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Determining the Role of Matrix Compliance in the Differentiation of Mammary Stem Cells

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Abstract

Multipotent stem cells maintain the structure and function of the mammary gland throughout its development and respond to the physiological demands associated with pregnancy and lactation. The ability of mammary stem cells to maintain themselves as well as to give rise to differentiated progeny is not only affected by soluble factors but has increasingly become linked to mechanical cues including the elastic modulus of the extracellular matrix (ECM). Here we describe a protocol for determining how the mechanical properties of the ECM regulate the fate of mammary stem or progenitor cells. This protocol includes detailed methods for the fabrication of substrata with varying stiffness, culture of mammary progenitor cells on synthetic substrata, pharmacological modulation of actomyosin contractility, and analysis of gene expression to define the resulting fate of human mammary stem cells.

Keywords: Differentiation, Lineage specification, Mammary stem cells, Matrix compliance, Mechanical stress, Mechanotransduction, Myoepithelial cells, Tissue morphogenesis

Abbreviations

αSMA	Alpha-smooth muscle actin
ESA	Epithelial-specific antigen
FAK	Focal adhesion kinase
K	Keratin
MLC	Myosin light chain
MSC	Mesenchymal stem cell
Muc1	Sialomucin-1
ROCK	Rho-associated kinase
TDLU	Terminal ductal lobular unit
2D	Two-dimensional
3D	Three-dimensional

1 Introduction

The mammary gland is a highly dynamic organ that undergoes dramatic morphogenetic changes during puberty, pregnancy, lactation, and post-lactational involution. During pregnancy, the ducts branch laterally to form an expanded tree with concomitant epithelial proliferation and differentiation; upon involution, the secretory epithelium undergoes apoptosis and the gland remodels back essentially to its virgin state (1–3). This regenerative and remodeling capacity of the gland requires multipotent stem or progenitor cells in the mammary epithelium. Mammary stem or progenitor cells provide dynamic and flexible attributes to the mammary gland and give rise to either the mature luminal epithelium or to the myoepithelium through a series of lineage specification and concomitant differentiation (3, 4). Human mammary stem or progenitor cells are characterized by the expression of both keratin (K)14 and K19, and generate the bi-layered terminal ductal lobular unit (TDLU), the basic functional structure of the human mammary epithelium (5, 6). Cells from the two epithelial layers of the TDLU express a number of specific proteins that are frequently used as lineage markers: the inner layer of luminal epithelial cells expresses K8, K18, K19, and sialomucin-1 (Muc1), and the outer layer of myoepithelial cells expresses K5, K14, and α-smooth muscle actin (αSMA) (5–7).

Maintenance and differentiation of mammary progenitor cells are governed by complex interactions between cell–cell, cell-extracellular matrix (ECM), and cell-soluble factors as well as by mechanical cues present within the tissues (2, 7–9). Although the regulatory roles of soluble signals, such as growth factors and cytokines, for stem cells have long been appreciated, recent studies demonstrate that the regulation of stem cell fate by these biochemical signals is also strongly affected by co-existing adhesive, mechanical, and topological cues (8, 10, 11). Moreover, regulation of stem cells in vivo normally occurs in the context of development, tissue regeneration, and wound healing, in which the mechanical environment surrounding the stem cells changes dynamically (10, 12). The modulus of elasticity (E) of the ECM, also commonly but incorrectly referred to as tissue stiffness, not only varies within the body from soft brain tissue (0.1 kPa) to rigid calcifying bone (>30 kPa) (13, 14), but also changes dynamically over the course of development (e.g., mammary branching morphogenesis), in response to function (e.g., mammary gland lactation), and during pathogenesis (e.g., tissue fibrosis and tumorigenesis) (14–18). Such changes in matrix elasticity have been shown to influence cellular behaviors including proliferation, locomotion, spreading, and differentiation of stem cells (13, 17, 19, 20). Culture of multipotent mesenchymal stem cells (MSCs) on synthetic matrix mimicking the elasticity of ECM within a tissue leads to tissue-specific gene

expression and promotes organ-specific differentiation (13). Matrix compliance also regulates the renewal and fate-switching of mammary progenitor cells (7).

Stem cells sense and respond to mechanical signals through integrin-mediated adhesions and the force balance between intracellular cytoskeletal contractility and resistance from the ECM (11, 21). Cell-ECM adhesions lead to the phosphorylation of focal adhesion kinase (FAK) and recruitment of vinculin to transmit forces between the ECM and the cytoskeleton, in which myosin-mediated contractility acts as a primary regulator of cellular contractile forces (11, 22, 23). These intracellular forces regulate signaling pathways involved in fundamental cellular processes which play a critical role in determining cell phenotype, such as stem cell differentiation (7, 13, 17). RhoA, a member of the Rho family of small GTPases, regulates signaling involved in cytoskeletal reorganization. RhoA stimulates cytoskeletal tension through its effector, Rho-associated kinase (ROCK), which directly phosphorylates myosin light chain (MLC) and MLC phosphatase to synergistically increase MLC phosphorylation and thus myosin II contractility (24–26). Inhibiting cytoskeletal tension using Y27632 (a ROCK inhibitor), blebbistatin (a myosin II inhibitor), or ML-7 (a MLC kinase inhibitor), or enhancing actomyosin contraction using calyculin A (a MLC phosphatase inhibitor) significantly affects the phenotype of mammary stem cells as well as MSCs (7, 21, 27, 28).

Here, we describe methods for examining how matrix compliance affects the differentiation of human mammary progenitor cells. This protocol presents technical details for the fabrication of synthetic matrices of varying stiffness, culture of mammary progenitor cells on these synthetic substrata, pharmacological modulation of actomyosin contractility, and cellular and molecular characterization of mammary progenitor cell fate in response to alterations in the mechanical properties of the ECM. This series of methods can be used to examine how changing the mechanical properties of the tissue microenvironment affects the phenotypes of stem and progenitor cells as well as differentiated cells (7, 17).

2 Materials

2.1 Cell Culture

1. D920 human mammary progenitor cells (6).
2. H14 medium: 1:1 DMEM:F12 medium supplemented with 250 ng/ml insulin, 10 μg/ml human transferrin, 2.6 ng/ml sodium selenite, 0.1 nM estradiol, 1.4 μM hydrocortisone, 5 μg/ml prolactin, and 0.1 mM gentamicin.
3. Non-pepsinized collagen type I (bovine; Koken).
4. Phosphate-buffered saline (PBS).

5. Trypsin-EDTA (0.05 %).

2.2 Synthesis and Functionalization of Synthetic Substrata

1. Coverglass (31.75 mm diameter, 17–25 mm thick; Fisher Scientific).
2. Germicidal UV lamp (365 nm).
3. Acrylamide (40 % solution).
4. *N*,*N*′-Methylene bisacrylamide (2 % solution).
5. Ammonium persulfate (APS).
6. *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine (TEMED).
7. 3-Aminopropyltrimethoxysilane (APTMS; Sigma).
8. Acetone.
9. Glutaraldehyde (50 % stock; Sigma).
10. Dichlorodimethylsilane.
11. Toluene.
12. Methanol.
13. *N*-Sulfosuccinimidyl-6-(4′-azido-2′-nitrophenylamino) hexanoate (Sulfo-SANPAH; Pierce).
14. 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; 50 mM, pH 8.5).
15. Non-pepsinized collagen type I (bovine; Koken).
16. 6-well plate.

2.3 Pharmacological Manipulation of Cell Contractile Machinery

1. Y27632 (Tocris).
2. Blebbistatin (Sigma).
3. Calyculin A (Calbiochem).

2.4 Immunofluorescence Staining and Microscopy Analysis

1. Phosphate-buffered saline (PBS).
2. Paraformaldehyde (4 % in PBS).
3. Triton-X-100 (0.3 % in PBS).
4. Goat serum (10 % in PBS).
5. Rabbit anti-keratin-14 antibody (Covance).
6. Mouse anti-keratin-8 antibody (AbCam).
7. Rabbit anti-phospho-focal adhesion kinase ($pFAK^{Tyr397}$) antibody (Invitrogen).
8. Rabbit anti-vinculin antibody (Cell Signaling).
9. Alexa Fluor 488 phalloidin (Invitrogen).
10. Alexa 594 goat-anti-rabbit (Invitrogen).
11. Alexa 488 goat-anti-mouse (Invitrogen).
12. Hoechst 33342 (Invitrogen).
13. Inverted fluorescence microscope.

Table 1
Primers used to determine mammary progenitor cell fate via qRT-PCR

Gene	Sequences
18S rRNA	Fwd: CGCCGACGACCCATTCGAAC Rev : GAATCGAACCCTGATTCCCCGTC
Keratin-8	Fwd: AGTTACGGTCAACCAGAG Rev : GTCTCCAGCATCTTGTTC
Keratin-14	Fwd: AACCACGAGGAGGAGATG Rev : GTTCAGAATGCGGCTCAG
Keratin-19	Fwd: GCGACTACAGCCACTACTAC Rev : GTCTCAAACTTGGTTCGGAAG
E-cadherin	Fwd: TGAAGGTGACAGAGCCTCTGGAT Rev : TGGGTGAATTCGGGCTTGTT
P-cadherin	Fwd: GCTGAACATCACGGACAAG Rev : CCTTCCTCGTTGACCTCTG
p63	Fwd: TTGTTGGAAAGTAACTGTGAGAAC Rev : CAAGGGAACTCTTCGTTTAAGTG
αSMA	Fwd: GAGTTACGAGTTGCCTGATG Rev : GGTCCTTCCTGATGTCAATATC

2.5 Quantitative Real-Time PCR (qRT-PCR) Analysis

1. Phosphate-buffered saline (PBS).
2. Trizol reagent (Invitrogen).
3. Diethylpyrocarbonate (DEPC) water.
4. cDNA synthesis kit (Thermo Scientific).
5. iQ SYBR Green Supermix (BioRad).
6. Primers (Table 1).
7. Real-Time PCR detection system (such as MiniOpticon™ Real-Time PCR detection system).
8. UV spectrophotometer.

3 Methods

3.1 Mammary Progenitor Cell Culture

D920 human mammary progenitor cells were derived from epithelial-specific antigen $(ESA)^{+}/Muc1^{-}$ human breast stem cells and immortalized with human papilloma virus (HPV) proteins E6 and E7 (5). Not only do D920 cells express K19, a marker for mammary stem cells, but clones give rise to cells expressing combinations of K19 and K14 in two-dimensional (2D) culture and generate discretely bi-layered TDLU-like structures in vivo in cleared fat pads of NOD/SCID mice and in three-dimensional

(3D) matrigel (5). The following protocol describes detailed methods to maintain D920 mammary progenitor cells.

1. Coat cell culture plate with collagen (50 μg/ml) and incubate at 4 °C overnight.
2. Rinse the collagen-coated plate twice with 1× PBS.
3. Trypsinize D920 cells using 2 ml of Trypsin-EDTA (0.05 %).
4. Collect cells with 10 ml H14 medium into a 15 ml tube.
5. To remove residual Trypsin-EDTA, centrifuge the suspension at 100 × *g* for 5 min, aspirate the supernatant, and resuspend cells with fresh H14 medium.
6. Plate D920 human mammary progenitor cells on the collagen-coated plate using H14 medium (see Note 1).
7. Change medium every 48 h.

3.2 Preparation of Synthetic Substrata of Varying Stiffness for Cell Culture

This protocol describes methods to create synthetic matrices with tunable elasticity from polyacrylamide (Fig. 1). Polyacrylamide provides several advantages as a cell culture substratum: its elasticity can be tuned precisely by changing the relative concentrations of acrylamide and bis-acrylamide (20); the surface chemistry of the gel can be kept constant while changing its mechanical properties (17, 20); nonspecific binding of proteins or cells are negligible and thus the adhesive molecules that are covalently attached to the surface serve as the primary ligands for cell attachment (29); the porosity of the gels allows for the flow of medium and provides a more physiological environment than do solid surfaces (30). Table 1 provides the expected elastic moduli for specific concentrations of acrylamide and bis-acrylamide. The viscoelastic properties of the polyacrylamide gels were determined using rheometry, and the Young's moduli of the gels were calculated from the shear modulus, G, using the following equation: $E = 2G(1 + \nu)$, where ν is the Poisson ratio ($\nu = 0.48$ for polyacrylamide) (17, 31). The following protocol is written for 31.75 mm-diameter coverglass for use in a 6-well plate. The volumes can be adjusted up or down to compensate for larger or smaller coverglass, as needed.

1. To render the surface hydrophobic, incubate coverglass in 0.1 N NaOH for 30 min (see Note 2). Rinse coverglass three times thoroughly with MilliQ dH_2O and after the last wash, dry completely using a vacuum aspirator (slides can be stored under nitrogen after this step). Follow steps 2 and 3 for the bottom slide and follow step 4 for the top slide.
2. [Bottom slide] In a chemical fume hood, incubate cover glass in 2 % 3-aminopropyl trimethoxysilane (APTMS) diluted in acetone for 30 min. Rinse each coverglass three times with acetone and air dry (see Note 3; slides can be stored under nitrogen after this step).

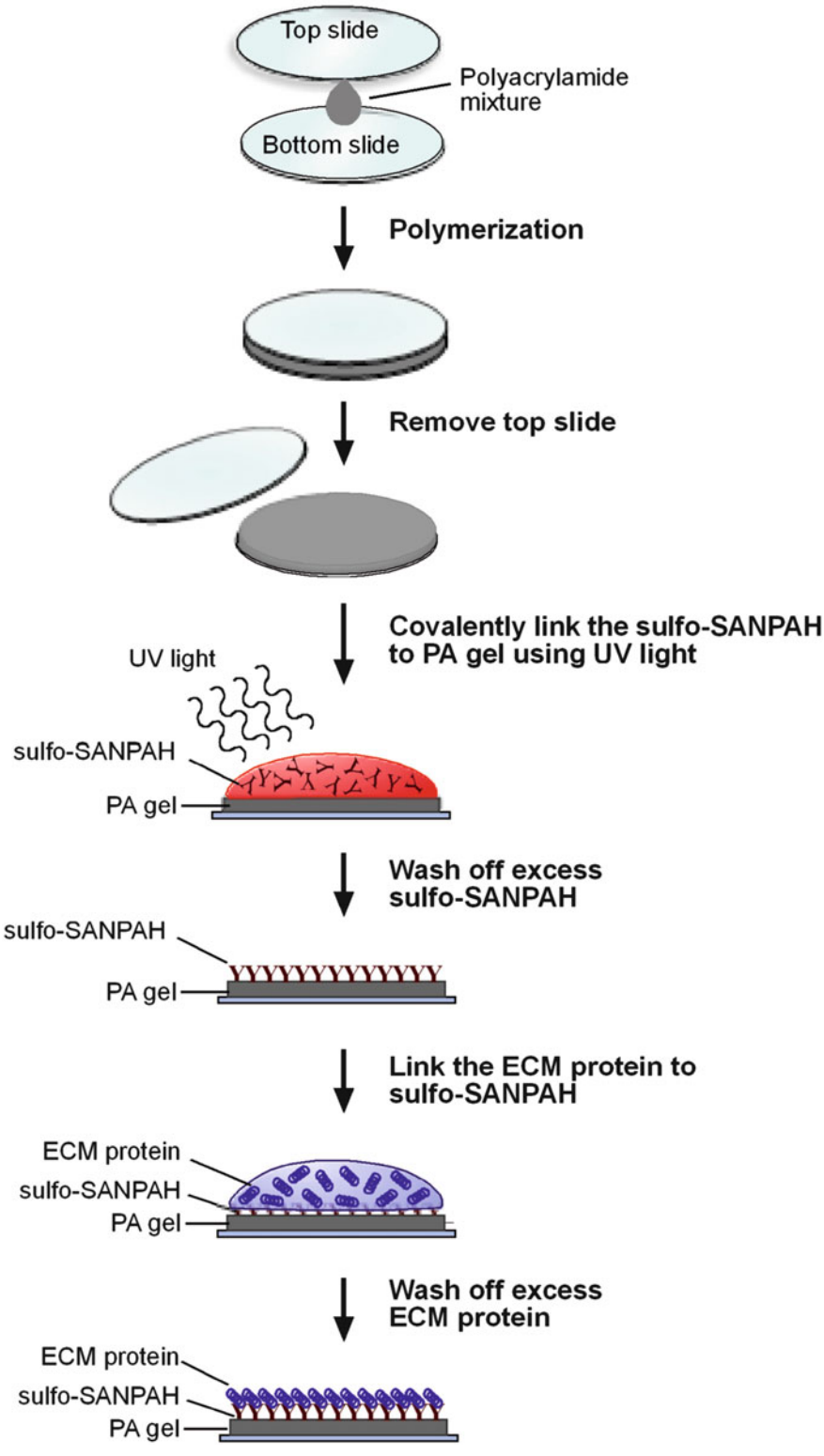

Fig. 1 Schematic overview of the setup for making synthetic substrata. The gel-glass composite contains the amino-silanated coverglass (*bottom slide*), polymerizing acrylamide solution, and chloro-silanated coverglass (*top slide*). The polyacrylamide gel is functionalized by cross-linking sulfo-SANPAH at 365 nm followed by attachment of desired ECM protein

Table 2
Polyacrylamide solutions to generate synthetic substrata of various compliances

Elasticity (Pa)	% Acrylamide	% Bis-acrylamide	Acrylamide (μl)	Bis-acrylamide (μl)	dH_2O (μl)	TEMED (μl)	10 % APS (μl)
130	5	0.01	125	5	864.5	0.5	5
910	5	0.03	125	15	854.5	0.5	5
2,030	5	0.06	125	30	839.5	0.5	5
4,020	5	0.35	125	175	694.5	0.5	5

3. [Bottom slide] In a chemical fume hood, incubate coverglass in 0.5 % glutaraldehyde diluted in 1× PBS for 30 min. Rinse coverglass three times with MilliQ dH_2O and dry completely with vacuum aspirator. (Slides can be stored under nitrogen after this step.)
4. [Top slide] In a chemical fume hood, incubate coverglass in 2 % dichlorodimethylsilane diluted in toluene for 30 min. Rinse coverglass three times with methanol and air dry. (Slides can be stored under nitrogen after this step.)
5. Prepare polyacrylamide solution using acrylamide and bis-acrylamide at desired final concentration. See Table 2 for concentrations and the corresponding elastic modulus. Combine acrylamide, bis-acrylamide, and MilliQ dH_2O in a 1.5 ml tube. Degas the mixture under vacuum for 30 min to remove dissolved oxygen (see Note 4). Just before removing the polyacrylamide solution from the vacuum, prepare 10 % solution of APS. Add appropriate volumes of TEMED and APS to the degassed polyacrylamide solution (Table 2).
6. Place 36 μl of the polyacrylamide mixture onto the center of the bottom slide and cover with the top slide (Fig. 1). The setup resembles a sandwich in which the polymerizing solution sits between the top and bottom coverglass (see Notes 5 and 6).
7. Allow the gel to polymerize for 30 min. After polymerization, place the gels in 1× PBS. These hydrogels can be stored for long periods of time without altering mechanical properties. To store them, immerse hydrogels in 1× PBS to keep them hydrated at 4 °C.
8. Remove the top slide carefully and rinse the gel twice with 1× PBS to remove unpolymerized acrylamide.

The following steps should be performed in a biosafety cabinet using sterilized materials:

9. Incubate the polyacrylamide gel with 100 % ethanol for 5 min and wash three times with 1× PBS. Incubate the gel with 50 mM HEPES, pH 8.5 for 5 min. During the incubation, prepare 2 mM of sulfo-SANPAH dissolved in sterilized MilliQ dH_2O (see Note 7).
10. Aspirate excess 50 mM HEPES and place 200 μl of 2 mM sulfo-SANPAH on top of the gel and swirl gently to coat the surface completely (see Note 8).
11. Expose the gel to a germicidal UV lamp (365 nm) for 10 min and rinse once with 50 mM HEPES, pH 8.5.
12. Repeat steps 10–11 and rinse the gel three times with 50 mM HEPES, pH 8.5.
13. Pipet 200 μl of 0.2 mg/ml of collagen diluted in 1× PBS on top of the gel and swirl gently to coat the gel. Incubate gel overnight at 4 °C (see Notes 9–11).

3.3 Culture of Mammary Progenitor Cells on Substrata with Varying Compliance

1. To remove extra collagen from the polyacrylamide surface, wash collagen-coated gels three times with 1× PBS, followed by incubation in culture medium at 37 °C for 1 h.
2. Place gels in a 6-well culture plate.
3. Trypsinize D920 mammary progenitor cells.
4. Plate 1–2 × 10^5 D920 mammary progenitor cells on each gel using H14 medium and culture for 24–72 h.

3.4 Pharmacological Manipulation of Cytoskeletal Contractility

1. Plate D920 mammary progenitor cells on the collagen-functionalized polyacrylamide gel or on a collagen-coated cell culture plate.
2. After 24 h, change medium to fresh H14 medium that includes the desired inhibitor. Y27632 (10 μM) and blebbistatin (12.5 μM) can be used to inhibit cytoskeletal tension. Conversely, calyculin A (2 nM) can be used to enhance actomyosin contraction. A detailed description of these inhibitors is provided above.

3.5 Immunofluorescence Staining to Determine the Fate of Mammary Progenitor Cells

Immunofluorescence staining allows the visualization of specific proteins in cells by binding a specific antibody chemically conjugated with a fluorescent dye such as Alexa fluor. These labeled antibodies directly or indirectly bind to the antigen of interest, which allows for detection of the protein through fluorescence techniques. The fluorescence can be visualized using widefield or confocal microscopy and quantified using a flow cytometer, automated imaging instrument, or imaging software.

1. Rinse cells cultured on collagen-coated gels with 1× PBS and fix in 4 % paraformadehyde solution diluted in 1× PBS for 15 min.

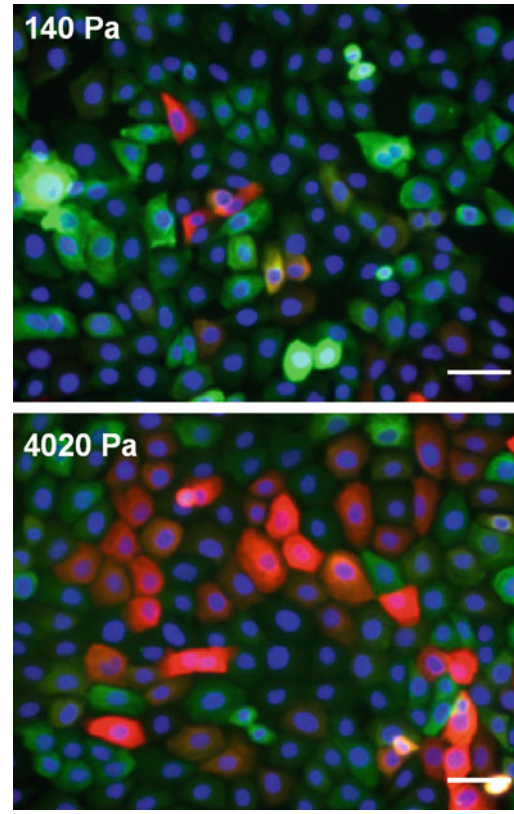

Fig. 2 Immunofluorescence analysis of mammary progenitor cells cultured on substrata of different compliances. Shown are staining for K14 (*red*) and K8 (g*reen*). Scale bars, 50 μm

2. Rinse samples twice with 1× PBS and permeabilize with 0.3 % Triton-X-100 in 1× PBS for 10 min.
3. Rinse samples twice with 1× PBS and incubate with blocking reagent (0.1 % Triton-X-100 in 10 % goat serum in PBS) for 6 h at room temperature or overnight at 4 °C.
4. Incubate with primary antibody diluted in 10 % goat serum at 4 °C overnight with shaking. Changes in cell fate can be determined using antibodies against cytokeratins (Fig. 2). Alterations in cell-ECM adhesions and cytoskeletal contraction in response to substratum stiffness can be determined using antibodies against FAK or vinculin, or by labeling F-actin with phalloidin. The roles of these proteins are described above.
5. Wash samples three times with 1× PBS for 15 min.
6. Incubate samples with secondary antibody conjugated with fluorescent dye (e.g., Alexa Fluor 488 conjugate) for 2 h at room temperature.
7. Wash samples three times with 1× PBS for 15 min.

8. Counterstain the nuclei of cells with Hoechst 33342 for 10 min prior to imaging.
9. Analyze using ImageJ to determine the number of cells expressing specific keratins.

3.6 Gene Expression Analysis to Determine Mammary Progenitor Fate

Quantitative real-time PCR (qRT-PCR) is a method used to detect relative levels of gene expression. SYBR green is frequently used as a fluorescent dye for qRT-PCR. SYBR intercalates with double-stranded DNA and this intercalation causes the SYBR to fluoresce, which can be detected with a qPCR machine and converted into Ct values from the intensity of the fluorescence. The following protocol covers the qRT-PCR technique using SYBR green methodology and overviews how to analyze data. Lineage specification of mammary progenitor cells can be determined by analyzing the expression levels of luminal epithelial markers (K8, K19, and E-cadherin) or myoepithelial markers (K14, P-cadherin, αSMA, and p63). Sequences for primers to amplify these genes are listed in Table 1.

3.6.1 RNA Isolation from Mammary Progenitor Cells

1. Collect cells by trypsinization and centrifuge at 100 × *g* for 5 min.
2. Lyse cells in Trizol reagent by repetitive pipetting. Use 1 ml of the reagent for 1–2 × 10^6 cells. Incubate the homogenized samples for 5 min at room temperature for the complete dissociation of the nucleoprotein complexes.
3. Add 0.2 ml of chloroform per 1 ml Trizol reagent and shake tubes vigorously for 20 s.
4. Centrifuge the sample at 18,000 × *g* for 15 min. Following centrifugation, the mixture separates into a lower red phase (phenol–chloroform), an interphase, and a colorless upper aqueous phase that contains RNA.
5. Transfer the aqueous phase into a 1.5 ml tube and precipitate the RNA by adding 0.5 ml isopropanol. Mix the sample well by inverting the tube and incubate for 10 min. The RNA precipitate is often invisible at this step.
6. Centrifuge the sample at 18,000 × *g* at 4 °C for 15 min. The RNA pellet may be visible after centrifugation.
7. Remove the supernatant and wash the RNA pellet twice with 1 ml of 75 % ethanol. Resuspend the RNA pellet by vortexing and centrifuge at 11,000 × *g* at 4 °C for 5 min.
8. Remove the supernatant (75 % ethanol) and air-dry the RNA pellet (see Note 12).
9. Dissolve RNA in 30–40 μl DEPC-treated water and measure the concentration of RNA by determining the absorbance at 260 nm in a spectrophotometer (see Note 13). Purified RNA can be maintained at −20 or −70 °C for long-term storage (see Note 14).

Table 3
Reverse transcription reaction mix

Reaction mix	Final concentration	Volume (μl)
5× Reaction buffer	1×	4
dNTPs mix (5 mM)	500 μM	2
Oligo dT	500 ng	1
RT enhancer		1
Reverse transcriptase		1
DEPC dH_2O		11 – *x* μl for 1 μg of RNA
RNA	1 μg	*x* μl for 1 μg of RNA
Total		20

Table 4
Thermal cycles for reverse transcription

Procedure	Temperature (°C)	Time (min)	Number of cycles
cDNA synthesis	42	30	1
Inactivation[a]	95	2	1

[a]After inactivation, place cDNA immediately on ice

3.6.2 cDNA Synthesis Using Reverse Transcription (RT) of RNA

1. Mix 1 μg of RNA with RT-reaction mix as described in Table 3.
2. Spin down reaction mix and follow thermal cycles as described in Table 4.

3.6.3 Quantitative Real-Time PCR (qRT-PCR) Using SYBR Green

1. Mix cDNA with SYBR green reaction mix in 0.2 ml qPCR tube as described in Table 5. Primers used for detecting human mammary progenitor cell fate are listed in Table 1.
2. Spin down reaction mix and run the thermal cycles as described in Table 6 using an optical qPCR thermal cycler.
3. Determine the fold-change in expression of each target mRNA relative to 18S rRNA based on the threshold cycle (Ct) as follows (see Note 15):

$$\text{Fold change} = 2^{-\Delta(\Delta Ct)}[Ct = Ct_{target} - Ct_{18S};\ \Delta(\Delta Ct) = \Delta Ct_{treatment} - \Delta Ct_{control}]$$

Table 5
qRT-PCR reaction mix

Reaction mix	Final concentration	Volume (μl)
2× SYBR Green super mix	1×	12.5
Primer (forward; 5 μM)	100 nM	0.5
Primer (reverse; 5 μM)	100 nM	1
cDNA		1
DEPC dH_2O		10
Total		25

Table 6
Thermal cycles for qRT-PCR

Procedure		Temperature	Time	Number of cycles
Polymerase activation and DNA denaturation		95 °C	3 min	1
Amplification	Denaturation	95 °C	10 s	40
	Annealing	55–60 °C	15 s	
	Extension	72 °C	10 s	
	Read plate			
Extension		72 °C	10 min	1
Melt curve analysis		65–98 °C increment 0.2 °C for 0.01 s		1
Read plate				
Final extension		72 °C	10 min	1

4 Notes

1. The plasticity of mammary progenitor cells may be affected by increasing time and passage in culture. To retain multipotency, D920 cells should be maintained within a narrow passage window and passaged approximately every 5 days when the cells are ~80 % confluent. The multipotency of D920 progenitor cells can be confirmed by expression of K14 and K19 in 2D culture.

2. For uniform gel attachment, coverglass should be coated with an even layer of 0.1 N NaOH. After evaporation, a thin semi-transparent film of NaOH will remain on the coverglass. If needed, repeat this step until a thin white film of NaOH is visible on the surface. Alternatively, this step can be performed by adding 0.1 N NaOH to the surface of the coverglass and heated at 80 °C until the liquid has evaporated.
3. It is important to completely rinse off any residual APTMS, for it will create a brown precipitate with glutaraldehyde that fluoresces under UV light and may thus interfere with later procedures.
4. Dissolved oxygen in the solution will act as a sink for the free radical polymerization. Degassing the solution will not only speed up polymerization but will also ensure more uniform polymerization.
5. Polymerization is initiated immediately after adding 10 % APS and TEMED. To ensure a uniform polymerization of the polyacrylamide gel, step 6 must be performed within a short period of time (couple of minutes).
6. Be careful to avoid air bubbles. The presence of bubbles creates discontinuous regions within the polyacrylamide gel that may cause the mechanical properties to vary spatially within the substratum.
7. Sulfo-SANPAH is light sensitive and should be shielded from light until use.
8. Complete coverage of sulfo-SANPAH is necessary to ensure even coating of ECM proteins on synthetic substrata.
9. Due to the aqueous instability of the sulfosuccinimidyl ester, conjugation of ECM proteins should begin immediately upon activation. If making large gels, place the gel on a rocker to ensure that the solution of ECM protein remains well mixed and that the gels are coated evenly.
10. The concentration of ECM protein can be optimized for each cell type. It is critical that the protein solution not precipitate for proper conjugation of the ECM protein to the polyacylamide gel. Increasing the collagen concentration within the solution to enhance binding may cause the collagen to precipitate once warmed to physiological temperature. We have successfully used concentrations of 0.2 mg/ml of bovine collagen and 0.2 mg/ml of fibronectin, which have previously been shown to support adhesion of D920 mammary progenitor cells (7) and mammary epithelial cells (17).
11. The amount of the ECM protein conjugated to the surface of the polyacrylamide gel can be measured using an enzyme-linked immunosorbent assay (ELISA) or fluorescently labeled

protein (13, 17). Fluorescent staining can also be used to confirm uniform coating of the protein.

12. Do not dry the RNA by centrifugation under vacuum. Overdrying the RNA will greatly decrease its solubility.
13. The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of RNA. A ratio of 260/280 greater than 1.8 is generally accepted as pure for RNA. The ratio of absorbance at 260 and 230 nm is also used as a secondary measure of RNA purity. The 260/230 ratio for pure RNA is often greater than 2.0. Significantly lower ratios may indicate the presence of protein, phenol, or other contaminants that absorb strongly at or near 280 or 230 nm.
14. Freeze and thaw RNA samples as little as possible. It may be better to keep samples on ice for a few hours than to refreeze them in between. Quickly thaw samples then place them on ice.
15. The Ct value is used for accurate quantification of gene expression by qPCR. Ct value should be measured when the level of fluorescence gives signal over the background and is in the linear portion of the amplification curve.

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