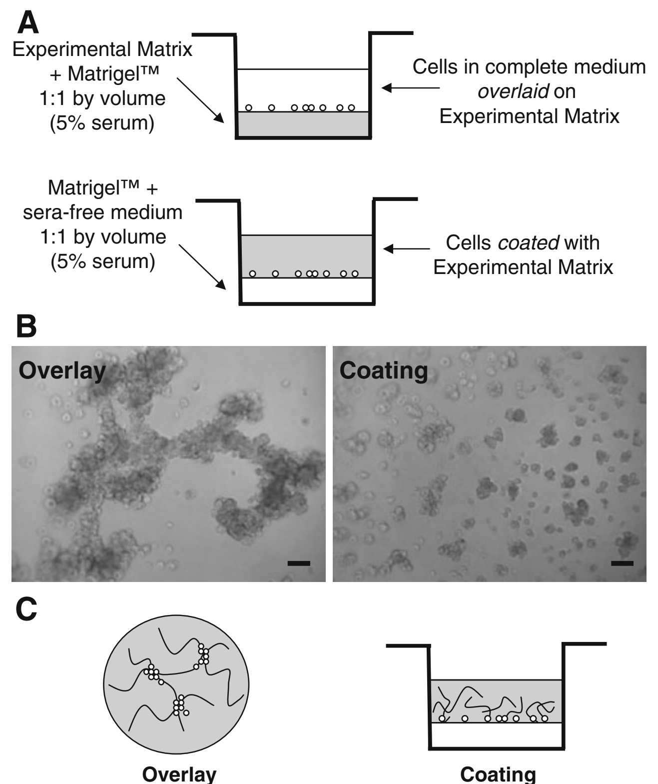

Figure 2 3D culture assays for study of acute cell-ECM interactions. **a** Overlay assay (*top*)—cells are plated in complete medium and *overlaid* onto an experimental matrix/Matrigel substratum. Coating assay (*bottom*)—cells are *coated* in experimental matrix and plated onto diluted Matrigel substratum. *Gray* = experimental matrix; *White* = culture medium (Overlay) or Matrigel™ (Coating) **b** Human immortalized, but non-transformed mammary epithelial MCF12A cells form larger, more complex organoids in the Overlay assay when compared to the Coating assay (24 h, nulliparous matrix). This difference in structuring is thought to be due to facilitation of cell movement by tracks of ECM fibers in the Overlay assay, and impediment of cell movement by surrounding intact ECM fibers in the Coating assay **c**. Scale bar=100 µm

Day 1

1. In a 96-well format, prepare ECM pads by pipetting 100 µl/well of a 1:1 dilution of 200 µg/ml experimental matrix:Matrigel™ plus 5% serum. Experimental matrix is diluted to 200 µg/ml in serum-free medium. Matrigel™ is undiluted. Serum and serum-free medium is cell-type specific.
2. Allow pads to solidify overnight at 37°C in a cell culture incubator.

Day 2

3. Harvest cells and rinse once in PBS. Resuspend the appropriate number of cells in 200 µl/well complete medium.

4. On top of each matrix pad, plate 200 μl/well of cell suspension.
5. Place plate in incubator at 37°C. Monitor every 12–24 h, for 24–72 h. Note: The initial interactions between cells and matrix are fragile, and it is recommended that cultures not be evaluated for at least 8 h after plating to avoid disrupting these interactions.

Protocol 2-2. Short-Term Coating Method

Day 1

1. In a 96-well format, prepare pads by pipetting 100 μl/well of a 1:1 dilution of serum-free medium:Matrigel™ plus 5% serum. Serum and serum-free medium is cell-type specific.
2. Allow pads to solidify overnight at 37°C in a cell culture incubator.

Day 2

3. Harvest cells and rinse once in PBS. Resuspend the appropriate number of cells in 200 μl/well of 200 μg/ml experimental matrix. Matrix should be diluted in serum-free medium appropriate for the cell type used.
4. Place plate in incubator at 37°C. Monitor every 12–24 h, for 24–72 h.

Protocol 2-3. Fixing Cells for Histological Analysis (Overlay and Coating Assays)

1. Without removing media (overlay) or matrix (coating), add 3-4 drops of 1% trypan blue (in PBS) to each well. Let sit for 10 min.
2. Gently remove trypan blue/media/matrix from well using a Pasteur pipette. Do not use vacuum aspiration, as it will disrupt cells and/or pad.
3. Slowly add methacarn (60% methanol, 30% chloroform, 10% glacial acetic acid) drop-wise to sufficiently cover each pad. Let sit for 5 min.
4. Gently remove methacarn with Pasteur pipette.
5. To further solidify pads, fill each well with 70% ethanol, wrap with Parafilm®, and let sit for at least 24 h.
6. Harvest pads from well for histological analysis. Gently dissociate pad from side of well using a dissection probe, and place pad in a tissue cassette between two biopsy sponges. Cassettes containing pads may be stored in 70% ethanol until time of processing. Process pads using a standard mouse tissue processing protocol, embed in paraffin and section at 4 μm.

Steps in Matrix Prep that Can Influence Composition and Function of Extracted Matrix

To achieve maximal recovery of intact ECM proteins, while minimizing the amount of cellular product present in the final matrix, variables at each step of the ECM isolation protocol have been optimized. The ratio of starting tissue to buffer volume is critical in determining both final protein concentration and composition of the matrix. Efforts to improve protein yield by doubling the amount of starting tissue while keeping the current buffer volumes constant resulted in a 2-fold increase in total protein (Table 1), with no improvement in ECM enrichment (data not shown). In an additional attempt to further increase the final matrix concentration, a longer centrifugation time of 2.5 h was tested, though this resulted in an undesirable increase in fibronectin degradation when compared to the current centrifugation time of 30 min (data not shown). Protein

Table 1 Parameters affecting protein concentration of the final ECM

Condition	Stage of gland	Protein concentration (μg/ml)
Current isolation method (regenerated cellulose dialysis tubing, spins 1 and 2 at 100,000×*g* for 30 min.)	Nulliparous	300–700
Current isolation method (regenerated cellulose dialysis tubing, spins 1 and 2 at 100,000×*g* for 30 min.)	Involution	1,749[a]
2× tissue:buffer	Nulliparous	1,271[b]
Spins 1 and 2 for 2.5 h at 100,000×*g*	Nulliparous	1,059
Cellulose ester dialysis tubing	Involution	833[a]
Spins 1 and 2 at 100,000×*g*	Nulliparous	431
Spins 1 and 2 at 10,000×*g*	Nulliparous	293

[a] Empirically, mammary ECM isolated from involution-stage mammary glands is more concentrated than matrix isolated from nulliparous-stage

[b] ECM protein concentration for corresponding 1× tissue:buffer preparation is 651 μg/ml

yield of the final matrix was also affected by the composition of the dialysis membrane. Higher protein concentrations were achieved through dialysis with regenerated cellulose membrane when compared to cellulose ester tubing (Table 1). In addition to trying to increase the overall protein concentration of the final matrix, a lower speed centrifugation of 10,000×*g* was compared to the current speed of 100,000 × *g* in an attempt to decrease the amount of cellular protein present in the final product. Reducing the speed to 10,000 × *g* did not decrease cellular contaminants, but did decrease total protein concentration of final matrix (Table 1), indicating the current speed of 100,000 × *g* is preferred for achieving a high ECM protein concentration without an excessive increase in the cellular component.

The ECM isolation protocol has also been optimized for maximum performance of cells in our short-term assays. A number of different Polytron generators and homogenization times have been tested to determine which conditions minimize fragmentation of ECM proteins and produce a matrix that robustly supports cellular organization in 3D culture. Homogenization efficiency can be improved using Polytron generators with serrated teeth, however to determine if these teeth promote non-biologic fragmentation of extracellular matrix proteins, generators with and without teeth were compared directly. By western blot, the integrity of ECM proteins fibronectin and laminin were equivalent between both generator types (data not shown), though cells in 3D culture formed more complex structures in Overlay assays when plated onto matrix isolated using the generator with teeth (Fig. 3a). To determine if increased cellular organization resulted from increased homogenization, different size Polytron generators and homogenization times were analyzed. Increased homogenization using a larger diameter generator and greater length of time than the current protocol led to an increase in the contaminating cellular component of the final matrix, as well as decreased organoid formation (Fig. 3b and c). Homogenizing for a shorter period of time similarly decreased cell structuring (data not shown). These data indicate that over- or under-homogenization has adverse effects on how cells organize on/in the final matrix, possibly due to changes in the viscoelastic properties of the ECM. The dialysis steps were also modified to result in ECM more supportive of organoid formation. The original ECM isolation protocols upon which this tissue-specific protocol is based include NEM in all three dialyses [5, 33]; however, limiting NEM to only the first dialysis greatly enhanced cell performance and subsequent cellular organization (Fig. 3d).

The concentration of the experimental matrix and the architecture of the pad in 3D cell culture assays can significantly impact organoid formation. To determine the ideal amount of experimental matrix to use in our short-term assays, matrix concentrations of 100, 200 and 600 μg/ml were utilized. Both 100 and 600 μg/ml led to decreased organization of MCF12A cells in Overlay assays (Fig. 4a) when compared to 200 μg/ml, highlighting the influence of matrix concentration on organization of cells. Organoid formation in these assays is also affected by the location of

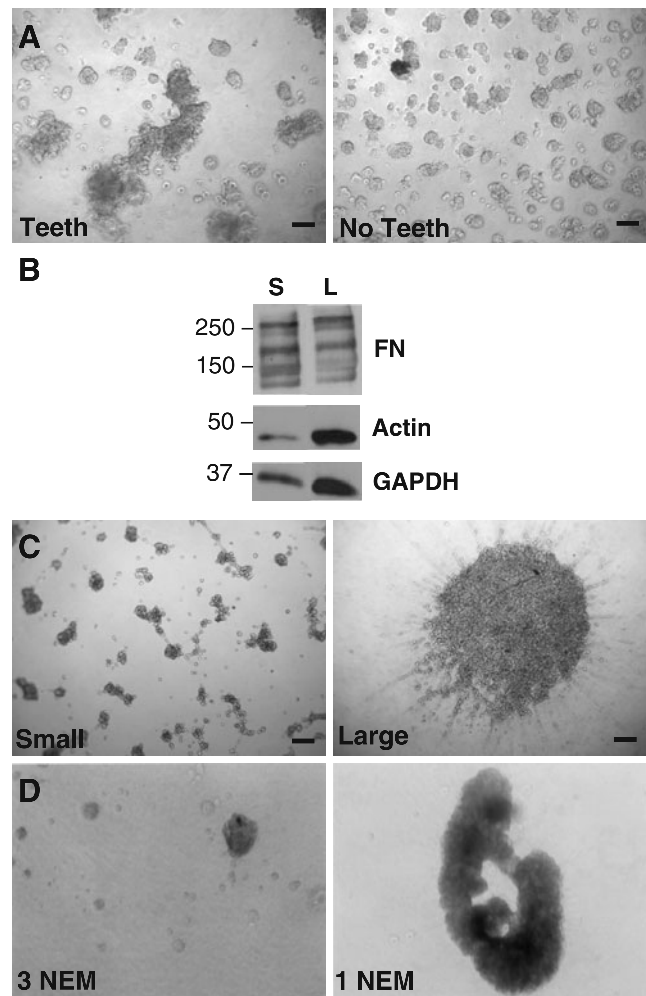

Figure 3 Optimization of ECM isolation procedure. **a** Non-transformed MCF12A epithelial cells plated in nulliparous ECM in an Overlay assay display more complex structuring on ECM homogenized using a Polytron generator (Kinematica PTA-7EC) with serrated teeth (*left*) compared to no teeth (*right*). Scale bar=100 μm, 24 h. **b** Western blot analyses reveal that although comparable levels of ECM protein fibronectin (FN) are observed, increased homogenization time using larger generator PTA-10EC (L) results in increased contamination of cellular proteins actin and GAPDH in the final matrix compared to standard homogenization times with smaller generator PTA-7EC (S). Numerical values in kilodaltons. **c** Ras-transformed MCF12A cells plated in nulliparous ECM in a Coating assay display decreased cellular organization on ECM homogenized using larger generator PTA-10EC compared to standard homogenization using smaller generator PTA-7EC. Scale bar=200 μm, 60 h. **d** FSK3 murine mammary epithelial cells plated in nulliparous ECM in an Overlay assay do not survive and display decreased cellular organization when NEM is included in all three dialysis steps (*left*) compared to NEM only in the first dialysis step (*right*). Scale bar=100 μm, 72 h

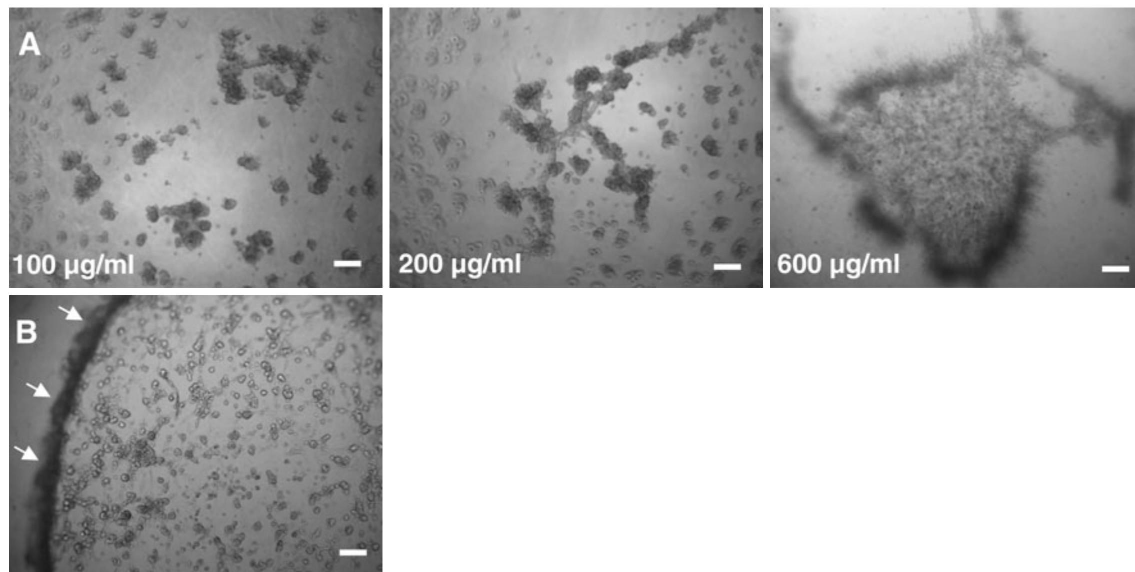

Figure 4 Optimization of acute ECM-cell interactions in 3D culture. **a** MCF12A epithelial cells plated in nulliparous mammary ECM in an Overlay assay display decreased cellular organization in 100 or 600 μg/ml ECM compared to 200 μg/ml ECM. Scalebar=200 μm, 48 h. **b** Clustering of cells along the edge of the well (*arrows*) can occur in 3D culture, as seen with MCF12A cells plated in nulliparous ECM in Coating assay. Scalebar=200 μm, 60 h

the cells within the well, and should be kept in mind when using 3D cell culture as a readout of ECM function (Fig. 4b). Concavity of the pad leads to differences in physical properties of the ECM across the pad, and is thought to contribute to varied structuring of cells across the well. One potential method for increasing uniformity of the substratum surface is to follow a two-step approach for plating the pad, in which ½ of the matrix is added to the center of the well and allowed to solidify before the addition of the second ½ of the matrix.

Discussion

The protocols described here for the isolation and utilization of mammary specific ECM permit investigation of important and fundamental questions regarding cell-ECM interactions. These include potential differences in ECM function between species and implications for current 3D culture models used for the study of human breast cancer, the role of ECM in breast density, cancer risk and progression, the influence of oncogenes and tumor suppressors, as well as the effects of lifestyle choices on ECM composition and function, such as exercise and diet. Though the described ECM isolation protocol has been optimized for mammary tissue, and subsequent use in 3D epithelial cell culture, the procedure may be adapted for different organs and cell types.

The isolation protocol may also be amended for potential downstream applications. For example, high molecular weight fibrillar collagens are relatively insoluble in high salt and urea [32]. Thus, fibrillar collagens are underrepresented in the final matrix and over represented in the collagen-rich pellet discarded after the 3rd centrifugation step in the isolation procedure (Step 19, Fig. 1c—P3). Because collagen is a major component of mammary ECM, attempts have been made to include the insoluble collagen-rich pellet in the final matrix to further recapitulate the viscoelastic and mechanosignaling properties of the in vivo environment in 3D culture. However, addition of the collagen pellet can interfere with live cell imaging (data not shown). Depending on the question and application, it may be advantageous to include this pellet in the final matrix.

In addition to the protocols described above for short-term assays, we have used rat mammary ECM in longer-term assays using a transwell 3D cell culture model developed by the Sonnenschein lab [46]. Further, cells can also be isolated from both short- and long-term assays and analyzed biochemically to elucidate pathways important in ECM-cell interactions. Also, mammary ECM can be utilized as a chemoattractant for motility, invasion and haptotaxis assays [11, 42, 45]. Each assay will likely require specific optimization in both the ECM isolation protocol and the cell-based assays.

ECM matrix obtained from whole tissues, regardless of tissue source, is enriched for ECM, and we show here a 20- to 60-fold increase in ECM proteins concurrent with a 90-fold decrease in soluble cellular proteins (Fig. 1e). Nonetheless, these ECM-enriched preparations are still highly complex mixtures of ECM proteins, matrix-associated growth factors and proteases, and cellular contaminants. In general, the

more soluble the cellular component is in high salt buffer, the more efficient its removal during the matrix isolation procedure. A recent proteomic analysis of Matrigel™ identified over 300 proteins in this widely used matrix [32]. Of the top 25 proteins identified in Matrigel™, only eight are in the category of ECM [32]. In the same report, proteomic analysis of nulliparous rat mammary matrix revealed differences in both individual ECM protein content and relative ratios between mammary matrix and Matrigel™ [32]. These data point to the complexity of isolated matrices as well as to the relevance of tissue-specific analyses. Another consideration is whether to evaluate isolated ECM matrices in the presence or absence of added growth factors. Our lab assesses the function of tissue-, stage- and disease-specific matrices in the presence of associated growth factors to more fully replicate the in vivo environment. The complex interplay between ECM and numerous signaling pathways demonstrated in the mammary gland [47] may not be recapitulated in an environment of growth factor reduction, though a growth factor reduced environment may aid in isolating the function of individual ECM proteins or preparations.

We have provided protocols for isolating tissue-specific matrix and for in vitro applications to model acute ECM-cell interactions. It is evident that the isolation of biologically-relevant ECM vastly expands our ability to study the intricate interplay between a cell and its microenvironment. The field of tissue-specific ECM function is in its infancy, and our objective here is to provide tools to permit expansion of this critically important area of research.

Acknowledgements We would like to thank Brittany Allen-Petersen and Jodie Fleming, PhD for critical review of the manuscript, and Shauntae McDaniel for her extensive contributions to protocol development.

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