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Epithelial and Mesenchymal Subpopulations Within Normal Basal Breast Cell Lines Exhibit Distinct Stem Cell/Progenitor Properties

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ABSTRACT

It has been proposed that epithelial–mesenchymal transition (EMT) in mammary epithelial cells and breast cancer cells generates stem cell features, and that the presence of EMT characteristics in claudin-low breast tumors reveals their origin in basal stem cells. It remains to be determined, however, whether EMT is an inherent property of normal basal stem cells, and if the presence of a mesenchymal-like phenotype is required for the maintenance of all their stem cell properties. We used nontumorigenic basal cell lines as models of normal stem cells/progenitors and demonstrate that these cell lines contain an epithelial subpopulation ("EpCAM+," epithelial cell adhesion molecule positive [EpCAM^{pos}]/CD49f^{high}) that spontaneously generates mesenchymal-like cells ("Fibros," EpCAM^{neg}/CD49f$^{med/low}$) through EMT. Importantly, stem cell/progenitor properties such as regenerative potential, high aldehyde dehydrogenase 1 activity, and formation of three-dimensional acini-like structures predominantly reside within EpCAM+ cells, while Fibros exhibit invasive behavior and mammosphere-forming ability. A gene expression profiling meta-analysis established that EpCAM+ cells show a luminal progenitor-like expression pattern, while Fibros most closely resemble stromal fibroblasts but not stem cells. Moreover, Fibros exhibit partial myoepithelial traits and strong similarities with claudin-low breast cancer cells. Finally, we demonstrate that Slug and Zeb1 EMT-inducers control the progenitor and mesenchymal-like phenotype in EpCAM+ cells and Fibros, respectively, by inhibiting luminal differentiation. In conclusion, nontumorigenic basal cell lines have intrinsic capacity for EMT, but a mesenchymal-like phenotype does not correlate with the acquisition of global stem cell/progenitor features. Based on our findings, we propose that EMT in normal basal cells and claudin-low breast cancers reflects aberrant/incomplete myoepithelial differentiation. STEM CELLS *2012;30:292–303*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

The ducts and lobules of the normal mammary gland are mainly composed of two different cell layers: an inner layer of secretory epithelial (luminal) cells and a basal layer of contractile myoepithelial cells. These glandular components are embedded in fibrous and adipose stromal tissue. Mammary stem cells are a specific subpopulation of basal cells with high self-renewal capacity and multilineage differentiation, and thus a single stem cell is able to generate a complete ducto-lobular tree containing functional luminal and myoepithelial cells [1, 2]. Stem cells generate this diversity of cell lineages via committed luminal or basal progenitors that have high colony forming ability in vitro but limited regenerative potential in vivo (reviewed in [3]).

In recent years, there has been increased interest in stem cells in the field of breast cancer research for two main reasons. First, it has been hypothesized that the distinct breast cancer molecular subtypes (luminal, basal, HER2, and claudin-low) originate from the transformation of specific cells at different levels of the differentiation hierarchy, and thus the phenotypic characteristics of breast tumors are reminiscent of those of their cell of origin [4, 5]. Second, it has been shown that specific cancer cells with enhanced tumor-initiating capabilities also exhibit stem cell properties such as self-renewal and differentiation, although the stem cell origin of these "Cancer Stem Cells" (CSCs) is unknown [6, 7]. Moreover, CSCs show enhanced motility, invasion, tumor-initiating ability, and resistance to chemotherapy, compared with more "differentiated" tumor cells (reviewed in [7]).

The epithelial–mesenchymal transition (EMT) is an essential developmental process that is frequently deregulated in

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diverse diseases including cancer [8]. Importantly, it has been hypothesized that acquisition of mesenchymal-like phenotype via EMT in normal breast cells and breast cancer cells associated with the acquisition of stem cell features, and that the presence of EMT characteristics in specific breast cancer molecular subtypes (i.e., claudin-low subtype) would be consistent with their stem cell origin and/or enrichment for cancer cells displaying a CSC phenotype (reviewed in [9]). This hypothesis is based on the following experimental findings: (a) claudin-low tumors, which show mesenchymal traits, exhibit gene expression patterns resembling those of breast stem cells [5, 10]; (b) similar to tumors, claudin-low breast cancer cell lines (also known as basal-B [11]) overexpress EMT transcriptional inducers such as Snail, Twist, and Zeb1, exhibit strong mesenchymal features, and show a breast stem cell-like cell surface maker profile and gene expression pattern [10, 12]; (c) tumor cells with mesenchymal/EMT traits are enriched in CSC properties including the $CD44^{high}/CD24^{neg}$ profile, enhanced motility, invasion, and tumor-initiating ability (reviewed in [13]); (d) induction of EMT via overexpression of Snail and Twist1 transcription factors or by transforming growth factor β treatment in human mammary epithelial cells (HMECs) results in the acquisition of a mesenchymal phenotype with $CD44^{high}/CD24^{neg}$ profile and enhanced stem cell features such as self-renewal and ability to form mammospheres in vitro [14, 15]. Whether the association of EMT with CSC properties in tumor cells implies a stem cell origin or acquisition of true stem cell features has proven difficult to assess due to the confounding effect of tumor dedifferentiation. Therefore, to assess the implication of EMT in breast cancer it is essential to elucidate first its functional role in normal stem cells. Specifically, it remains to be determined if EMT is an intrinsic and specific property of normal basal stem cells, and if the acquisition of mesenchymal-like phenotype via EMT is required for the generation and/or maintenance of all stem cell properties, including their potential to differentiate.

To address these questions, we have used immortalized nontumorigenic breast cell lines with a basal phenotype as they have been proposed to be models of normal stem cells [16] and can be experimentally induced to undergo EMT [15, 17, 18]. We demonstrate that these cell lines exhibit intrinsic phenotypic plasticity and spontaneously generate mesenchymal-like cells through EMT. Our results demonstrate, however, that the majority of stem cell/progenitor properties are associated with an epithelial state not with a mesenchymal-like phenotype. Consequently, we propose that normal basal cells have intrinsic capacity for EMT, but acquisition of a mesenchymal phenotype is not required for the acquisition all their stem cell/progenitor properties.

Materials and Methods

Detailed procedures are provided in the Supporting Information Methods.

Cell Culture

The nontumorigenic basal cell lines MCF10A, MCF10-2A, and MCF12A were obtained from the American Type Culture Collection (ATCC; LGC Standards, Middlesex, UK, www.lgcstandards-atcc.org). These cells were short tandem repeat (STR) profiled by the provider and used within the first 20 passages. The basal/myoepithelial Myo1089 cell line [19], originated from basal cells of normal breast tissue and immortalized by SV40 large T antigen, was provided by Drs. Louise Jones and Michael Allen (Queen Mary's University of London). These cell lines were grown in Dulbecco's modified Eagle's medium (DMEM):F12 medium supplemented with 5% horse serum (Invitrogen, Life Technologies, Paisley, UK, www.invitrogen.com), 20 ng/ml epidermal growth factor (PreproTech, London, UK, www.peprotech.com), 500 ng/ml hydrocortisone (Sigma, Poole, UK, www.sigmaaldrich.com), 100 ng/ml cholera toxin (Sigma), and 10 μg/ml insulin (Sigma). T47D, MCF7, MDA-MB-157, SUM159, BT-20, and HCC1954 breast cancer lines were obtained from ATCC (STR profiled), grown following ATCC's recommendations, and used within the first 10 passages. Three primary human breast fibroblast (HBFs) cultures were obtained from reduction mammoplasties and grown in DMEM plus 10% fetal bovine serum.

Flow Cytometry Analyses and Fluorescence-Activated Cell Sorting

Single cell suspensions were subjected to flow cytometry analysis on a LSRII instrument (BD Biosciences, Oxford, UK, www.bdbiosciences.com) using standard protocols. For sorting experiments, 2×10^4 cells from each subpopulation were purity sorted using a fluorescence-activated cell sorting (FACS) Aria instrument (BD Biosciences). Activity of aldehyde dehydrogenase 1 (ALDH1) was measured by the Aldefluor assay (StemCell Technologies, Grenoble, France, www.stemcell.com) following the manufacturer's instructions.

Mammosphere Formation and Morphogenetic Potential on Matrigel

Mammosphere experiments were performed in triplicate following the protocol previously described [20] by plating 2×10^3 cells per well onto ultralow-attachment six-well plates (Corning Life Sciences, Amsterdam, The Netherlands, www.corning.com/lifesciences). After 1 week, mammospheres were photographed with an AxioCam Icc1 camera (Zeiss, Hertfordshire, UK, www.zeiss.co.uk) coupled to a Leica DMIL microscope (Leica, Milton Keynes, UK), and spheres more than 100 μm in diameter were quantified with AxioVision-Rel 4.7 software. To test the differentiation potential of mammospheres, approximately 20 spheres were plated onto 24-well tissue culture plates coated with collagen I and cultured for 4 days in complete DMEM:F12 medium plus 5% horse serum. Colonies originated from attached spheres were stained for epithelial cell adhesion molecule (EpCAM) and vimentin (VIM). Formation of three-dimensional (3D) acinar structures was performed as described previously [21] by plating 4×10^3 sorted cells per well onto eight-well chambers (Corning) coated with growth factor-reduced Matrigel (Invitrogen). After 20 days, 3D structures were fixed with 4% paraformaldehyde, stained with Alexa488-phalloidin and 4′,6-diamidino-2-phenylindole. Fluorescent images were captured on a Leica Microsystems (www.leica-microsystems.com) TCS-SP2 confocal microscope.

Small Interfering RNA (siRNA) Transfection and Quantitative Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

A total of 1×10^4 sorted EpCAM+ cells and Fibros were transfected in quadruplicate experiments with 50 nM siRNA SMARTpools (Dharmacon, Lafayette, CO, www.dharmacon.com) targeting *SNAI2* (Slug), *ZEB1*, or nontargeting control using Dharmafect 3 reagent. Cells were grown for 4 days, and RNA was extracted with RNeasy mini kit (QIAGEN, West Sussex, UK, www.qiagen.co.uk). Quantitative RT-PCR (qRT-PCR) was performed using Assays-on-Demand Taqman probes (Applied Biosystems, Life Technologies, Paisley, UK, www.appliedbiosystems.com) on an ABI Prism 7900HT sequence detection system (Applied Biosystems). Relative expression was measured in reference to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). List of probes are provided in Supporting Information methods.

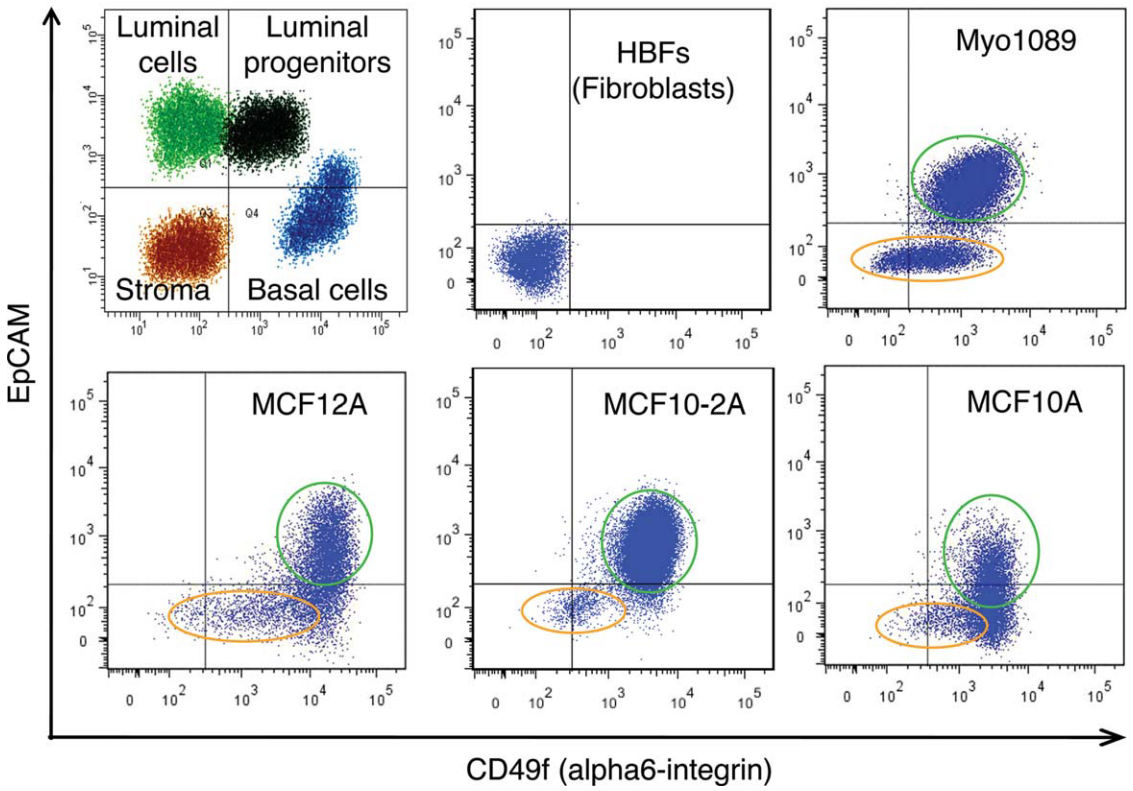

Figure 1. Flow cytometry analysis using EpCAM and CD49f identifies distinct cell subpopulations in nontumorigenic basal cell lines. Upper left panel: Representation of the four different cell subpopulations identified in the normal human mammary gland (adapted from Lim et al. [5]) (see text for details). Nontumorigenic basal cell lines contain two separate cell subpopulations, $EpCAM^{pos}/CD49f^{high}$ (green oval) and $EpCAM^{neg}/CD49f^{medium\text{-}to\text{-}low}$ (orange). The profile of HBFs is shown as control. Abbreviations: EpCAM, epithelial cell adhesion molecule; HBFs, human breast fibroblasts.

Gene Expression Profiling and Statistical Analysis

A total of 2×10^4 EpCAM+ cells and Fibros were sorted in triplicate (>98% purity) from MCF12A and Myo1089 cell lines and grown for 4 days until they reached 90% confluency. RNA was isolated using Trizol reagent (Invitrogen). As comparators, RNA was extracted from unsorted T47D cells and a pool of three human primary breast fibroblasts cultures. Three RNA samples for each cell population and cell line were hybridized onto whole genome HumanHT12_v3 Expression BeadChips (Illumina, San Diego, CA, www.illumina.com). Microarray experiments were performed by the Genomics Services Group from the Wellcome Trust Centre for Human Genetics, Oxford, UK. Microarray data were read using BeadStudio and normalized with the lumi (1.14.0) package in R by applying robust spline normalization and variance stabilization transformation to log transformed bead intensities. Microarray data have been deposited in ArrayExpress database (accession E-MTAB-804) and in the in-house ROCK database (http://rock. icr.ac.uk/collaborations/Mackay/Basal_lines/). Pairwise significance analyses of microarray (SAM) comparisons were performed with the samr package [22] using an overall false discovery rate (FDR) <1%. To obtain the list of genes that differentially expressed >1.5-fold between the distinct populations in both Myo and MCF12A cell lines, each cell line was analyzed by SAM individually and the common genes were selected. The nearest centroid correlation method was performed as described before [23]. The association of EpCAM+ and Fibros-specific gene lists with previous gene expression signatures was performed essentially as described before [10, 24]. For these analyses the following data/signatures were used: genes differentially expressed in normal breast cell populations [5], genes associated to EMT in HMECs [24], the 9-cell line predictor of claudin-low breast cancer cell lines [10], genes differentially expressed between basal-A and basal-B/claudin-low cancer cell lines [11, 25]. Gene Ontology analysis was performed with ROCK (http://rock. icr.ac.uk/).

Results

Nontumorigenic Basal Cell Lines Contain Subpopulations with Distinct Phenotypic Features

To interrogate if normal basal cells exhibit intrinsic stem cell/progenitor properties, we used three nontumorigenic cell lines with strong basal phenotype (MCF10A, MCF10-2A, and MCF12A) [11, 25] and immortalized cells with basal/myoepithelial features (Myo1089) [19]. We first assessed their differentiation status by flow cytometry analysis using EpCAM and CD49f (α6-integrin), as these two markers are able to identify the four distinct cell populations of the human normal mammary gland (Fig. 1): differentiated luminal cells ($EpCAM^{high}/CD49f^{neg}$), luminal progenitors ($EpCAM^{pos}/CD49f^{pos}$), basal/stem cell-enriched ($EpCAM^{low}/CD49f^{high}$), and stromal cells ($EpCAM^{neg}/CD49f^{neg}$) [5, 26]. Primary normal HBFs, used as a control, exhibited the expected stromal phenotype ($EpCAM^{neg}/CD49f^{neg}$). Surprisingly, the four nontumorigenic basal cell lines showed a remarkable intrinsic heterogeneity and contained two separate subpopulations: $EpCAM^{pos}/CD49f^{high}$ (luminal progenitor-like) and $EpCAM^{neg}/CD49f^{med/low}$ (basal-like to stromal-like; Fig. 1). Moreover, after isolation by FACS (Fig. 2A, Supporting Information Fig. S1) or differential trypsinization (Supporting Information Fig. S2) these two distinct cell

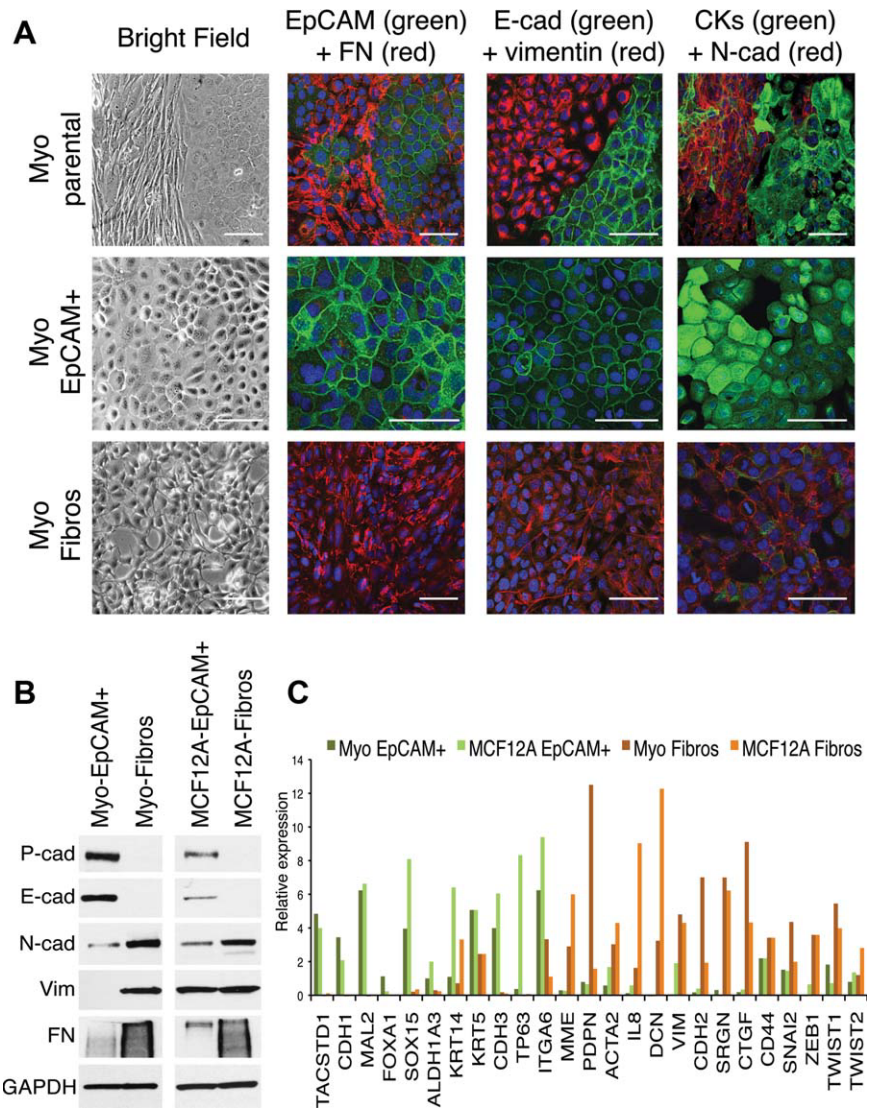

Figure 2. Phenotypic characterization of the distinct subpopulations within nontumorigenic basal cell lines. (**A**): EpCAM^{pos}/CD49f^{high} (EpCAM+) and Fibros (EpCAM^{neg}/CD49f$^{medium\text{-}to\text{-}low}$) subpopulations were isolated by fluorescence-activated cell sorting from Myo1089 cells and analyzed by immunofluorescence. Scale bar = 100 μm. (**B**): Immunoblotting confirms that isolated EpCAM+ cells from MCF12A and Myo cell lines express epithelial markers whereas Fibros cells overexpress mesenchymal markers. (**C**): A total of 25 genes differentially expressed between EpCAM+ and Fibros subpopulations from the gene expression profiling were validated by quantitative RT-PCR. Gene expression levels are relative to *GAPDH* and normalized (baseline) to a reference sample containing a pool of mRNAs from six breast cancer cell lines. Abbreviations: CKs, cytokeratins; E-cad, E-cadherin; EpCAM, epithelial cell adhesion molecule; FN, fibronectin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; N-cad, N-cadherin; P-cad, P-cadherin; RT-PCR, reverse-transcription polymerase chain reaction; Vim, vimentin.

populations showed notable phenotypic differences. Sorted EpCAM^{pos}/CD49f^{high} cells from Myo1089 (Myo-EpCAM+), MCF12A (MCF12A-EpCAM+), and MCF10-2A (MCF10-2A-EpCAM+) cell lines showed the typical cobblestone epithelial morphology, whereas sorted EpCAM^{neg}/CD49f$^{med/low}$ cells (Myo-Fibros, MCF12A-Fibros, and MCF10-2A-Fibros) exhibited a spindle morphology (Fig. 2A, Supporting Information Fig. S1). Importantly, Fibros, either within the parental cell lines or after their isolation, overexpressed mesenchymal markers (fibronectin, VIM, N-cadherin) and had decreased expression of epithelial markers (E-cadherin, cytokeratins; Fig. 2A, 2B, Supporting Information Fig. S1). Similar results were observed in sorted populations from MCF10A cells (not shown).

To delineate the molecular differences between these two populations, we performed gene expression profiling on freshly sorted EpCAM+ and Fibros subpopulations from Myo1089 (Myo) and MCF12A cell lines. Using significance analysis of microarrays (FDR <1%), 1,957 genes (represented by 2,492 probes) were differentially expressed between EpCAM+ and Fibros in both cell lines (Supporting Information Table S1). According to gene ontology analysis, a significant fraction of the genes upregulated in Fibros were functionally associated to extracellular matrix remodeling (e.g., *FN1*, *Col5A1*, and *LOX*) and cell migration (e.g., *THBS1*, *SPOCK1*), whereas EpCAM+ cells showed enrichment in genes involved in intercellular adhesion (e.g., *CLDN7*, *JUP*, and *DSC3*) and epithelial differentiation (*FOXA1*, *ELF3*) (Supporting Information Table S1). Microarray data and qRT-PCR validation demonstrated that EpCAM+ cells coexpressed luminal (e.g., *CDH1*, *MAL2*, and *FOXA1*) and basal/myoepithelial (*CDH3*, *TP63*, and *KRT5*) markers (Fig. 2C,

Supporting Information Table S1), a phenotype that is consistent with their progenitor-like FACS profile. By contrast, Fibros showed high expression of mesenchymal markers (e.g., *CDH2*, *FN1*, *CTGF*, and *DCN*), and genes functionally associated with EMT, such as *TGFB2*, *IL8* [27], and the EMT-transcriptional inducers *TWIST1*, *TWIST2*, *SNAI2*/*Slug*, *ZEB1*, and *ZEB2* [28] (Fig. 2C, Supporting Information Table S1). Of note, Fibros exhibited partial myoepithelial traits, as they expressed low but detectable mRNA and protein levels of basal keratins (*KRT5* and *KRT14*) and high expression of *VIM*, CD10 (*MME*), and podoplanin (*PDPN*) (Fig. 2C, Supporting Information Fig. S3) but did not have detectable protein expression of other bona fide myoepithelial markers such as P-cadherin (*CDH3*) (Fig. 2B), p63 (*TP63*), and α-smooth muscle actin (*ACTA2*) (Supporting Information Fig. S3). Therefore, whereas EpCAM+ cells have an undifferentiated phenotype, Fibros exhibit characteristics of cells that have undergone EMT and display partial myoepithelial features.

Spontaneous EMT in Basal Cells Does Not Increase All Stem Cell/Progenitor Properties

As it has been reported that generation of mesenchymal-like cells via experimentally induced EMT in HMECs promotes stem cell features [14, 15], we first assessed if Fibros arise from EpCAM+ cells via EMT or vice versa. EpCAM+ and Fibros subpopulations were sorted from MCF12A and Myo1089 with high purity (>99%) and cultured for six passages. Flow cytometry analysis demonstrated that the EpCAM+ cells from both cell lines were able to continuously generate Fibros, but the reverse rarely occurred (Fig. 3A, Supporting Information Fig. S4). The emergence of E-cadherin^{neg}/vimentin^{pos}/fibronectin^{pos} mesenchymal-like cells from purity sorted EpCAM+ cells, and the manifest similarity between Fibros isolated from the parental cell lines and those originated from sorted EpCAM+ cells (Supporting Information Fig. S5), demonstrate that mesenchymal-like cells are spontaneously generated from EpCAM+ cells via EMT. Moreover, the fact that Myo-Fibros did not revert to an epithelial phenotype (even after cultured >35 passages; data not shown) and MCF12A-Fibros showed only minimal generation of EpCAM+ cells after six passages (Supporting Information Fig. S4) also indicates that the stem cell property of regeneration in vitro (capacity to regenerate the phenotypic heterogeneity of the parental cell line) predominantly resides within the EpCAM+ population. To determine further whether EpCAM+ cells or the spontaneously generated Fibros showed enhanced stem cell/progenitor properties, we used four additional approaches.

First, we tested if any of these populations exhibited the CD44^{high}/CD24^{neg} profile, which has been observed in cell populations with stem cell-like or CSC features (reviewed in [6]). While Myo-EpCAM+ and MCF12A-EpCAM+ were mainly CD44^{pos}/CD24^{pos}, Myo-Fibros and MCF12A-Fibros predominantly exhibited the CD44^{high}/CD24^{neg} profile but also contained CD44^{high}/CD24^{pos} cells (Fig. 3B, Supporting Information Fig. S4B). No EpCAM^{pos}/CD44^{high}/CD24^{neg} cells (another maker combination used to identify CSCs [29, 30]) were observed in these cell lines. Within the Fibros, isolated CD44^{high}/CD24^{neg} and CD44^{high}/CD24^{pos} populations interconverted in culture but both populations retained a fibroblastic morphology and did not express EpCAM (Supporting Information Fig. S6). Thus the CD44^{high}/CD24^{neg} profile associates with a mesenchymal-like phenotype but it alone does not identify a population capable of regenerating the heterogeneity observed in their parental cell lines.

Second, ALDH1 enzymatic activity was measured using the Aldefluor assay [31]. Within the parental Myo1089 (Fig. 3C) and MCF12A cells (Supporting Information Fig. S4C), Aldefluor activity was consistently higher in the EpCAM+ compared with the Fibros subpopulations. In addition, Myo-Fibros, MCF12A-Fibros, and MCF10-2A-Fibros after sorting and being passaged in vitro maintained lower Aldefluor expression than passaged EpCAM+ cells (Supporting Information Fig. S7). Consistent with this observation, EpCAM+ cells displayed higher mRNA expression of *ALDH1A3* gene (Fig. 2C), the isoform responsible for ALDH1 activity in stem cells [32], than Fibros.

Third, the formation of mammospheres in anchorage-independent conditions was analyzed. Myo-Fibros showed a higher frequency of mammosphere formation compared with Myo-EpCAM+ cells, whereas in the MCF12A cell line no significant difference between the two populations was observed (Fig. 4A). A key feature of mammary stem cells grown as spheres is their ability to differentiate to diverse lineages when plated onto collagen [20]. The EpCAM+-derived mammospheres attached poorly to collagen, but those that did attach formed either EpCAM+ or mixed colonies. By contrast, the Fibros-derived mammospheres attached easily but consistently gave rise to only EpCAM^{neg}/vimentin^{pos} mesenchymal-like colonies (Fig. 4A). Hence, although Fibros cells may have increased survival ability in anchorage-independent conditions they lack differentiation capabilities.

Finally, mammary stem cells when grown on a bed of Matrigel show morphogenetic potential and form acinar structures containing polarized epithelial cells [33]. As illustrated in Figure 4B, MCF12A-EpCAM+ and Myo-EpCAM+ cells generated acinar-like structures with hollow lumen whereas Fibros mostly formed solid structures. Moreover, approximately 25% of the Fibros structures showed strands of cells invading into the matrix, providing further evidence that spontaneous EMT results in the generation of invasive mesenchymal-like cells but not cells with morphogenetic potential.

Taken together, our results demonstrate that the spontaneous EMT in basal cell lines results in the generation of mesenchymal-like cells with invasive behavior and capacity to form mammospheres. However, other stem cell/progenitor properties such as the ability to regenerate the heterogeneity of the parental cell line, morphogenetic potential, and ALDH1 activity predominantly reside within the epithelial EpCAM^{pos}/CD49f^{high} cells, which is consistent with their stem cell/progenitor FACS marker profile [33–35] and their phenotypic characteristics (Fig. 2). Nonetheless, in freshly isolated primary breast cells, stem cells have also been identified as the EpCAM$^{neg/low}$/CD49f^{high} population [5, 26]. To test this, we isolated EpCAM$^{neg/low}$/CD49f^{high} cells from MCF12A and Myo1089 cell lines and demonstrated that they do not represent a distinct cell population and do not show increased stem cell/progenitor properties in these in vitro models (Supporting Information Figs. S8, S9).

EpCAM+ Cells Show Similarities with Luminal Progenitors of the Normal Mammary Gland While Fibros Resemble Stromal Cells

To investigate the phenotypic characteristics of EpCAM+ cells and Fibros, we compared their expression patterns with those of freshly isolated normal human mammary gland cell populations (differentiated luminal cells, luminal progenitors, basal/stem cell-enriched, and stromal cells) profiled by Lim et al. [5] using the method of nearest centroid correlation [23]. As controls we also profiled and analyzed primary HBFs

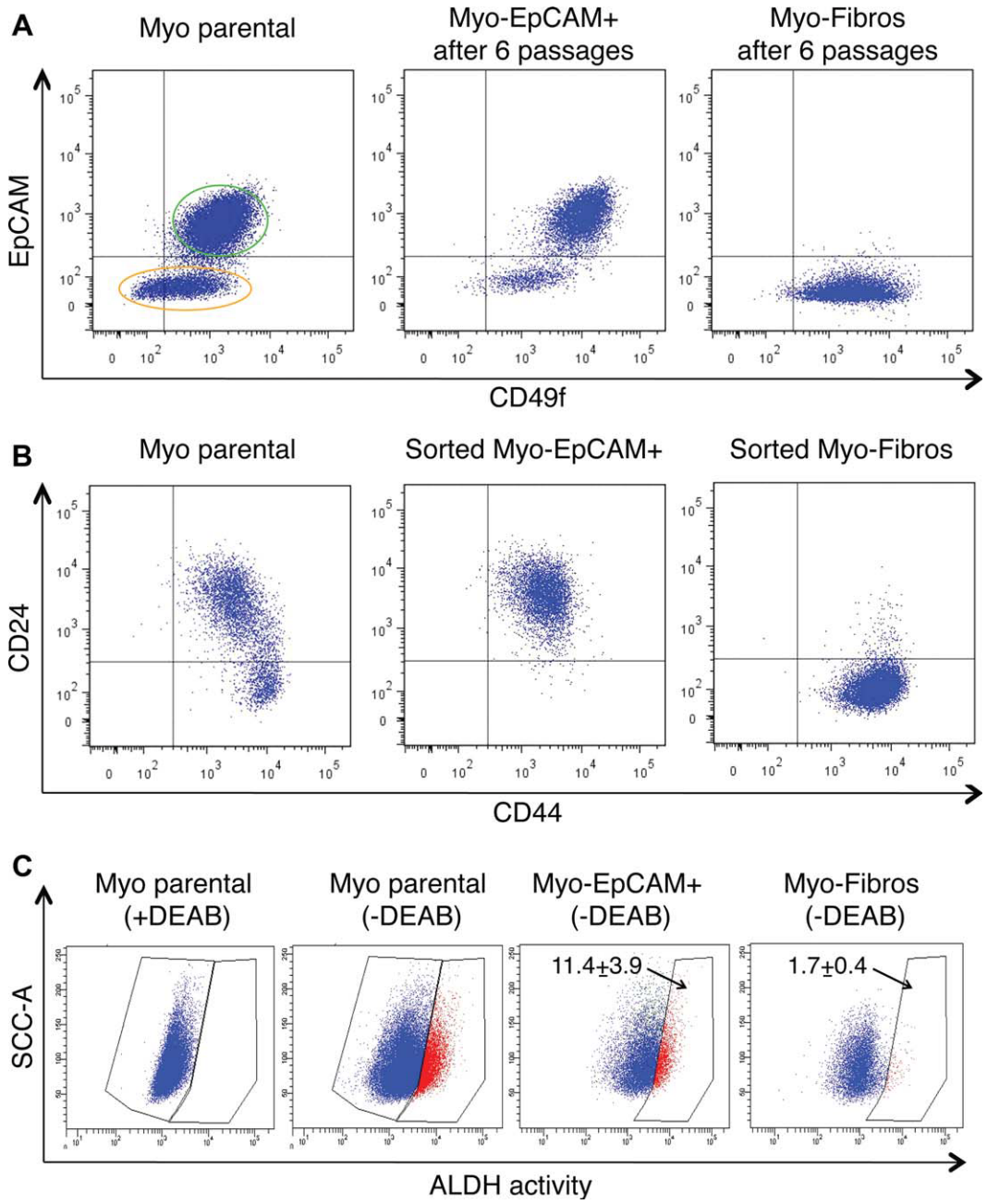

Figure 3. Evaluation of stem cell features in the distinct subpopulations within the Myo1089 cell line. (**A**): Analysis of the repopulation activity of different cell subpopulations. EpCAM+ (green oval) and Fibros (orange) were isolated by fluorescence-activated cell sorting, cultured for six passages, and reanalyzed by flow cytometry. (**B**): Analysis of the CD44/CD24 profile in the sorted subpopulations. (**C**): Quantification of ALDH1 activity by Aldefluor assay. Cells were triple labeled with Aldefluor and antibodies for EpCAM and CD49f. Left panels: Aldefluor staining of parental Myo cells in the presence or absence of the DEAB inhibitor. Right panels: EpCAM+ and Fibros were gated and the proportion of Aldefluor positive cells within each population quantified. Data represent mean number of Aldefluor positive cells ± SEM in five independent experiments. Differences were statistically significant (t test $p < .05$). Abbreviations: ALDH, aldehyde dehydrogenase 1; DEAB, diethylaminobenzaldehyde; EpCAM, epithelial cell adhesion molecule; SSC-A, side scatter-area.

and the luminal breast cancer cell line T47D (Materials and Methods section). Using this method, HBFs, Myo-Fibros, and MCF12A-Fibros were classified as stromal cells, T47D cells as luminal differentiated, and Myo-EpCAM+ and MCF12A-EpCAM+ cells as luminal progenitors (Supporting Information Table S2). To assess further the similarity of EpCAM+ cells and Fibros with luminal progenitors and stromal cells of the normal breast, we first identified the genes that were differentially expressed >1.5-fold between EpCAM+ and Fibros in both MCF12A and Myo cell lines ($n = 512$, Supporting Information Table S3). The expression of these 512 genes was interrogated in the normal breast cell subpopulations [5], using the method previously reported [10, 24]. As shown in Figure 5A, the expression of the EpCAM+-upregulated genes was higher in luminal progenitors than basal/stem cell-enriched and stromal cell populations, while the genes upregulated in Fibros showed the highest expression in stromal cells (Fig. 5A).

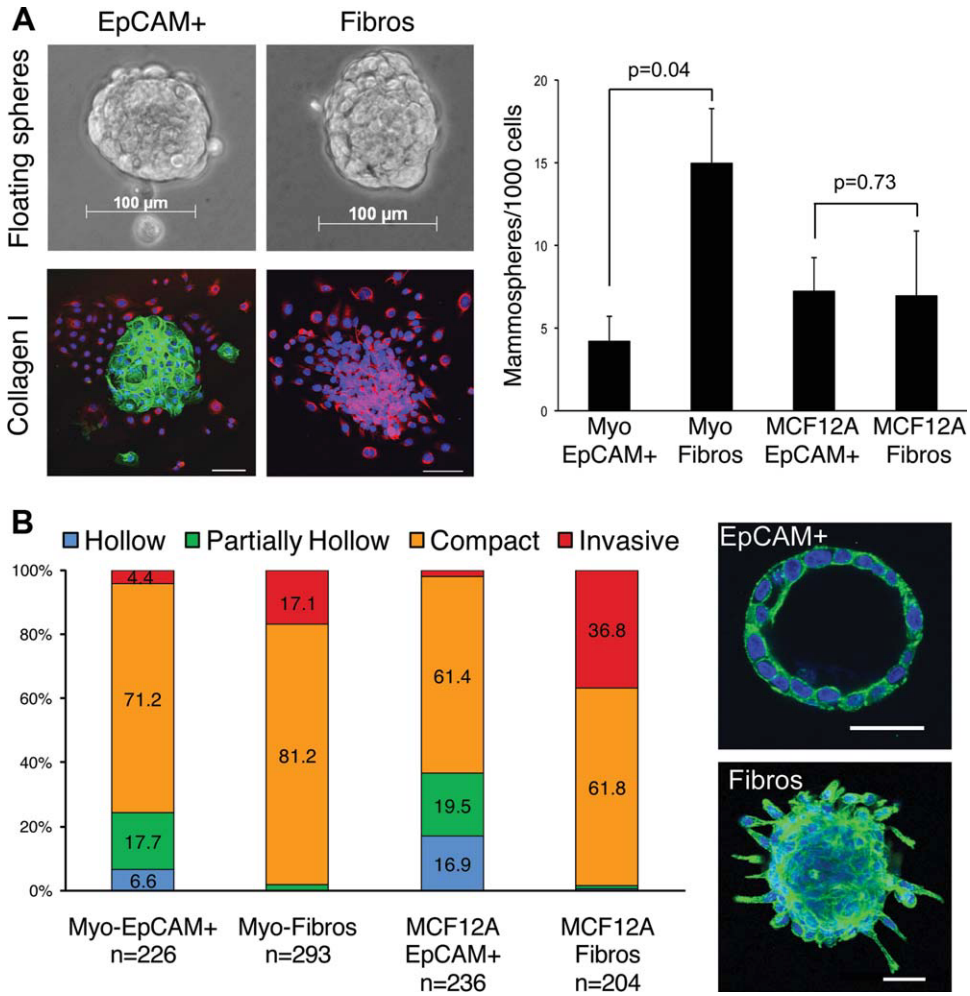

Figure 4. Analysis of stem cell/progenitor properties of distinct populations within MCF12A and Myo1089 cell lines. (**A**): Mammosphere formation. Upper left panels: Representative images of mammospheres generated from Myo-EpCAM+ cells and Myo-Fibros after 1 week in culture. Right panel: Average number ±SEM of mammospheres (>100 μm). Data shown are from three independent experiments (*t* test *p* values). Bottom left panels: Mammospheres were cultured on collagen I in differentiation medium for 4 days. Colonies were stained for EpCAM (green), vimentin (red), and DAPI (blue). Scale bar = 100 μm. (**B**): Morphogenetic potential of EpCAM+ cells and Fibros to form three-dimensional acini. Sorted cells were cultured for 20 days on Matrigel, and the 3D structures were stained with phalloidin-488 (green) plus DAPI (blue) and classified as hollow lumen, partially hollow, compact, or invasive. *n*, number of colonies analyzed for each subpopulation. Right panels: Representative confocal images of EpCAM+ cells generating hollow acinar structures and Fibros cells forming invasive cell strands into the Matrigel. Scale bar = 100 μm. Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; EpCAM, epithelial cell adhesion molecule.

Spontaneous EMT Generates Cells That Resemble Claudin-Low Cancer Cell Lines

The data presented here demonstrate that Fibros exhibit a mesenchymal-like phenotype, $CD44^{high}/CD24^{low}$ profile, invasive behavior, and capacity to form mammospheres. As these properties have also been associated with claudin-low breast cancers [10, 12, 24] and HMECs experimentally forced to undergo EMT [14, 15], we examined whether the gene expression changes during spontaneous EMT in the basal cell lines parallel those associated with EMT in breast cancer cell lines. For this we assessed in our basal cell populations the expression of the gene signature associated to experimentally induced EMT in HMECs [24] and the 9-cell line predictor of claudin-low breast cancer cell lines [10]. Additionally, two independent gene lists distinguishing basal-A and mesenchymal/basal-B/claudin-low cancer cell lines [11, 25] were also evaluated (Supporting Information Fig. S10A). The genes upregulated during EMT and in claudin-low cell lines were significantly higher in Fibros compared with EpCAM+ cells (Fig. 5B), whereas the genes downregulated during EMT or highly expressed by basal-A relative to mesenchymal/basal-B/claudin-low cell lines were elevated in EpCAM+ (Fig. 5B, Supporting Information Fig. S10A). As expected, HBFs showed the highest expression of genes induced during EMT (Fig. 5B). We next analyzed the expression of our 512-gene list (Supporting Information Table S3 shows the degree of overlap between this gene list and previously reported gene signatures [10, 11, 25]) in the collection of breast cancer cell lines profiled by Neve et al. [11]. Again, the same correspondence between Fibros and claudin-low cell lines and between EpCAM+ cells and basal-A cell lines was found (Fig. 5C). Moreover, an unsupervised hierarchical cluster analysis using our 512-gene list revealed that claudin-low, basal-A, and luminal cell lines segregated into distinct dendrogram branches (Supporting Information Fig. S10B).

Together the results of these transcriptomic analyses indicate that EpCAM+ cells resemble luminal progenitors and

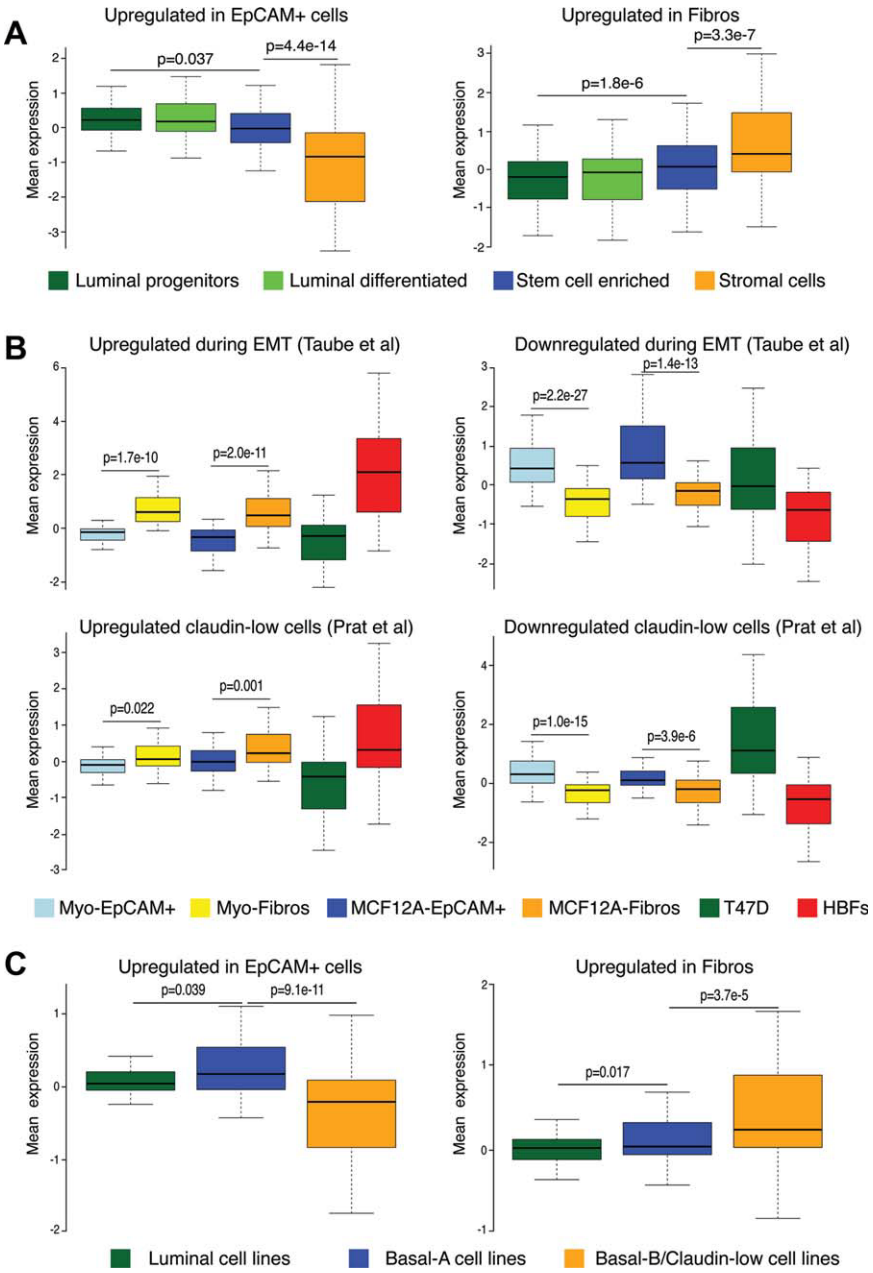

Figure 5. Meta-analysis of gene expression signatures from EpCAM+ cells and Fibros, cell subpopulations of the normal mammary gland and breast cancer cell lines. The expression of the upregulated and downregulated genes from the indicated signatures is represented as box-and-whisker plots. *p* values were calculated by comparing gene expression means between the indicated samples using two-tailed unpaired *t* test [10, 24]. (**A**): The expression of the 512 genes differentially expressed >1.5-fold between EpCAM+ and Fibros in both Myo and MCF12A cell lines (upregulated in EpCAM+ $n = 191$, in Fibros $n = 391$; Supporting information Table S3) was evaluated in the four cell populations of the normal mammary gland profiled by Lim et al. [5]. (**B**): EpCAM+ and Fibros subpopulations from MCF12A and Myos, T47D, and HBFs were analyzed for the average expression of the following gene signatures: EMT experimentally induced in human mammary epithelial cell (HMECs; upregulated genes $n = 91$; downregulated genes $n = 158$) [24], the 9-cell line predictor of claudin-low breast cancer cell lines (upregulated $n = 426$; downregulated $n = 361$) [9]. (**C**): Expression of the 512 genes differentially expressed >1.5-fold between EpCAM+ and Fibros assessed in 49 breast cancer cell lines profiled by Neve et al. [11]. Cell lines were classified as luminal, basal-A, and basal-B according to Neve et al. [11]. Abbreviations: EpCAM, epithelial cell adhesion molecule; EMT, epithelial–mesenchymal transition; HBFs, human breast fibroblasts.

basal-A cancer cell lines, while Fibros show strong similarities with normal breast stromal cells, HMECs that have undergone EMT, and claudin-low breast cancer cells. Consistent with this, flow cytometry analysis of six breast cancer cell lines confirmed the phenotypic correlation between Fibros and claudin-low cancer cells and between EpCAM+ cells and basal-A breast cancer cell lines (Supporting Information Fig. S11).

Slug and Zeb1 Transcription Factors Inhibit Luminal Differentiation in Basal Cell Lines

Finally, to interrogate the molecular mechanisms that control the phenotype of EpCAM+ cells and Fibros, we tested the role of specific transcription factors in regulating the differentiation of these two populations. Of the transcription

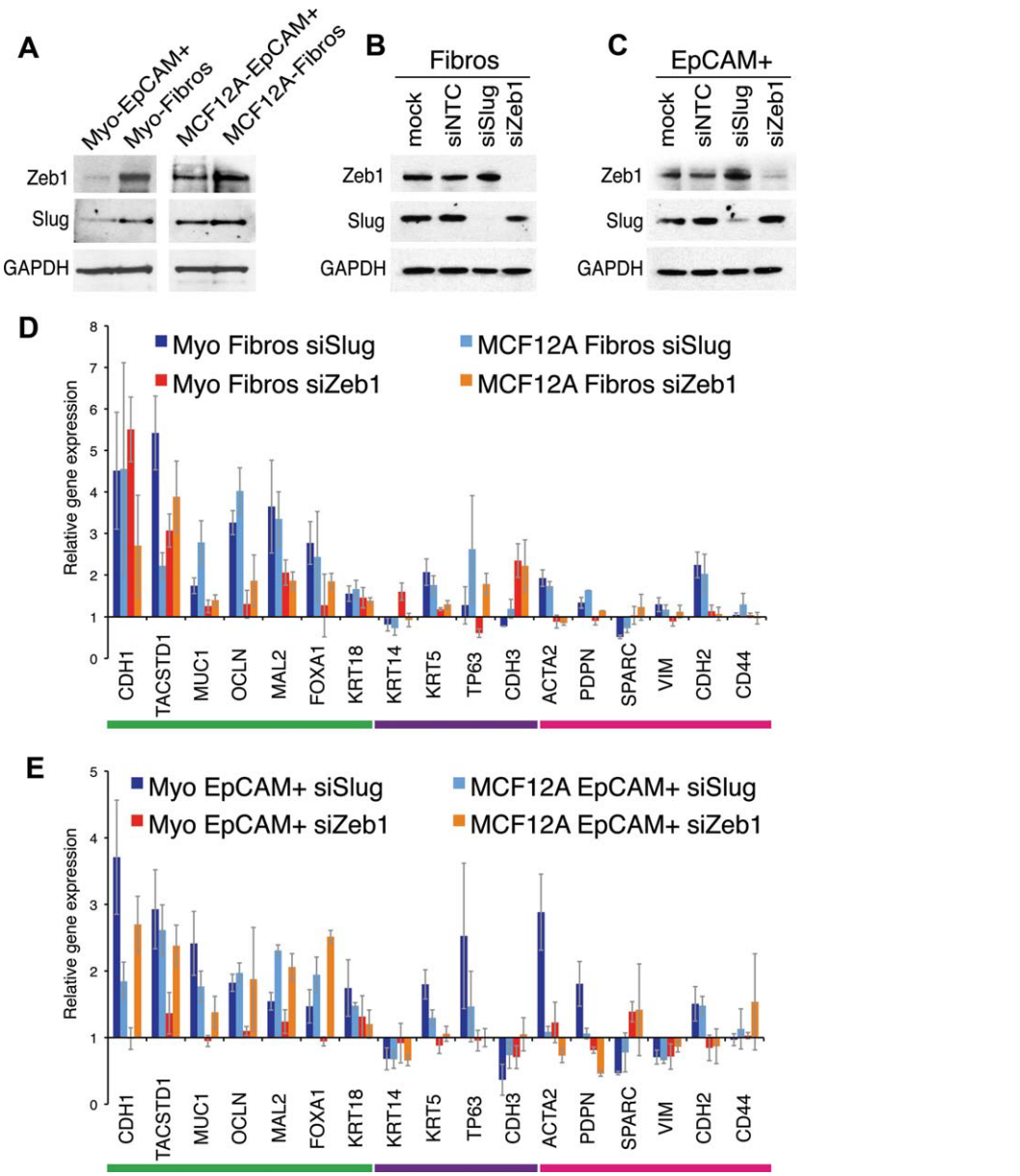

Figure 6. SNAI2 (Slug) and ZEB1 regulate the differentiation of EpCAM+ and Fibros cell populations in basal cell lines. (**A**): Endogenous expression of Zeb1 and Slug proteins in Myo1089 and MCF12A EpCAM+ and Fibros subpopulations. Data are representative of four independent experiments. (**B, C**): Representative examples of Slug and Zeb1 knockdown by siRNA on freshly sorted Fibros and EpCAM+ cells, respectively. (**D, E**): Effect of Slug and Zeb1 silencing on the differentiation of Fibros and EpCAM+ cells, respectively, assessed by quantitative RT-PCR. Bars represent average fold change ±SEM of gene expression in four independent siRNA transfections normalized to the expression value in the corresponding siNTC-transfected cell populations (baseline value = 1). Luminal epithelial genes are underlined in green, basal genes in purple and markers shared by myoepithelial cells and mesenchymal cells in pink. Abbreviations: EpCAM, epithelial cell adhesion molecule; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RT-PCR, reverse-transcription polymerase chain reaction; siNTC, nontargeting control siRNA; siRNA, small interfering RNA.

factors differentially expressed between EpCAM+ and Fibros (Fig. 2C, Supporting Information Table S1), the EMT regulators Snai2 (Slug) and Zeb1 were selected for further analysis due to their reported association with stem cell properties in diverse tissue types [36, 37]. By immunoblotting, Slug and Zeb1 protein levels were higher in Fibros than EpCAM+ cells from Myo and MCF12A cell lines (Fig. 6A). siRNA-mediated silencing of either *Slug* or *Zeb1* expression in the sorted Fibros from Myo and MCF12A cells resulted in an upregulation of luminal gene expression and a variable effect on basal and mesenchymal markers (Fig. 6B, 6D) but was not sufficient to completely reverse them to an epithelial morphology (not shown). Similarly, silencing of *Slug* in Myo-EpCAM+ cells and inhibition of either *Slug* or *Zeb1* in MCF12-EpCAM+ consistently increased the expression of luminal-specific genes while the effect on basal and mesenchymal markers was variable (Fig. 6C, 6E). *Zeb1* downregulation in Myo-EpCAM+ cells had only a minor effect on the differentiation markers, consistent with the low endogenous expression of *Zeb1* in these cells (Fig. 6A). These data indicate that Slug and Zeb1 regulate the progenitor-like phenotype of EpCAM+ cells and the mesenchymal-like phenotype of Fibros primarily by inhibiting luminal differentiation.

Discussion

In recent years, there has been increased interest in EMT in stem cell biology due to its proposed role in promoting the reversion of differentiated cells to a primitive stem cell-like phenotype [9, 14]. This function of EMT implies that normal stem cells would have mesenchymal-like features and that, conversely, the acquisition of differentiated epithelial traits via a mesenchymal-to-epithelial transition (MET) would decrease stem cell properties. Whether gaining a mesenchymal-like phenotype is a prerequisite for the acquisition of all stem cell properties and whether EMT–MET occur intrinsically within normal stem cells remain to be determined.

Using immortalized nontumorigenic basal cell lines as models for normal stem cell/progenitors, we demonstrate that these cells exhibit inherent phenotypic plasticity and intrinsic proclivity to generate mesenchymal-like EpCAM^{neg}/CD44^{high}/CD24^{low} cells via EMT. These data are consistent with the finding that primary and immortalized HMECs, which are known to exhibit strong basal/myoepithelial traits [38], also contain mesenchymal-like cell populations [39, 40]. Consequently, generation of mesenchymal-like cells is indeed part of the plasticity repertoire of basal cells with stem cell features. However, where our data differ is that previous studies in HMEC reported that phenotypic and gene expression similarities to normal stem cells correlate with acquisition of mesenchymal-like features [14, 15, 41], whereas such associations are not observed in our study. Here we provide evidence that these discrepancies reflect the plasticity of stem cell properties in distinct phenotypic states. Based on these findings, we also propose a new interpretation of the relationship between EMT and the acquisition of a stem cell gene expression profile.

Our data demonstrate that specific stem cell/progenitor properties in vitro are differentially associated with distinct phenotypic states of normal basal breast cells. Specifically, the EpCAM^{neg} mesenchymal-like state exhibits properties of CSCs (CD44^{high}/CD24^{neg} profile, invasiveness, and formation of mammospheres), while properties of normal stem cells/progenitors (differentiation, morphogenetic potential and high ALDH1 activity) associate to the more epithelial EpCAM^{pos}/CD49f^{high} cell state (or EpCAM$^{pos/low}$/CD49f^{high} in noncultured primary mammary cells [5, 26]). Consistent with this, it has recently been reported in squamous cell carcinomas that different cell states exhibit specific CSC properties cells, being epithelial EpCAM^{pos} cells more proliferative and Aldefluor-positive but mesenchymal-like EpCAM^{neg} cells more invasive [42]. Moreover, in contrast to the original reports that assigned the differentiation potential exclusively to the mesenchymal-like state in HMECs and breast cancer cells (reviewed in [13, 43]), recent studies demonstrate that these cells can switch between mesenchymal-like CD24^{neg} and epithelial CD24^{pos} populations in vitro and in vivo [41, 44–46]. Hence, together these data indicate that stem cell potential in normal HMECs and CSC features in breast tumors are not fixed to the mesenchymal-like cell state but are dynamically modulated depending on in vitro and in vivo conditions. In human tumors, this interconversion between cellular states with different CSCs properties has clinical implications, as anticancer therapies that exclusively target the invasive mesenchymal-like population would potentially lead to a tumor regrowth from the remaining epithelial CD24^{pos} cells. In normal breast, however, the functional relevance of the association of mesenchymal-like cells with CSC characteristics (CD44^{high}/CD24^{neg} profile, invasiveness, mammospheres) is unclear, as these features are not unambiguous signs of stemness. For example, to date there is no definitive evidence demonstrating that normal breast stem cells can acquire a mesenchymal-like phenotype in vivo or that they are invasive under physiological conditions. Moreover, the CD44^{high}/CD24^{neg} profile does not unequivocally distinguish stem cells in the normal mammary gland [34] but a heterogeneous mix of cells [17]. Finally, although the CD44^{high}/CD24^{neg} mesenchymal-like cells generally showed enhanced mammosphere formation, the possibility that the mammosphere assay favors the survival of mesenchymal-like cells over other epithelial populations with stem cell/progenitor potential cannot be ruled out.

Together these data suggest that the EMT process is not an end-point mechanism to generate normal stem cells or CSCs but a mechanism to increase phenotypic and functional diversity in normal and cancer cells. If this model is correct it has to be reconciled with the reports showing a similarity between the gene expression profiles of normal human breast stem cells, HMECs that have undergone EMT and of claudin-low breast cancers [5, 10, 24], a finding that led to the proposal that tumors with claudin-low/EMT features originate from basal stem cells [9].

The reported similarity between EMT and stem cells gene expression profiles can, however, be interpreted differently based on our results and after reanalyses of microarray datasets. It should be noted that, when compared to the four cell populations of the normal breast, the Fibros population described here, HMECs induced to undergo EMT [24], and claudin-low cancer cell lines and tumors most closely resemble stromal cells, and to a much lesser extent the basal stem cell-enriched population [5, 10]. Although an interpretation of these data is that EMT and claudin-low tumors represent complete transdifferentiation of basal stem cells to fibroblasts, we show here that claudin-low mesenchymal-like cells can originate from EpCAM^{pos}/CD49f^{high} luminal progenitor-like cells. Hence we propose the alternative novel hypothesis that EMT in normal and cancer breast cells reflects altered/incomplete myoepithelial differentiation from progenitor cells rather than a reversion to a stem cell state. This hypothesis is supported by the following evidence: (a) the basal/stem cell-enriched population profiled by Lim et al. [5] that was associated with the EMT/claudin-low phenotype [10, 24] was predominantly composed of myoepithelial cells [5, 47]; (b) there are important phenotypic and functional similarities between myoepithelial cells and (myo)fibroblasts [48] that may account for the partial overlap observed between the gene expression profiles of basal/myoepithelial and stromal cells [5]; (c) during EMT in our cell systems and in HMECs [24] there is increased expression of the markers shared by myoepithelial cells and fibroblasts such as αSMA, CD10, and VIM.

Finally, our hypothesis that EMT reflects aberrant myoepithelial differentiation offers new insights on the possible cell of origin of claudin-low and basal cancers. First, we suggest that mesenchymal and basal/myoepithelial characteristics observed in claudin-low cancers do not reflect a stem cell origin. Second, consistent with recent evidence demonstrating that basal tumors originate from luminal progenitors with blocked/altered luminal differentiation potential [5, 49, 50], we propose that EpCAM^{pos}/CD49f^{high} cells in immortalized nontumorigenic basal cell lines represent preneoplastic models of such altered luminal progenitors. Accordingly, the capacity of EpCAM^{pos}/CD49f^{high} epithelial cells to generate claudin-low cells via EMT would reflect the inability of luminal progenitors to undergo complete myoepithelial differentiation when luminal commitment is blocked. Therefore, we suggest that EMT processes are involved in the regulation of luminal

and myoepithelial differentiation in stem cell/progenitors. Consistent with this, we showed a role for the Slug and Zeb1 transcription factors in maintaining the luminal-progenitor and mesenchymal phenotypes by inhibiting luminal differentiation. Although complete luminal or myoepithelial differentiation likely involves multiple crosstalk with other transcription factor families (e.g., FOXA1, p63), the data presented here, together with the recently reported role of Slug in regulating lineage commitment of *BRCA1*-mutated cells [50], support a model in which EMT transcription factors control lineage differentiation of breast cells in normal and pathological conditions.

Conclusions

Our results demonstrate that EMT is part of the plasticity repertoire of mammary cells with basal features, and specific stem cell properties are differentially associated with the epithelial and mesenchymal-like states. Although studies on primary normal human stem cells/progenitors are required to fully address the association of EMT and stemness, our data indicate that EMT process is not an endpoint mechanism to generate normal stem cells. We propose instead that generation of mesenchymal-like cells via EMT in normal basal breast cells and claudin-low breast cancers reflects altered/incomplete myoepithelial differentiation.

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

References

1 Stingl J, Eirew P, Ricketson I et al. Purification and unique properties of mammary epithelial stem cells. Nature 2006;439:993–997.
2 Shackleton M, Vaillant F, Simpson KJ et al. Generation of a functional mammary gland from a single stem cell. Nature 2006;439:84–88.
3 Visvader JE. Keeping abreast of the mammary epithelial hierarchy and breast tumorigenesis. Genes Dev 2009;23:2563–2577.
4 Stingl J, Caldas C. Molecular heterogeneity of breast carcinomas and the cancer stem cell hypothesis. Nat Rev Cancer 2007;7:791–799.
5 Lim E, Vaillant F, Wu D et al. Aberrant luminal progenitors as the candidate target population for basal tumor development in BRCA1 mutation carriers. Nat Med 2009;15:907–913.
6 Chang CC. Recent translational research: Stem cells as the roots of breast cancer. Breast Cancer Res 2006;8:103.
7 Visvader JE, Lindeman GJ. Cancer stem cells in solid tumours: Accumulating evidence and unresolved questions. Nat Rev Cancer 2008;8:755–768.
8 Thiery JP, Acloque H, Huang RY et al. Epithelial–mesenchymal transitions in development and disease. Cell 2009;139:871–890.
9 May CD, Sphyris N, Evans KW et al. Epithelial–mesenchymal transition and cancer stem cells: A dangerously dynamic duo in breast cancer progression. Breast Cancer Res 2011;13:202.
10 Prat A, Parker JS, Karginova O et al. Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer. Breast Cancer Res 2010;12:R68.
11 Neve RM, Chin K, Fridlyand J et al. A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. Cancer Cell 2006;10:515–527.
12 Blick T, Hugo H, Widodo E et al. Epithelial mesenchymal transition traits in human breast cancer cell lines parallel the CD44(hi/)CD24 (lo/−) stem cell phenotype in human breast cancer. J Mammary Gland Biol Neoplasia 2010;15:235–252.
13 Hollier BG, Evans K, Mani SA. The epithelial-to-mesenchymal transition and cancer stem cells: A coalition against cancer therapies. J Mammary Gland Biol Neoplasia 2009;14:29–43.
14 Mani SA, Guo W, Liao MJ et al. The epithelial–mesenchymal transition generates cells with properties of stem cells. Cell 2008;133:704–715.
15 Morel AP, Lievre M, Thomas C et al. Generation of breast cancer stem cells through epithelial–mesenchymal transition. PLoS One 2008;3:e2888.
16 Pauley RJ, Soule HD, Tait L et al. The MCF10 family of spontaneously immortalized human breast epithelial cell lines: Models of neoplastic progression. Eur J Cancer Prev 1993;2 (suppl 3):67–76.
17 Bloushtain-Qimron N, Yao J, Snyder EL et al. Cell type-specific DNA methylation patterns in the human breast. Proc Natl Acad Sci USA 2008;105:14076–14081.
18 Sarrio D, Rodriguez-Pinilla SM, Hardisson D et al. Epithelial–mesenchymal transition in breast cancer relates to the basal-like phenotype. Cancer Res 2008;68:989–997.
19 Holliday DL, Brouilette KT, Markert A et al. Novel multicellular organotypic models of normal and malignant breast: Tools for dissecting the role of the microenvironment in breast cancer progression. Breast Cancer Res 2009;11:R3.
20 Dontu G, Abdallah WM, Foley JM et al. In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. Genes Dev 2003;17:1253–1270.
21 Debnath J, Muthuswamy SK, Brugge JS. Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures. Methods 2003;30:256–268.
22 Tusher VG, Tibshirani R, Chu G. Significance analysis of microarrays applied to the ionizing radiation response. Proc Natl Acad Sci USA 2001;98:5116–5121.
23 Weigelt B, Mackay A, A'Hern R et al. Breast cancer molecular profiling with single sample predictors: A retrospective analysis. Lancet Oncol 2010;11:339–349.
24 Taube JH, Herschkowitz JI, Komurov K et al. Core epithelial-to-mesenchymal transition interactome gene-expression signature is associated with claudin-low and metaplastic breast cancer subtypes. Proc Natl Acad Sci USA 2010;107:15449–15454.
25 Charafe-Jauffret E, Ginestier C, Monville F et al. Gene expression profiling of breast cell lines identifies potential new basal markers. Oncogene 2006;25:2273–2284.
26 Eirew P, Stingl J, Raouf A et al. A method for quantifying normal human mammary epithelial stem cells with in vivo regenerative ability. Nat Med 2008;14:1384–1389.
27 Hwang WL, Yang MH, Tsai ML et al. SNAIL regulates interleukin-8 expression, stem cell-like activity, and tumorigenicity of human colorectal carcinoma cells. Gastroenterology 2011;141:279–291.
28 Peinado H, Olmeda D, Cano A. Snail, Zeb and bHLH factors in tumour progression: An alliance against the epithelial phenotype? Nat Rev Cancer 2007;7:415–428.
29 Al-Hajj M, Wicha MS, Benito-Hernandez A et al. Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci USA 2003;100:3983–3988.
30 Fillmore CM, Kuperwasser C. Human breast cancer cell lines contain stem-like cells that self-renew, give rise to phenotypically diverse progeny and survive chemotherapy. Breast Cancer Res 2008;10:R25.
31 Ginestier C, Hur MH, Charafe-Jauffret E et al. ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. Cell Stem Cell 2007;1:555–567.

32 Marcato P, Dean CA, Pan D et al. Aldehyde dehydrogenase activity of breast cancer stem cells is primarily due to isoform ALDH1A3 and its expression is predictive of metastasis. Stem Cells 2011;29:32–45.
33 Villadsen R, Fridriksdottir AJ, Ronnov-Jessen L et al. Evidence for a stem cell hierarchy in the adult human breast. J Cell Biol 2007;177:87–101.
34 Raouf A, Zhao Y, To K et al. Transcriptome analysis of the normal human mammary cell commitment and differentiation process. Cell Stem Cell 2008;3:109–118.
35 Stingl J, Eaves CJ, Zandieh I et al. Characterization of bipotent mammary epithelial progenitor cells in normal adult human breast tissue. Breast Cancer Res Treat 2001;67:93–109.
36 Wellner U, Schubert J, Burk UC et al. The EMT-activator ZEB1 promotes tumorigenicity by repressing stemness-inhibiting microRNAs. Nat Cell Biol 2009;11:1487–1495.
37 Sun Y, Shao L, Bai H et al. Slug deficiency enhances self-renewal of hematopoietic stem cells during hematopoietic regeneration. Blood 2010;115:1709–1717.
38 Ince TA, Richardson AL, Bell GW et al. Transformation of different human breast epithelial cell types leads to distinct tumor phenotypes. Cancer Cell 2007;12:160–170.
39 Scheel C, Eaton EN, Li SH et al. Paracrine and autocrine signals induce and maintain mesenchymal and stem cell states in the breast. Cell 2011;145:926–940.
40 Walia V, Elble RC. Enrichment for breast cancer cells with stem/progenitor properties by differential adhesion. Stem Cells Dev 2010;19:1175–1182.
41 Chaffer CL, Brueckmann I, Scheel C et al. Normal and neoplastic nonstem cells can spontaneously convert to a stem-like state. Proc Natl Acad Sci USA 2011;108:7950–7955.
42 Biddle A, Liang X, Gammon L et al. Cancer stem cells in squamous cell carcinoma switch between two distinct phenotypes that are preferentially migratory or proliferative. Cancer Res 2011;71:5317–5326.
43 Creighton CJ, Chang JC, Rosen JM. Epithelial–mesenchymal transition (EMT) in tumor-initiating cells and its clinical implications in breast cancer. J Mammary Gland Biol Neoplasia 2010;15:253–260.
44 Keller PJ, Lin AF, Arendt LM et al. Mapping the cellular and molecular heterogeneity of normal and malignant breast tissues and cultured cell lines. Breast Cancer Res 2010;12:R87.
45 Meyer MJ, Fleming JM, Ali MA et al. Dynamic regulation of CD24 and the invasive, CD44posCD24neg phenotype in breast cancer cell lines. Breast Cancer Res 2009;11:R82.
46 Gupta PB, Fillmore CM, Jiang G et al. Stochastic state transitions give rise to phenotypic equilibrium in populations of cancer cells. Cell 2011;146:633–644.
47 Lim E, Wu D, Pal B et al. Transcriptome analyses of mouse and human mammary cell subpopulations reveal multiple conserved genes and pathways. Breast Cancer Res 2010;12:R21.
48 Petersen OW, Lind Nielsen H, Gudjonsson T et al. The plasticity of human breast carcinoma cells is more than epithelial to mesenchymal conversion. Breast Cancer Res 2001;3:213–217.
49 Molyneux G, Geyer FC, Magnay FA et al. BRCA1 basal-like breast cancers originate from luminal epithelial progenitors and not from basal stem cells. Cell Stem Cell 2010;7:403–417.
50 Proia TA, Keller PJ, Gupta PB et al. Genetic predisposition directs breast cancer phenotype by dictating progenitor cell fate. Cell Stem Cell 2011;8:149–163.

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