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Stromagenesis during tumorigenesis: characterization of tumor-associated fibroblasts and stroma-derived 3D matrices

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Abstract

It is increasingly recognized that interactions between cancer cells and their surrounding stroma are critical for promoting the growth and invasiveness of tumors. For example, cancer cells alter the topography and molecular composition of stromal extracellular matrix by increasing paracrine regulation of fibroblastic stromal cells during early tumor development. In turn, these physical and biochemical alterations of the stroma, profoundly affect the properties of the cancer cells. However, little is known about the cross-talk between stroma and cancer cells and it is mainly due to the lack of a suitable *in vitro* system to mimic the stroma *in vivo*. We present an *in vivo*-like 3-D stromal system derived from fibroblasts harvested from tissue samples representing various stages of stroma progression during tumorigenesis. The chapter describes how to isolate and characterize fibroblasts from a plethora of tissue samples. It describes how to produce and characterize fibroblast-derived 3-D matrices. Finally, it describes how to test matrix permissiveness by analyzing the morphology of cancer cells cultured within various 3-D matrices.

Keywords

Extracellular matrix; tumor (or cancer)-associated fibroblasts; fibroblast-derived 3-D matrix; cell morphology; tumor stroma

1. Introduction

One of the fundamental differences between transformed and normal cells is the manner in which they interact with their immediate environment. Benign epithelial tumors are constrained by a surrounding stroma (1,2) consisting, in part, of fibroblastic cells and fibrillar extracellular matrix (ECM). This normal stroma inhibits or contains tumorigenicity (3,4). However, at a critical point in transformation, tumors overcome this stromal barrier, inducing changes that promote rather than impede tumor progression (1,5–13). Changes in the stroma accompanying tumor progression include appearance of discontinuities in the basement membrane surrounding the growing tumor, several immune responses, and the formation of new blood vessels (angiogenesis). Among these host responses are additional alterations to the mesenchymal connective tissue in the vicinity of the tumor (14). Mesenchymal alterations, known as ‘stromagenesis,’ occur in parallel to tumorigenesis and resemble tissue responses during wound healing or fibrosis (12,14–19). Quiescent fibroblasts (also known as stellate cells in pancreatic and hepatic stromas (20,21)) are the predominant cell type within a ‘normal stroma’, and secrete an ECM that is believed to provide a natural barrier that constrains tumor progression (1,16,22–25). In contrast, the ECM produced by a primed stroma either genetically or epigenetically modified, can provoke, stimulate, and support (instead of constraining) tumor progression (5,8,12,25–29). The primed fibroblasts engage in paracrine and autocrine feedback

signaling with the developing tumor cells (11,30), causing the eventual loss of normal tissue homeostasis (11). In the course of this parallel progression, differentiated myofibroblastic stromal cells, now termed activated cancer- or tumor-associated fibroblasts (TAFs) begin to express a set of proteins including collagen-I, fibronectin (15,31), desmin, α -smooth muscle actin (α -SMA) (16,32), and others, grossly altering the protein constituents and architecture of the ECM. During this later ‘activated stroma’ phase or desmoplasia, the tumor becomes invasive and metastatic (33,34).

As result of the interactions between stroma and cancer cells, the cancer cells modify their morphology and, thus, their migratory mechanism (35–37). Examples of these modifications include, among others, epithelial to mesenchymal and epithelial to amoeboid transitions (35, 38). The cancer cells that present amoeboid morphology present a ‘lymphocytic’ type of movement that is driven by weak interactions with the ECM, it is independent of proteases and is controlled by the small GTPase RhoA and its effector ROCK to generate cortical tension, stiffness and the maintenance of round cell morphology (36,39). However, cells that undergo epithelial to mesenchymal transition present mesenchymal or spindle morphology and migrate guided by the matrix fibers or strands. The migration of cells with a mesenchymal morphology is dependent on integrin mediated adhesion and ECM degradation by proteases (40).

In this chapter, we describe protocols to isolate stromagenic fibroblasts from various tissue samples and to obtain three-dimensional (3-D) matrices derived from these fibroblasts. The chapter includes methods for characterizing both fibroblasts and their derived matrices in order to sort them as normal, primed or activated, also known as desmoplastic (tumor-associated). The last part of the chapter is dedicated to the analysis of the morphology that is acquired by cancer cells when cultured within the various fibroblast-derived 3-D matrices.

2. Materials

NOTE: All solutions and equipment coming into contact with tissue samples or living cells must be sterile. Therefore, aseptic techniques should be used accordingly.

2.1. Isolation of Fibroblasts from Normal and/or Tumor Tissue Samples

2.1.1. General equipment

- Cell culture hood (e.g., Thermo Scientific).
- Scalpel.
- Scissors.
- Fine-pointed forceps (e.g., Dumont 4).
- Tissue Culture Incubator: 37°C, 5–10% (v/v) humidified CO₂ incubator.
- T-75 tissue culture flask (at least three for every time that the isolated cells are subcultured, e.g. Nunc).
- Tissue culture inverted microscope.
- Tissue culture centrifuge.
- 0.22- μ m stericup PES filter units (e.g, Millipore).

2.1.2. General Reagents

- PBS: Add 8 g of NaCl, 0.2 g KCl, 1.44 g of Na₂HPO₄, and 0.25 g of KH₂PO₄ to a final volume of 1L of distilled H₂O and dissolve. Adjust pH using 1M HCl and/or 1M NaOH until obtaining a stable pH of 7.4. Filter through a 0.22- μ m stericup filter

(see above) following manufacturer's instructions. Store at room temperature and verify the lack of phosphate precipitates prior to usage.

- Penicillin/Streptomycin stock solution of (e.g., Mediatech, Inc.): 10,000 U/ml Penicillin and 10,000 µg/ml Streptomycin.
- Trypsin- EDTA (ethylene diamine tetra-acetic acid) solution ((This solution can be purchased ready and sterile) e.g., Mediatech, Inc.): 2.5 g of trypsin, 0.2 g EDTA, 8 g NaCl, 0.4 g of KCl, 1g of glucose, 0.35 g of NaHCO₃, and 0.01 g phenol red dissolved into H₂O to a final volume of a 1L. Sterilize solution by filtration through a 0.22-µm stericup filter (see above) and store up to 3 months at -20°C.
- DMEM: High-glucose Dulbecco's modified Eagle medium (e.g., Mediatech, Inc.).
- Fibroblast medium: DMEM (see above) supplemented with 10% Fetal Bovine Serum (FBS) (Hyclone), 100 units/ml penicillin, and 100 µg/ml of streptomycin (Mediatech, Inc.). Store at 4°C for up to 1 month.
- Cell-freezing medium: Fetal Bovine Serum (FBS) containing 10% Dimethyl Sulfoxide (DMSO, Sigma-Aldrich). Store at 4°C for up to 1 month.

2.1.3. Isolation of Fibroblasts from Freshly Minced Normal and/or Tumor Tissue Samples

- 12-well tissue culture plates (one plate for each tissue sample). (e.g., BD Falcon).
- PBS pen/strep: PBS (see above) containing 100 U/ml Penicillin (Mediatech, Inc.) and 100 µg/ml Streptomycin (Mediatech, Inc.). Store at 4°C for up to 1 month.
- 60-mm tissue culture dishes. (two for each tissue sample) (e.g., Corning).
- Fungizone (Mediatech, Inc.): Stock concentration 250 µg/ml.
- Ciprofloxacin or Cipro (Mediatech, Inc): Stock concentration 10mg/L.

2.1.4. Isolation of Fibroblasts from Collagenase-Digested Normal and/or Tumor Tissue Samples

- 10-cm tissue culture dishes (three for each tissue sample) (e.g., Fisher)
- DMEM pen/strep: DMEM containing 100 U/ml Penicillin and 100 µg/ml Streptomycin. Store at 4°C up to 1 month.
- BSA (e.g., Fraction V, Sigma-Aldrich).
- DMEM-3%BSA-pen/strep: add 3g of BSA (see above) to 100 ml DMEM, warm it up to 37°C in order to dissolve, let it cool to room temperature, filter through a 0.22-µm stericup filter (see above), following manufacture's instructions, and add 100 U/ml penicillin and 100 µg/ml streptomycin. Store at 4°C up to 1 week.
- 0.2-µm syringe filter (e.g., Whatman).
- Collagenase-3 (Worthington) (10X): add 150 mg of collagenase-3 to 10 ml DMEM (serum free). Filter solution through a 0.2-µm syringe filter (see above) following manufacture's instructions. Store at 4°C up to 1 week.
- 150 ml sterile Erlenmeyer (one for each tissue sample).
- Orbital agitator.
- 50 ml polypropylene tubes (e.g., Corning).

- Nylon mesh: 500 μm (Sefar America Inc.) nylon mesh and cell strainers of 100 μm and 40 μm (BD Falcon). Before using the 500 μm nylon mesh, it should be sterilized by soaking the membrane on 100% ethanol and evaporate/dry inside cell-culture hood.

2.2. Characterization of Isolated Fibroblasts

2.2.1. Lysis of Isolated Cells

- Cell culture hood (e.g., Thermo Scientific).
- Tissue Culture Incubator: 37°C, 5–10% (v/v) humidified CO₂ incubator.
- 0.22- μm stericup- PES filter units (e.g., Millipore).
- Trypsin- EDTA (ethylene diamine tetra-acetic acid) solution ((This solution can be purchased ready and sterile) e.g., Mediatech, Inc.): 2.5 g of trypsin, 0.2 g EDTA, 8 g NaCl, 0.4 g of KCl, 1g of glucose, 0.35 g of NaHCO₃, and 0.01 g phenol red dissolved into H₂O to a final volume of a 1L. Sterilize solution by filtration through a 0.22- μm stericup filter (see above) and store up to 3 months at –20°C.
- Inverted microscope.
- Fibroblast medium: DMEM (see above) supplemented with 10% Fetal Bovine Serum (FBS) (e.g., Hyclone), 100 units/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ of streptomycin (e.g., Mediatech, Inc.). Store at 4°C for up to 1 month.
- Hematocytometer.
- 35-mm tissue culture dishes (e.g., Corning).
- PBS: Add 8 g of NaCl, 0.2 g KCl, 1.44 g of Na₂HPO₄, and 0.25 g of KH₂PO₄ to a final volume of 1L of distilled H₂O and dissolve. Adjust pH using 1M HCl and/or 1M NaOH until obtaining a stable pH of 7.4. Store at room temperature and verify the lack of phosphate precipitates prior to use.
- RIPA Buffer: 5 ml of 1 M Tris (pH 8.0), 1 ml of 5M NaCl, 1g Deoxycholate salt, 0.2 g NaF, 1 ml of Glycerol and 1 ml of Triton X-100 add distilled H₂O to attain final volume of 100 ml. Store at 4°C.
- 1.5 ml microcentrifuge tubes (e.g. Eppendorf).
- Cell scraper (e.g., BD Falcon).
- Sonicator equipped with a small enough probe to fit in 1.5 ml microcentrifuge tubes (e.g., Branson).
- Refrigerated microcentrifuge.
- Isopropanol (e.g., Sigma-Aldrich).

2.2.2. Western blotting to test the expression of specific Cell Markers

- β -mercaptoethanol (e.g., Sigma-Aldrich).
- 5X SDS Sample Buffer: 3.125 ml of 1 M Tris-Cl (pH 6.8), 0.3 g of EDTA (100 mM in 5X), 1.0 g of SDS, 5 ml of Glycerol and 0.05 g of Bromphenol Blue dissolve in distilled H₂O to a final volume of 10 ml. Store at 4°C without reducing agents. To effectively reduce disulfides, add 10% β -mercaptoethanol to the 5X stock just prior to use.
- Pre-cast 8–16% gradient Tris-glycine gel (e.g., Invitrogen).

- Pre-labeled molecular weight marker (e.g., Invitrogen).
- 10X Running Buffer: Add 144 g of Glycine, 30 g of Tris and 10 g of SDS to a final volume of 1L using distilled H₂O. Store at room temperature. To make 1X solution, add 100 ml to 900 ml ddH₂O.
- 1X Transfer Buffer: Add 14.4g of Glycine, 3.0g of Tris and 200 ml of Methanol to a final volume of 1L using distilled H₂O. Store at 4°C up to 1 month.
- PVDF membranes (two membranes per cell type) (e.g., Millipore).
- Methanol (e.g., Sigma-aldrich).
- Ponceau's Solution (e.g., Sigma-Aldrich).
- TBS: Add 100ml of 1M Tris and 60 ml of 5M NaCl to 840 ml of distilled H₂O (final concentration of 10 mM Tris-HCl (pH 8.0) and 150 mM NaCl). Store at 4°C and verify the lack of phosphate precipitates prior to use.
- TBST: TBS (see above) containing a final concentration of 0.05% Tween-20 (Polyoxyethylenesorbitanmonolaurate). Store at 4°C and verify the lack of phosphate precipitates prior to use.
- Blocking Solution: TBS (see above) containing a final concentration of 5% BSA (e.g., Sigma-Aldrich). Dissolve completely the BSA using a magnetic bar and stirrer. Store at 4°C up to 1 month, confirm the lack of precipitates prior to use.
- Primary antibodies: mouse anti-vimentin (Sigma-Aldrich, #V-5255), mouse anti-pan-keratin (Abcam, #Ab8068), mouse anti- Glutaraldehyde-3-Phosphate Dehydrogenase (GADPH) (Chemicon, #MAB374).
- Incubation Solution: TBST containing a final concentration of 3% BSA. Dissolve completely the BSA using a magnetic bar and stirrer. Store at 4°C up to 1 month, confirm the lack of precipitates prior to use.
- Secondary antibody: peroxidase-conjugated goat anti-mouse (Sigma-Aldrich, # A4416).
- ECL Detection Kit: ECL plus Western Blotting Detection System (Amersham # RPN2132).
- Heat sealable polyester pouches (e.g., Kapak).
- Heat plastic-pouch sealer (e.g., Kapak)
- Plastic wrap (e.g., Saran).
- Autoradiography film (Kodak).
- X-ray film processor (Kodak).

2.3. Production of Fibroblast-Derived 3-D Matrices

2.3.1. Preparation of Fibroblast-Derived 3-D Matrices

- Cell culture hood (e.g., Thermo Scientific).
- 35-mm tissue culture dish (e.g., Corning).
- 22-mm circular high quality coverslips (Carolina Biological Supply, # 63-3035).
- Anhydrous absolute ethanol (Pharmco-AAPER).
- 0.22-μm stericup-PES filter unit (Millipore).

- PBS: Add 8 g of NaCl, 0.2 g KCl, 1.44 g of Na₂HPO₄, and 0.25 g of KH₂PO₄ to a final volume of 1L of distilled H₂O and dissolve. Adjust pH using 1M HCl and/or 1M NaOH until obtaining a stable pH of 7.4. Filter through a 0.22-μm stericup filter (see above) following manufacturer's instructions. Store at room temperature and verify the lack of phosphate precipitates prior to use.
- 0.2% gelatin: add 1 g of gelatin (e.g., Fisher) to 500 ml of PBS. Autoclave the solution, let it cool at room temperature and sterilize by filtration using a 0.22-μm stericup filter. This solution can be stored at 4°C up to 6 months.
- Tissue Culture Incubator: 37°C, 5–10% (v/v) humidified CO₂ incubator.
- 1% glutaraldehyde: dilute 1ml (25% stock) glutaraldehyde (e.g., Sigma-Aldrich) into 24 ml PBS (see above) to obtain 1% solution. Sterilize by passing through a 0.22 μm stericup filter unit and store in aliquots at −20°C.
- 1M Ethanolamine: prepare 1M solution of ethanolamine (e.g., Sigma-Aldrich) in sterile H₂O by adding 0.062 ml of ethanolamine per ml of H₂O. Sterile filter passing through a 0.22 μm stericup filter unit.
- Hematocytometer.
- Trypsin- EDTA (ethylene diamine tetra-acetic acid) solution ((This solution can be purchased ready and sterile) e.g., Mediatech, Inc.): 2.5 g of trypsin, 0.2 g EDTA, 8 g NaCl, 0.4 g of KCl, 1g of glucose, 0.35 g of NaHCO₃, and 0.01 g phenol red dissolved into H₂O to a final volume of a 1L. Sterilize solution by filtration through a 0.22-μm stericup filter (see above) and store up to 3 months at −20°C.
- Inverted microscope.
- Penicillin/Streptomycin (Mediatech, Inc.): 10,000 U/ml Penicillin and 10,000 μg/ml Streptomycin.
- Fibroblast medium: DMEM (see above) supplemented with 10% Fetal Bovine Serum (FBS) (e.g., Hyclone), 100 units/ml penicillin, and 100 μg/ml of streptomycin (e.g., Mediatech, Inc.). Store at 4°C for up to 1 month.
- 0.2-μm syringe filters (e.g. Whatman)
- L-ascorbic acid sodium salt: prepare a stock solution of 50mg/ml of tissue culture grade L-ascorbic acid in PBS (e.g., Sigma-Aldrich) and sterilize through a 0.2μm syringe filter. This stock solution cannot be stored and should be prepared just prior to use.
- Small sterilized fine-pointed tweezers.
- 6-well bacterial (not treated for tissue culture) plate: one plate is needed for each two cell lines (e.g., Greiner bio-one).
- Parafilm.
- 50mM of Ethylenediamine tetra-acetic acid (EDTA) (e.g., Fisher).
- Sterile MilliQ ddH₂O.
- Extraction buffer: PBS containing 0.5% Triton X-100 (e.g., Sigma-Aldrich) and 20mM NH₄OH (e.g., Sigma-Aldrich). The solution of 0.5% Triton X-100 in PBS can be stored at 4°C for up to 1 month, while NH₄OH needs to be added just prior to use.
- PBS pen/strep: PBS (see above) containing 100 U/ml Penicillin (e.g., Mediatech, Inc.) and 100 μg/ml Streptomycin (Mediatech, Inc.). Store at 4°C for up to 1 month.

2.4. Characterization of Unextracted Fibroblast-derived 3-D Matrices by Indirect Immunofluorescence

2.4.1. Indirect multi-channel Immunofluorescent Labeling of Unextracted 3-D Stromal Matrices

- Chemical hood
- 0.22- μ m stericup PES filter units (Millipore).
- PBS: Add 8 g of NaCl, 0.2 g KCl, 1.44 g of Na₂HPO₄, and 0.25 g of KH₂PO₄ to a final volume of 1L of distilled H₂O and dissolve. Adjust pH using 1M HCl and/or 1M NaOH until obtaining a stable pH of 7.4. Filter through a 0.22- μ m stericup filter (see above) following manufacturer's instructions. Store at room temperature and verify the lack of phosphate precipitates prior to use.
- Fixing Solution: inside a chemical hood, add 2 g of sucrose (Fluka) and 10 ml (1 vial) of EM grade 16% para-formaldehyde (Electron Microscopy Sciences) to a final volume of 40 ml of PBS and dissolve. Store in the dark at room temperature for up to one week.
- Fixing and Permeablizing Solution: Add 100 μ l of Triton X-100 (Sigma-Aldrich) to 20 ml of Fixing Solution (see above). Store in the dark at room temperature for up to one week.
- PBST: Add 50 μ l Tween-20 (e.g., Sigma-Aldrich) into 100 ml PBS (final concentration 0.05%). Store at 4°C and verify the lack of phosphate precipitates prior to use.
- 100% Donkey serum stock (e.g., Jackson ImmunoResearch Laboratories).
- 20% Donkey serum: Add 0.4 ml of Donkey serum stock (see above) to 1.6 ml PBS. Store at 4°C.
- Block Vector Solution: Add one drop of M.O.M Mouse IgG Blocking Reagent (from M.O.M Kit BMK-2202, Vector Laboratories) to 1.25 ml 20% donkey serum (see above). Store at 4°C.
- Antibody Incubation Media: PBS containing a final concentration of 8% M.O.M Protein Concentrate (from M.O.M Kit BMK-2202, Vector Laboratories) and 5% donkey serum. Add 80 μ l of M.O.M Protein Concentrate and 50 μ l of 100% donkey serum stock (see above) to a final volume of 1 ml of PBS.
- Primary Antibody Solution: Add 1 μ l of mouse anti α -Smooth Muscle Actin (α -SMA) (1:400) (Sigma-Aldrich, #A2547) and 4 μ l rabbit anti-fibronectin (for human samples use Sigma, #F3648, for murine samples use abcam #ab23750 (1:100)) to 400 μ l of Antibody Incubation Media (see above).
- PBST containing 10% donkey serum: Add 20 μ l of 100% Donkey serum stock (see above) to 180 μ l of PBST (see above).
- Refrigerated microcentrifuge.
- Secondary Antibody Solution: For nuclei staining use SYBR green reagent (Invitrogen, #S7567) (1:8000) (*see* Note 1). For fibronectin and α -SMA detection, use anti-rabbit Cy5-conjugated (1:100) and anti-mouse Rhodaminered-conjugated

Note 1 In order to stain the Nuclei, other dyes can be used (e.g., Hoechst or DAPI) but since most confocal microscopes do not have UV filters to detect the signal of these fluorescent dyes, we proposed to use the fluorescent SYBR Green dye, which absorbs blue light ($\lambda_{\text{max}} = 498$ nm) and emits green light ($\lambda_{\text{max}} = 522$ nm) and whose fluorescence staining can be analyzed using the 488 nm channel available in most of the confocal microscopes.

(1:100) affinity purified F(ab')₂ donkey fragments (Jackson ImmunoResearch Laboratories, # 54557 and #54831, respectively). Antibody and SYBR green dilutions are made in PBST containing 10% of donkey serum (see above). Before usage, pre-clear the secondary antibody solution (to remove insoluble material) by centrifugation at 13,200 rpm for 15 min at 4°C.

- Forceps.
- Prolong Gold anti-fade reagent (Invitrogen).
- Microscope glass slides (e.g., Fisher)
- Confocal microscope (e.g., Nikon) equipped with a Krypton/Argon laser with three lines, 488, 568 and 647 nm, for fluorescence excitation of dye-labeled samples. This will allow the use of fluorescein-like (e.g., SYBR green for nuclei), TRITC-like (e.g., Rhodamine-red for α -SMA) and far-red-like (e.g., Cy5 for fibronectin) (see Note 2).

2.5. Morphological Analyses of Cancer Cells Re-Plated within Various Stromagenic Fibroblast-Derived 3-D Matrices

2.5.1. Fluorescent Labeling of Normal and/or Cancer Cell-bodies and Nuclei

- Cell culture hood (e.g., Thermo Scientific).
- Tissue Culture Incubator: 37°C, 5–10% (v/v) humidified CO₂ incubator.
- 0.22- μ m stericup PES filter units (e.g., Millipore).
- PBS: Add 8 g of NaCl, 0.2 g KCl, 1.44 g of Na₂HPO₄, and 0.25 g of KH₂PO₄ to a final volume of 1L of distilled H₂O and dissolve. Adjust pH using 1M HCl and/or 1M NaOH until obtaining a stable pH of 7.4. Filter through a 0.22- μ m stericup filter (see above) following manufacturer's instructions. Store at room temperature and verify the lack of phosphate precipitates prior to use.
- Heat-denatured 2% bovine serum albumin (BSA): dissolve 2 g of Bovine Serum Albumin (Fraction V, Fisher) in 100 ml distilled H₂O and sterilize using the 0.22 μ m filter. This solution can be stored indefinitely at 4°C. Just prior to use, aliquot the amount needed into a 50-ml polypropylene tube and heat for 7 min in boiling water, allow to cool back to room temperature. Heat-denatured BSA should appear translucent but not opaque or milky. Heat-denatured BSA cannot be stored.
- PANC1 (pancreatic cancer cell line) or any normal epithelial and/or cancer cell to be analyzed.
- Penicillin/Streptomycin (Mediatech, Inc.): 10,000 U/ml Penicillin and 10,000 μ g/ml Streptomycin.
- PANC1 medium: 440 ml of RPMI-1640 (e.g., Mediatech, Inc.), 50 ml of FBS (e.g., Hyclone), 5ml of Penicillin/Streptomycin (see above) and 5 ml of L-glutamine (e.g., Mediatech, Inc.). Note that if a different cell-line is to be used appropriate media needs to be prepared.
- 60-mm tissue culture dish (one for each cell line to be analyzed) (e.g., Corning).
- Trypsin- EDTA (ethylene diamine tetra-acetic acid) solution ((This solution can be purchased ready and sterile) e.g., Mediatech, Inc.): 2.5 g of trypsin, 0.2 g EDTA, 8 g NaCl, 0.4 g of KCl, 1g of glucose, 0.35 g of NaHCO₃, and 0.01 g phenol red dissolved

Note 2²Instead of a confocal microscope, an epifluorescent microscope equipped with a motorized Z-motor and deconvolution software can be used.

into H₂O to a final volume of a 1L. Sterilize solution by filtration through a 0.22- μ m stericup filter (see above) and store up to 3 months at -20°C .

- Sterile 15 ml polypropylene conical tubes (two per each cell line) (e.g., Corning)
- Centrifuge dedicated to cell culture equipped with adaptors for both 15 ml and 50ml polypropylene tubes.
- 0.22- μ m stericup PES filter units (e.g., Millipore).
- 35-mm dishes containing the desired fibroblast-derived 3-D matrix samples for analyses (minimum of two for each cell line) (see, Section 3.3.1.1).
- 35-mm tissue culture dishes, minimum of two for each cell line (e.g., Corning).
- CFDA-SE dye (carboxyfluorescein diacetate, succinimidyl ester dye): Prepare a 20mM stock solution of CFDA-SE dye (Vybrant^R CFDA-SE Cell Tracer Kit (Invitrogen)) using the high-quality DMSO that is included with the kit. Store in the dark at -20°C .
- Hoechst 33342: Bisbenzimidazole H 33342 fluorochrome, trihydrochloride (Calbiochem). Prepare 2 mM (Stock) solution in water. Store at 4°C protected from light.
- Chemical hood
- Fixing Solution: inside a chemical hood, add 2 g of sucrose (Fluka) and 10 ml (1 vial) of EM grade 16% para-formaldehyde (Electron Microscopy Sciences) to a final volume of 40 ml of PBS and dissolve. Store in the dark at room temperature for up to one week.
- Parafilm.
- PBS pen/strep: PBS (see above) containing 100 U/ml Penicillin (Mediatech, Inc.) and 100 $\mu\text{g}/\text{ml}$ Streptomycin (Mediatech, Inc.). Store at 4°C for up to 1 month.
- Epifluorescence microscope, equipped with UV (excitation 360–370 nm and emission 420 nm) and FITC (excitation 460–500 nm and emission 510–560 nm) filters, CCD camera and acquisition software.

2.5.2. Digital Analysis to Measure Cell Morphology

- .tiff format image-files to be analyzed.
- Analysis software: Microsoft Excel and/or MetaMorph 7.0r1 (Molecular Devices, Sunnyvale, CA).
- Girded, see-through paper.
- Stage Micrometer.

2.5.3. Statistical Analysis—GraPhpad Instant software (or any other simple statistical software).

3. Methods

NOTES: All solutions and equipment coming into contact with tissue samples or living cells must be sterile and aseptic techniques should be used accordingly. All cell-culture incubations are performed using a 37°C , 5–10% CO₂ humidified incubator.

3.1. Isolation of Fibroblasts from Normal and/or Tumor Tissue Samples

Fibroblasts synthesize and maintain the ECM of mesenchymal tissues. The main function known for fibroblasts is maintaining the structural integrity of all connective tissues by continuously secreting components (e.g., soluble cytokines, latent factors and matrix glycoproteins) and actively incorporating them to the ECM. The composition of a given ECM determines the specific physical and biochemical properties of each connective tissue.

This section describes two methods used to harvest primary fibroblasts from normal and/or neoplastic tissue samples (*see* Note 3). The first method, minced tissue method (Section 3.1.1), is based on the capability of fibroblasts to crawl out of tissue samples, thus facilitating their harvest. Minced tissue method provides fairly homogenous fibroblastic cell-cultures. Therefore, this method assures the isolation of a variety of different subpopulations of fibroblasts present in a given (normal or tumor-associated) tissue. The second method, enzymatic digestion method (Section 3.1.2), is faster and yields a higher recovery rate of cells from the tissue. Since this is a much faster method, contaminations are less common than in the first method. Fibroblast cultures obtained using the second method, are heterogeneous and probably better represent the fibroblastic population of a given tissue. Unfortunately, the heterogeneous aspect of this procedure often results in cultures that contain additional types of cells (e.g., epithelial). This method is adequate for fibroblasts that are impaired in their motile capabilities, or when heterogeneous cultures are needed.

In the minced tissue method (first method), the tissue samples are cut into small pieces and each of these pieces is separately placed in a tissue culture plate until fibroblasts migrate out of the tissue. In the enzymatic method (second method), tissue samples are actively digested and cells sorted by size exclusion. Once the fibroblast cultures reach confluence (in both methods) they can be frozen for later or expand for immediate experimental use.

3.1.1. Isolation of Fibroblasts from Freshly Minced Normal and/or Tumor Tissue Samples

1. Using sterile dissecting scissors and/or sterile needles make several scratches on the plastic surface of a 12-well tissue culture plate in a star-like or grid configuration. These rough surfaces will facilitate tissue adherence to the plates allowing the fibroblasts to crawl out into the culturing plates. The scratches need to be made while the plate is inside a tissue-culture hood thus avoiding contaminations and the plates need to be rinsed with sterile PBS, following scratching of the surface, to eliminate the plastic-debris.
2. Rinse the human or murine normal or tumor tissue samples (obtained fresh immediately after surgery) in a 60-mm tissue culture dish that contains cold (4°C) sterile PBS pen/strep.
3. Using scissors and assisting with tweezers put the rinsed tissue in a second 60-mm dish and chop into 1mm² pieces using a sterile scalpel (*see* Note 4).
4. Place the tissue pieces into the indentations created by the scratches in step 1 (one piece per well).

Note 3 Primary fibroblast cultures may be obtained from different tissues, normal or tumor, including but not limited to human tumor tissue from ovary, pancreas, lung or breast and from tissues of other species such as mouse and rat.

Note 4 The tissue tumor sample can be cut into two pieces, one can be used to obtain fibroblasts and the other can be frozen for further analysis, such as protein localization using immunohistochemistry or immunofluorescence techniques. To freeze half of the sample, put the tissue sample inside a plastic mold and cover with embedding medium (e.g. Tissue-Tek). Using tweezers, freeze the sample by floating the mold on liquid N₂, avoiding the liquid N₂ to directly contact the sample, once frozen, store at -80°C.

5. Allow samples to dry by leaving the plates uncovered inside the hood in the proximity of an open Bunsen-burner flame for a period of 5–8 minutes. This will ensure adherence of the tissue samples to the scratched bottom of the dishes.
6. Carefully, add 1ml of fibroblast medium to each well containing a minced piece of tissue, preventing the tissue samples from detaching (*see* Note 5). Place dishes inside incubator.
7. Replace half of the fibroblast medium with fresh fibroblast medium three times per week until primary fibroblasts migrate (or crawl out) of the tissue pieces. This process should be regularly monitored using an inverted microscope and it normally takes 2–8 weeks depending on the tissue source (e.g., ovarian tissues take about 2 weeks while pancreatic samples vary between 4–8 weeks depending whether tumors were untreated or irradiated prior to surgery).
8. When the fibroblasts occupy most of the dish surface, remove the piece of tumor with sterile tweezers, trypsinize (*see* Note 6) and re-plate into a T-75 tissue culture flask. The piece of tissue removed can be re-placed on a new scratched plastic dish to obtain more fibroblasts repeating procedures starting from step 4.
9. Once fibroblasts reach confluence within the T-75 tissue culture flask, they can be expanded (passaged) into additional dishes at a ratios between 1:3 and 1:5 (*see* Note 6 above) for experimental analyses and be used for production of fibroblast-derived matrices (between passages 2 and 6, see sections below) or be frozen using freezing medium (*see* Note 7).

NOTE: The isolated fibroblasts can be immortalized (e.g., using SV40 large T antigen). If the fibroblasts are immortalized, cultures would need to be re-characterized later on (see section 3.2).

3.1.2. Isolation of Fibroblasts from Collagenase-Digested Normal and/or Tumor Tissue Samples

1. Place human (or murine) normal or tumor tissue samples (fresh from surgery) into a 10-cm plastic dish containing 10 ml DMEM pen/strep and rinse briefly.
2. Transfer samples into a 10-cm plastic dish containing 10ml DMEM-3%BSA-Pen/Strep.
3. Using a sterile scalpel and assisting with tweezers cut the tumor tissue in small pieces to facilitate the enzymatic digestion that will follow.
4. Transfer the material into a sterile 150 ml Erlenmeyer. Add 8 ml DMEM-3%BSA-Pen/Strep into the 10-cm plastic dish and collect the remaining tissue pieces transferring them into the same 150 ml Erlenmeyer.
5. Add 2 ml of Collagenase-3 (10X) the samples thus diluting the collagenase 10 fold.
6. Place the Erlenmeyer in an orbital shaker for 1h and agitate at 200 rpm at 37°C (*see* Note 8). After 1h, most of the tumor pieces should be digested, if undigested tumor-

Note 5 If the tumor tissue is detached from the dish, it can be placed in a new scratched dish repeating steps 4–6 of section 3.1.1.

Note 6 When subculture or recovery of the cells from a plate is required, remove the medium from the flask and rinse cells briefly using pre-warmed (37°C) trypsin-EDTA to remove trypsin inhibitors contained in the serum used for culturing the cells. Then, add enough trypsin-EDTA to slightly cover the cells on the flask and observe under an inverted microscope until the cells detach from the culture dish and become rounded and not clustered to each other (1 to 3 min). Once the cells have completely detached from the bottom of the flask, add 10ml of fibroblast medium to neutralize the trypsin and collect the cells. Pipette up and down carefully to mechanically disrupt the remaining cell aggregates. At this point, cells can be sub-cultured or frozen.

Note 7 Before freezing, the fibroblasts should be actively proliferating therefore ensuring that no contaminations will be held within the frozen samples.

pieces still remain continue the agitation until complete digestion is evident (*see* Note 9).

7. Transfer the digested tissue into 50 ml polypropylene tubes and centrifuge it at 200 g for 10 min to collect the cells (*see* Note 10).
8. Remove the supernatant containing the collagenase in order to prevent further degradation, thus avoiding damaging the cells.
9. Re-suspend the cell-pellet in 10 ml fibroblast medium.
10. Filter the cell-suspension through 500 μ m nylon mesh followed by filtrations through 100 μ m and 40 μ m cell strainers, thus removing remaining tissue pieces. Transfer the final filtrate into a 10-cm tissue culture dish and incubate for 2 h inside the incubator (*see* Note 11).
11. Following the 2 h incubation change the medium to remove non-adherent cells and other material.
12. Once fibroblasts reach confluence within the T-75 tissue culture flask, they can be expanded (passaged) into additional dishes at a ratios between 1:3 and 1:5 (*see* Note 6 in section 3.1.1) for experimental analyses and be used for production of fibroblast-derived matrices (between passages 2 and 6, *see* sections below) or be frozen using freezing medium (*see* Note 7 in section 3.1.1).

NOTE: The isolated fibroblasts can be immortalized (e.g., using SV40 large T antigen). If the fibroblasts are immortalized, cultures would need to be re-characterized later on (*see* below).

3.2. Characterization of the Isolated Fibroblasts

Although most contaminant cells (epithelial and/or endothelial) perish after a few passages, it is always recommended to assure that the harvested cell population contains only fibroblastic cells. This can be assessed using specific cell markers, as well as by phenotypic analysis. Typically, fibroblasts are large, flat and relatively elongated cells with branched bodies that surround an oval and speckled nucleus. Fibroblasts express, among others, vimentin. In contrast, cytokeratin is an epithelial (and epithelial tumor) cell marker. In this section, we describe how to assess the homogeneity (under microscope) and the exclusivity (using a Western blot technique, *see* Note 12), of the harvested fibroblastic cell population. The isolated cells are plated onto 35-mm dishes and incubated overnight, their morphology and homogeneity is analyzed under a microscope, while cellular proteins are extracted and separated by SDS PAGE, followed by transfer into PVDF membranes and submitted to Western blot analyses using marker-specific antibodies.

NOTE: All solutions and equipment coming into contact with tissue samples or living cells must be sterile, and aseptic techniques should be used accordingly.

Note 8 If the starting amount of tissue sample is large, the volumes can be scaled up; use a bigger Erlenmeyer assuring that the solution will not be spilled out while stirring. For instance, if the final volume of DMEM will be 40 ml, a 250 ml Erlenmeyer should be used and 4 ml Collagenase-3 (10X) should be added (for details *see* section 3.2.1).

Note 9 When the pieces of the tissue are not dissolved after a period of 1h agitation, fresh collagenase-3 can be added or, alternatively, the tissue pieces can be mechanically dissociated by carefully pipetting up and down.

Note 10 Prior to centrifugation, the centrifuge should be equilibrated by preparing another 50 ml polypropylene tube that weighs the same as the 50 ml polypropylene tube containing the digested sample and then, samples should be placed on opposite sides of the centrifuge.

Note 11 This method renders an heterogeneous fibroblastic population. If homogenous clones are needed, then dilute cell concentration at this point, and plate them within multi-well tissue culture plate (96 or more wells), for sub-clonal selection.

Note 12 The Western blot technique described to determine the cell expression of specific markers is based on the use of a modified secondary antibody linked to a reporter enzyme, which when exposed to an appropriate substrate drives a colorimetric reaction and produces a color or precipitate, or a luminescent reaction (ECL) allowing detection by exposing onto photographic films. Nowadays, a variety of conjugated secondary antibodies are commercially available and blotted proteins can even be detected by infrared emitted fluorescence (e.g., using the Li-cor's Odyssey Infrared Imaging System and its pre-conjugated secondary antibodies).

3.2.1. Lysis of Isolated Cells

1. If the harvested cells to be characterized are from a stock that was frozen, quickly thaw the cells by placing the vial, containing frozen cells, in a 37°C waterbath. Immediately after thawing, dry the vial and rinse it with ethanol. Inside the hood, open the vial and transfer the cells into a T-75 tissue culture flask containing 10 ml pre-heated (37°C) fibroblast medium. In order to remove the DMSO that is present in the freezing medium, let the cells attach for about 2–3 hours then remove the medium and add 10 ml of fresh and pre-heated (37°C) fibroblast medium. For cells already in culture start from step 2.
2. Once cells reach 70–80% confluence, the homogeneity of the cell culture can be assessed using an inverted microscope.
3. For further characterization, split cells (*see* Note 6 in section 3.1.1) and count using the hemacytometer. Calculate the cell concentration and dilute with fibroblast medium to a final concentration of 1×10^5 cells/ml.
4. Add 2 ml of cell suspension (2×10^5 cells) into each of three 35-mm dishes. Place the three dishes in the incubator overnight.
5. Remove the dishes from the incubator and place them onto a tray containing ice (*see* Note 13). Wash the cells twice using ice cold PBS. Following the second wash, tilt the plate slightly to remove all liquid. This will minimize final-volume variability.
6. Add 250 μ l of cold RIPA Buffer onto each plate (*see* Note 14).
7. Incubate on ice for 5 minutes while gently rocking.
8. Scrape the dish using a cell scraper (keeping the dish on ice) to collect all the protein-lysate and transfer all the material into a 1.5 ml tube pre-placed on ice.
9. While on ice, sonicate the sample for 30 sec, using a small probe at medium power. OPTIONAL: Leave the tubes on ice for 30 additional sec and repeat step 9.
10. Centrifuge the lysates at 13,200 rpm for 15 minutes at 4°C.
11. Collect the supernatant and transfer into a clean 1.5ml tube. If not used immediately, for Western blot analysis, quickly freeze the lysates by placing the tubes within a pre-cooled isopropanol bath on dry-ice and store at –80°C for up to 2 weeks (*see* Note 15).

3.2.2. Western blotting to test the expression of specific Cell Markers

1. Add a fifth of the final volume of 5X Sample Buffer containing 10% of β -mercaptoethanol to the cell lysate sample to be analyzed (e.g., 20 μ l 5X Sample Buffer to 80 μ l cell lysate thus obtaining a final volume of 100 μ l).
2. Mix and then boil the samples at 100°C for 5 min.

Note 13 From this point on, no sterile conditions are required. Instead, it is very important to keep all material on ice, at all times, in order to minimize protease activity thus avoiding protein degradation.

Note 14 For larger dishes, scale up the volume of RIPA buffer. (e.g., for 60 mm dishes use 500 μ l instead of the suggested 250 μ l for 35 mm dishes).

Note 15 To quickly freeze cell lysates, prepare a dry-ice/isopropanol bath by placing a 400 ml beaker containing about 100 ml isopropanol within an ice bucket filled with dry ice. For safety, do this inside the chemical hood. Allow the isopropanol to cool for 30 minutes. Place the tubes containing freshly prepared and aliquoted lysates to a tube-rack and slowly lower the rack into the isopropanol assuring that the lysate volume is immersed in the isopropanol. The lysates should be frozen almost immediately (smaller aliquots are better). Finally, quickly place the tubes on dry-ice for immediate transfer to a –80°C freezer. Samples should remain stable for 2 weeks.

3. For sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), load about 30µl of each sample (*see* Note 16) and 10µl of pre-labeled molecular weight marker onto separate wells (8–16% Tris-glycine gel (*see* Note 17)).
4. Repeat step 3 to obtain a second identical gel (*see* Note 18).
5. Using 1X Running Buffer, separate the proteins in both gels by electrophoresis at 100V for 2 h or until the bromophenol blue from the samples reaches the bottom of the gels.
6. Activate the PVDF membranes (one for each gel) by soaking in methanol for 30 sec, then ddH₂O for 1 min and incubate in Transfer Buffer until ready to transfer.
7. Transfer the proteins from the gels to the PVDF membrane at 4°C using a transfer apparatus containing Transfer Buffer for 2 hr at 25V or overnight at 15V (*see* Note 19).

OPTIONAL: to visualize and assess the quality of the proteins transferred rinse the PVDF membranes with ddH₂O and place in 10ml Ponceau's Solution while rocking gently for 5 min. Wash the membrane with ddH₂O for about 5 min to remove the excess of Ponceau's solution (at this point, the membrane can be scanned or photographed for your records). Finally, wash membranes with PBS to remove the remaining dye.

8. Block the membranes by incubating in Blocking Solution for 1h at room temperature or overnight at 4°C while rocking gently (*see* Note 20).
9. Remove the Blocking Solution and add 10ml of Incubation Solution containing: 2µl anti-GAPDH (to be used as a total protein control) and 2µl anti-Vimentin (used as specific fibroblastic marker) to one of the membranes. To the other membrane, add 10ml of Incubation Solution containing: 2µl anti-GAPDH (to be used as a total protein control) and 2µl anti-pan-keratin (used as specific epithelial marker; it recognizes most types of keratins). Incubate each membrane with its specific antibody-cocktail, by gently rocking for 1h at room temperature (or overnight at 4°C with, *see* Note 20 above).
10. Wash the PVDF membrane 4 times, 5 min each time, using extensive amounts of TBST.
11. Incubate each membrane with secondary antibody; 10ml Incubation Solution containing 2µl of goat peroxidase-conjugated anti-mouse. Rock gently at room temperature for 1 hr.
12. Wash membranes 4 times, 5 min each time, using copious amounts of TBS.
13. Drain excess TBS by dabbing the edge of the PVDF membranes onto a paper towel, then position the PVDF membrane onto plastic wrap with the protein side facing up.

Note 16 In order to assure that the Western blot technique has properly worked, known fibroblastic and epithelial cell lysates should be loaded as positive controls (*see* Figure 1).

Note 17 In these protocols, we propose to use commercially available pre-cast gels. Nevertheless, gels can be poured at the lab just prior to use. For more information about SDS-PAGE technique consult a student's biochemistry text book or a molecular cloning laboratory manual.

Note 18 Since the molecular weight of vimentin is similar to keratins, it is necessary to use two gels. One of them will be used to detect the presence of vimentin and GAPDH and the other to detect the keratins. If Li-cor's Odyssey Infrared Imaging System is used, only one gel will be required since vimentin and keratin could be labeled by different fluorophores. In that case, the anti-vimentin antibody recommended is one that was generated in rabbit (e.g., Biovision, # 3634-100).

Note 19 Depending on the equipment used, different voltages, times and/or buffers may be required. Therefore, check the manufacturer's instructions prior of using any SDS-PAGE or transfer equipment.

Note 20 Following a 4°C overnight step, membranes should be brought back to room temperature before moving on to the next step.

14. Use Amersham Detection ECL Kit adding 50µl Solution B into 2ml Solution A. Add 1ml of this Detection Solution (drop wise) onto each PVDF membrane and incubate for 30 sec to 1min.
15. Drain off excess Detection Solution by dabbing edge onto paper towel and transfer membranes onto a clean plastic wrap or plastic pouch.
16. Place the covered membrane into an X-ray film cassette. In the dark, expose the membrane to autoradiography film and develop using a X-ray film processor in order to visualize the protein bands.
17. The individual protein bands corresponding to Vimentin, Keratin, and GADPH can be identified within the samples in question by comparing their molecular weights to the positive control samples (see example in Figure 1).

3.3. Production of Fibroblast-Derived 3-D Matrices

This section describes a method for generating cell-derived 3-D matrices produced by a plethora of primary fibroblasts. The resultant matrices can be used as substrates for culturing cells, since they closely resemble *in vivo* mesenchymal matrices (41,42). Utilizing *in vivo*-like 3-D matrices as substrates allows to study, in a physiologically relevant manner, how cells interact with their natural ECMs, as well as their microenvironmental induced structures, functions, and signaling, which differ from observations obtained by culturing cells on conventional 2D substrates *in vitro* (43–48).

In the protocols described in this section, the fibroblasts used are the primary fibroblasts harvested from the assorted normal and tumor tissue-samples described in previous sections 3.1.1 and 3.1.2. Following the description for 3-D matrix production, we describe how to extract cellular debris from the matrices.

3.3.1. Preparation of Fibroblast-Derived 3-D Matrices—The basic approach is to allow fibroblasts to produce their own 3-D matrices. For this, fibroblasts are plated and maintained at a confluent state, while fresh ascorbic acid (stabilizing the secreted matrices) is added every other day for a period of five to nine days. Matrices are extracted, using an alkaline detergent treatment, thus removing cellular debris and leaving the 3-D matrices intact and stably attached to the culture dishes. The fibroblast-derived 3-D matrices contained within the dishes are then stored at 4°C for a couple of months.

3.3.1.1. Primary Fibroblast-Derived 3-D Matrices: *NOTE:* All solutions and equipment coming into contact with tissue samples or living cells must be sterile and aseptic techniques should be used accordingly.

OPTIONAL: For immunofluorescence experiments 22-mm coverslips are to be used. Coverslips need to be sterilized by flaming after dipping with absolute anhydrous ethanol. Proceed by placing the coverslips at the bottom of a 35-mm tissue culture dishes and rinse with PBS. Remove the PBS and continue to step 1.

1. Add 2 ml of 0.2% gelatin solution to each 35-mm tissue culture dish to be used (*see* Note 21) and incubate for 1 h at 37°C or overnight at 4°C.
2. Aspirate gelatin and wash with 2 ml PBS.

Note 21 The desired final number of plates to be used should be calculated before starting. The protocols describe the amount of volume needed for an individual 35-mm dish. Final volumes need to be calculated in respect to the final amount of desired 3-D matrix-coated plates and their types. For example, for 12-, 24- or 48-well tissue culture plates scale down the volumes of all reagents from 2 ml (35-mm dish) to 1, 0.5 and 0.250 ml per well, respectively. Alternatively, for the use of 60-mm or 10-cm dishes scale up the volumes of added reagents from 2 ml per dish to 4 and 10 ml, respectively.

3. Cross-link the gelatin by adding 2 ml 1% glutaraldehyde to each dish and incubate plates for 30 min at room temperature.
 4. Aspirate glutaraldehyde and wash plates 3 times, 5 minutes each time, with 2 ml of PBS.
 5. Add 2 ml of 1M ethanolamine to each dish and incubate at room temperature for 30 minutes. This will block the remaining glutaraldehyde, thus avoiding damaging the cells later on.
 6. Aspirate ethanolamine and wash plates 3 times, 5 minutes each time, with 2 ml PBS.
 7. Remove PBS and replace with 2 ml of fibroblast medium. If the medium appears purple, repeat steps 6 and 7 to remove any trace amounts of ethanolamine.
 8. Remove media from a T-75 semi-confluent tissue culture flask (80% confluent) containing the primary fibroblasts harvested following protocols in sections 3.1.1 or 3.1.2. These fibroblasts will be used for matrix production and should be at a passage between 2 to 6. A single semi-confluent T-75 flask should contain enough fibroblasts for the production of matrices coating about 11 to 14 35-mm dishes. As many as five flasks, containing primary fibroblasts, can be used in this procedure, rendering 55 to 70 35-mm matrix-covered plates (*see* Note 21 above).
 9. Trypsinize cells (*see* Note 6, in section 3.1.1), count and dilute to a final concentration of 2.5×10^5 cells/ml.
 10. Add 2 ml of cell suspension (5×10^5 cells) to each 35-mm dish where matrices will be produced.
 11. Place the dishes overnight in the incubator (*see* Note 22).
 12. Confirm that cells are confluent, remove the media and replace with fibroblast media containing freshly added 50 μ g/ml of ascorbic acid. Place cells back into the incubator and count this day as “2.” Repeat this step on the mornings of days, “4” and “6” (*see* Note 23).
1. On the morning of day 8 (*see* Note 24), carefully wash the dish with 2 ml PBS then remove the PBS.
 2. Slowly and very carefully add (drop wise) 1 ml of pre-heated (37°C) extraction buffer and observe under a tissue culture microscope until the fibroblasts are completely lysed (5–10 min).

OPTIONAL: When the matrices produced are too thick (more than 17 μ m thick), it is possible that following the above steps will not suffice for complete extraction. In that case, one of two possible approaches can be used. On the first approach, after removing the PBS from step 1, add 1ml of 50 mM EDTA and incubate at 37°C for 10 min, wash twice with PBS and proceed to step 2. On the second approach, after removing the PBS from step 1, add 2ml of sterile Millipore ddH₂O and incubate for 5–10 min at room temperature, carefully remove the ddH₂O and add additional 2ml

Note 22 Do not proceed to next step if cultures have not reached 100% confluence the following morning. If, after 24h, the dish does not appear completely confluent, change medium and wait until cultures reach 100% confluence. The lack of confluence prior to the next step can cause poor matrix production or quality or avoid matrix production all together.

Note 23 If coverslips are used, transfer the coverslip to a 6-multiwell bacterial (instead of a regular tissue-culture) petri dish before adding the ascorbic acid. This will minimize the growth of fibroblasts on the plate area outside of the coverslip and will, especially, facilitate lifting the coverslip thus avoiding tearing off the matrices at the final steps.

Note 24 Leave at least two dishes unextracted in order to use in section 3.4.1 for the characterization of matrices resulting in categorizing the fibroblasts (that produced the matrices) as normal, primed or tumor-associated.

of fresh sterile Millipore ddH₂O and incubate for 30 min at room temperature. Carefully discard the ddH₂O and continue to step 2.

3. Very carefully, without removing the extraction buffer, add 2 ml PBS avoiding turbulence as much as possible.

OPTIONAL: At this point the matrix can be placed at 4°C overnight. This will assure the stability of the newly extracted matrices and will minimize matrix detachment. Make sure to warm matrices back to room temperature before continuing to the next step.

4. To avoid damaging the matrices, slowly tilt the plate and carefully, without touching them, remove approximately 2.5ml of the solution.
5. Slowly and carefully add 2.5ml of PBS.
6. Repeat steps 4 and 5 twice.
7. Remove 2.5ml of the PBS and add 2ml of PBS strep/pen, seal the dish using parafilm strips and store matrices at 4°C for up to 3 months.

3.4. Characterization of Unextracted Fibroblast-Derived 3-D Matrices by Indirect Immunofluorescence

Tumor-associated (e.g., desmoplastic) stroma has been associated with a variety of invasive cancers (15,49). This stroma presents a scar-like phenotype, is highly fibrotic and can constitute more than 50% of the tumor mass. The desmoplastic stroma is characterized by the presence of activated myofibroblasts, which are highly proliferative and express alpha-smooth muscle actin (α -SMA). Three-dimensional matrices derived from primary fibroblasts harvested at different stages of tumor development differ in their orientation of fibronectin fibers, expression and organization of α -SMA and the morphology of both their cell body and nucleus (42). Therefore, characterization of the above-mentioned features can be used to sort unextracted 3-D matrix cultures as normal, primed or tumor-associated. For example, matrices derived from primary tumor-associated fibroblasts that are desmoplastic, present a parallel patterned matrix with high and homogenous α -SMA expression localized on stress fibers and elongated elliptical nuclei morphology (Figure 2). Matrices produced by primary primed fibroblasts present a more random organization of fibronectin fibers, relatively rounded nuclei and either lack α -SMA expression or express α -SMA at relatively low levels. Primary fibroblasts isolated from normal tissues normally produce very thin matrices and the majority of these do not overcome growth inhibition by contact, therefore, cultures are mono-layered. Nevertheless, some normal fibroblasts isolated from specific sites, such as normal ovaries, grow multi-layers when maintained *in vitro* as confluent cultures. Similarly to primed matrices, 3-D matrices obtained from normal primary fibroblasts are greatly disorganized and the unextracted nuclei also appear relatively round. However, in comparison to primed unextracted cultures, normal unextracted 3-D cultures vary on the expression of α -SMA from cultures that homogeneously express high levels of α -SMA (e.g., normal ovarian-derived 3-D cultures (50)) to heterogeneous or low homogenous expression levels of this protein (e.g., skin and pancreas-derived 3-D cultures).

The classification of unextracted *in vivo*-like 3-D stromal matrices produced by assorted isolated fibroblasts (sections 3.1.1 and 3.1.2) as normal, primed, or tumor-associated (desmoplastic), can be questioned using indirect immunofluorescent staining. In this section, unextracted 3-D stromal matrices, prepared onto coverslips (see section 3.3.1.1), are fixed and permeabilized, and subjected to multi-channel simultaneous fluorescent labeling of matrices (e.g., fibronectin), nuclei and α -SMA. Following multi-channel fluorescent labeling, the unextracted 3-D cultures are analyzed under a scanning or spinning disk confocal microscope.

3.4.1. Indirect multi-channel Immunofluorescent Labeling of Unextracted 3-D Stromal Matrices

1. Wash coverslips containing unextracted 3-D matrices (cultures obtained from step 1 in section 3.3.1.2) with 2ml of PBS (*see* Note 25).
2. Add 0.8ml Fixing/Permeablizing Solution to each coverslip and incubate at room temperature for 3min.
3. Carefully (without damaging the sample), aspirate solution and add 0.8ml Fixing Solution to each coverslip and incubate at room temperature for 20 min.
4. Replace solution with 2ml of PBST and incubate for 5–10 min.
5. Remove all solution by slightly tilting the plate containing the samples and carefully aspirate all liquid and replace with 50 μ l Block Vector Solution adding directly onto the sample (drop wise). Incubate coverslips for 1h at room temperature while assuring that samples stay moist (*see* Note 26).
6. Rinse twice, 2 min each time, with 2 ml of PBST.
7. Similar to the procedure described in steps 4 and 5, remove all liquid and add 50 μ l of Antibody Incubation Media directly onto each coverslip. Let sit for 5 min at room temperature.
8. Tip-off the excess of Antibody Incubation Media, remove and replace with 50 μ l of Primary Antibody Solution directly onto each coverslip and incubate for 1h at room temperature.
9. Wash coverslips 3 times, for 8 min each time, using 2 ml of PBST at room temperature.
10. Carefully replace solution with 50 μ l of Secondary Antibody Solution and incubate 30 min at room temperature.
11. Wash coverslips 3 times, for 8 min each time, using 2 ml of PBST at room temperature.
12. Rinse once using 2ml PBS and once using 2 ml ddH₂O.
13. Carefully, remove coverslips (one by one) from ddH₂O, get rid of excess of liquid and mount coverslips face down onto a microscope slide using a drop (12 μ l) of prolong mounting medium.
14. Let samples dry for 1–2 hr (or overnight), in the dark and store at –80°C until ready to analyze.
15. For analysis, use a confocal microscope (or epifluorescence microscope that can capture images along the z-axis). Acquire an image for each color (channel) and repeat on different locations per sample at least five times (*see* Note 27).

3.4.1.1. Image Analysis for Sorting *in vivo*-like 3-D cultures as normal, primed or tumor-associated: Listed below are some characteristics that will assist to classify fibroblastic unextracted 3-D cultures as “normal,” “primed” or “tumor-associated” (42) by using the three channel images acquired in the previous section (3.4.1) (*see* Note 28 and Figure 2):

Note 25 At least two coverslips containing unextracted 3-D matrices should be analyzed for each isolated fibroblastic cell line.

Note 26 Before adding the Block Vector Solution make sure that the coverslips are not touching the well walls. This will prevent loss of blocking solution due to capillarity, which could result in sample drying. If samples appear to be drying compensate with Block Vector Solution and continue with the incubation.

Note 27 Before acquiring pictures make sure samples are at room temperature.

Note 28 The classification described in this section is based on the characteristics of the unextracted cultures (42), which are matrix-dependent and, therefore, are not evident in 2-D cultures.

- Unextracted 3-D “tumor-associated” and “primed” cultures are multi-layered and, therefore, they produce matrices that range between 7–25 μm in thickness (see Note 29).
- “Normal” unextracted cultures range between mono- (e.g., human and murine skins) (42) and multi-layers (e.g., human ovary) and, therefore, their average thickness vary from just a few microns to thickness similar to the one seen in primed and tumor-associated cultures.
- Matrices that present a patterned parallel organization (revealed by fibronectin staining) are matrices-derived from “tumor-associated” (e.g., myofibroblastic) fibroblasts.
- “Normal” and “primed” matrices are mesh-like and disorganized.
- “Tumor-associated” cultures are characterized by high expression of endogenous α -SMA containing stress fibers.
- “Primed” 3-D cultures either do not express α -SMA or express very low homogenous levels of α -SMA; these cells are also known as proto-myofibroblastic cells, which down-regulate α -SMA expression prior to myofibroblastic differentiation associated with desmoplastic reactions.
- “Normal” 3-D cultures vary on their levels of α -SMA expression ranging from non-expressing cells, to heterogeneous (e.g., some cells express high levels while others do not express α -SMA) or homogeneous, expressing high levels of α -SMA (e.g., normal human ovary).
- “Normal” and “primed” cells have relatively rounded nuclei.
- “Tumor-associated” myofibroblastic cells have elliptical and condensed nuclei, which are organized in parallel patterns following the same orientation as both α -SMA positive stress fibers and fibronectin rich 3-D matrices.

Some examples of the above-mentioned characteristics for “tumor-associated” 3-D cultures are shown in Figure 2.

3.5. Morphological Analyses of Cancer Cells Re-Plated within Various Stromagenic Fibroblast-Derived 3-D Matrices

One of the main features of cancer progression resides in the fact that when tumors become invasive the basement membrane that normally isolates epithelium from mesenchyme becomes degraded and, therefore, invasive cancer cells directly interact with the mesenchymal stromal components both prior to intravasation and after extravasation at the secondary sites. The specific morphological phenotype acquired by invasive cancer cells while migrating within mesenchymal tissues is predictive of their invasive strategy behavior (35–37). For example, it is well known that tumor cell migration and metastasis can occur by multiple mechanisms (e.g., epithelial-mesenchymal transition, or mesenchymal-amoeboid transition (35–37). These various mechanisms require different signaling pathways, directly induced by the stroma and are clearly distinguished by specific cell morphologies (e.g., mesenchymal vs. amoeboid). Therefore, testing whether cells acquire differential morphologies within different stromagenic staged 3-D matrices could predict how the specific cells would invade within specific microenvironmental settings (e.g., normal, primed or tumor-associated stroma). Amoeboid cells are relatively rounded, while mesenchymal cells are spindled-shaped. Mesenchymal cell

Note 29^{Matrix} thickness can vary in different cultures of the same cell-line. It often depends on passage-number and quality of reagents used (e.g., FBS).

invasion requires the function of integrins and specific matrix-proteases while amoeboid invasion is independent of integrins and matrix-proteases functions and instead requires the activation of the ROCK pathway (39,40).

In this section, we provide a method for evaluating 3-D matrix-induced epithelial cancer cell morphology. Prior of cell re-plating within the assorted 3-D matrices, the nuclear and cytosolic compartments of the epithelial cancer cells are fluorescently labeled. Then cells are re-plated within the assorted matrices overnight and their morphologies are measured following the acquisition of several representative double-channeled monochromatic images using an epifluorescence microscope equipped with filters for the acquisition of the specific fluorophores used.

3.5.1. Fluorescent Labeling of Normal and/or Cancer Cell-bodies and Nuclei

1. Block the matrices by adding 2 ml of heat-denatured 2% BSA onto a 35-mm plates containing fibroblast-derived 3-D matrices (see section 3.3.1.2) and incubate at 37°C for 1h (*see* Note 30).
2. After incubation, carefully rinse the matrices twice with 2 ml of PBS.
3. Use a semi-confluent 60-mm culture dish containing PANC-1 (or any other epithelial cells for analysis (e.g., normal or cancer cells)) and aspirate the media.
4. Add 4 ml of corresponding medium containing 4 µl of Hoechst 33342 Stock Solution. Incubate 15 min at 37°C.
5. Rinse 5 times with PBS.
6. Trypsinize the cells (*see* Note 6 in section 3.1.1) and centrifuge at 1200 rpm for 3 min at room temperature obtaining a cell pellet.
7. Remove the supernatant and re-suspend cells by gently pipetting up and down, using 1ml of pre-heated (37°C) PBS containing 1µl CFDA SE dye stock solution.
8. Incubate at 37°C for 15 minutes.
9. Centrifuge cells at 1200 rpm for 3 min at room temperature and remove the supernatant.
10. Carefully re-suspend the pellet by gently pipetting with 5 ml PBS.
11. Repeat steps 8 and 9 three times thus removing residual free dye.
12. Re-suspend cells using 3 ml of their corresponding epithelial medium (e.g., PANC-1 medium), count cells using a hemacytometer and dilute to a final concentration of 1×10^4 cells/ml.
13. Carefully remove the PBS from a 35-mm plate containing fibroblast-derived 3-D matrix and add 2 ml of cells from the previous step.

OPTIONAL: regular, uncoated 2-D plates can be used in parallel as controls in order to assess the effects that mesenchymal 3-D matrices have on the epithelial normal or cancer cell morphology.
14. Place cells in the incubator overnight.
15. Rinse cells carefully with 2ml of PBS.

Note 30 Make sure that matrices are pre-warmed to room temperature after storage at 4°C by placing the plates containing extracted matrices at room temperature for at least one hour prior to use.

16. Aspirate the PBS, add 2 ml Fixing Solution and incubate for 20 min at room temperature, in the dark.
17. Remove the Fixing Solution and wash with 5 ml PBS.
18. Aspirate the PBS, add 2 ml of fresh PBS pen/strep and seal the dishes using parafilm. Store at 4°C in the dark until ready for image acquisition (up to one week).
19. Place the dishes under the epifluorescence microscope and acquire three simultaneous monochromatic images using the 10X or 20X objectives. The first image should be acquired using filters for CFDA-SE dye (similar to FITC, or green dyes). The second image should be acquired at exactly the same position using the filter for Hoechst 33342 (similar to DAPI, or blue dyes). The third image (again at exactly the same position) should be acquired using transmitted, instead of fluorescent, light (e.g., phase contrast). A minimum of 5 random locations should be acquired for each sample and samples should be prepared in duplicate rendering a total of 10 images per condition analyzed. All images should be saved as .tiff files for digital analysis (see section 3.5.2).

3.5.2. Digital Analyses to Measure Cell Morphology—The digital analysis can be carried out manually or automatically, this section describes how to measure cell morphology using the MetaMorph 7.01 software (Molecular Devices):

1. Using the Metamorph software, open the three files containing the monochromatic images acquired in the previous section corresponding to the cytosolic (CFDA-SE) and nuclear (Hoechst) compartments, as well as the transmitted light channel (e.g., phase contrast) image.
2. Go to the “Apps” menu and select “Cell scoring.” Select the image corresponding to the Hoechst staining as the “W1 Source image” (All nuclei) and the CFDA-SE staining for “W2 Source image” (Positive marker). Select a representative nucleus and cytoplasm, respectively.
3. Determine the minimum and maximum width of the nuclei and cytoplasm, using the single line tool, while pointing at it to determine the intensity above the background for both images.
4. Click on “Preview” for each image to assess if your selected areas depict the correct nuclei (Hoechst positive) and cytosolic (CFDA-SE positive) fractions. If correct, continue to step 5; otherwise, modify the parameters until accurate previews are obtained.
5. Select “Apply;” an accurate image named “segmentation” will appear and both nuclei and cytosols will be evident in it (see Figure 3).
6. “Threshold” the resulting “segmentation” image by selecting “Auto Threshold for Light Objects” (localized on the left toolbar of the image).
7. Open the “Measure” menu and select “Integrated Morphology Analysis.”
8. In the list of parameters to be measured, select “Area,” “Breadth,” “Length,” and “Elliptical Form Factor.” Make sure that these parameters are selected under the Show/Log Data section.
9. Log the data to an Excel file.
10. Analyze the Excel data by averaging and questioning the variation of the samples. Cells that present amoeboid like morphologies will present elliptical form factors near 1 while mesenchymal spindle-shape factors will render smaller number results.

OPTIONAL: for manual measurements print each image onto girded (see-through) paper and determine the length (the span of the longest chord of the cell) and the breadth (the caliper width of the cell, perpendicular to the longest chord) for each cell. Then, calculate length over breadth and estimate as in step 10.

3.5.3. Statistical Analysis

1. A minimum of 12 cells are needed for statistical analyses (many more are expected). Each experimental condition should have been repeated at least once more.
2. The elliptical form factors should be compared using a Welch corrected t-test. The unpaired t-test assumes that the two populations have the same variances. Since the variance equals the standard deviation squared, this means that the populations have the same standard deviation. A modification of the t-test (developed by Welch) can be used when you are unwilling to make that assumption. These values can be calculated using any statistical software (e.g., Instat).
3. The resultant number will be indicative of the p value:
 - a. A p value greater than 0.05 should be stated not significant.
 - b. A p value between 0.05 and 0.001 is regarded as statistically significant.
 - c. A p value between 0.001 and 0.0001 should be stated “very” significant.
 - d. A p value equal or lower to 0.0001 should be regarded as statistically “extremely” significant.

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Figure 1. Western blot analysis for the characterization of fibroblastic and/or epithelial cell populations

Protein lysates were loaded on two identical gels and proteins were separated by SDS-PAGE and then transferred to PVDF membranes. Membranes were blotted using antibodies against epithelial marker, pan-keratin (top) or fibroblastic marker, vimentin and loading control, GAPDH (bottom). Positive controls for both fibroblastic and epithelial cells, as well as three samples characterized as fibroblastic are shown. Molecular weight markers are stated.

Figure 2. Characteristic tumor-associated unextracted 3-D matrix culture

Reconstituted confocal images obtained from indirect immunofluorescence of a tumor-associated unextracted 3-D culture. **A**, homogenous α -SMA expression along stress fibers (red). **B**, parallel patterned matrix labeled with fibronectin (green). **C**, organized, condensed and elliptically shaped nuclei characteristic of myofibroblastic cells (blue). **D**, is a merged image of all three monochromatic images. Note that monochromatic images were arbitrarily designated with the colors shown.

Figure 3. Example of nuclei and cytosolic images rendering segmented file for morphometry analyses

Left panel shows the nuclei staining, while the middle panel the cytosolic compartment of the same cells. The resultant right panel image is the computer version of the cells selected for analyses that will render the cell's morphometric quantifications.