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REGULAR ARTICLE

In vitro multipotent differentiation and barrier function of a human mammary epithelium

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Abstract As demonstrated by a variety of animal studies, barrier function in the mammary epithelium is essential for a fully functioning and differentiated gland. However, there is a paucity of information on barrier function in human mammary epithelium. Here, we have established characteristics of a polarizing differentiating model of human mammary epithelial cells capable of forming a high-resistance/low-conductance barrier in a predictable manner, viz., by using MCF10A cells on permeable membranes. Inulin flux decreased and transepithelial electrical resistance (TEER) increased over the course of several days after seeding MCF10A cells on permeable membranes. MCF10A cells exhibited multipotent phenotypic differentiation into layers expressing basal and lumenal markers when placed on permeable membranes, with at least two distinct cell phenotypes. A clonal subline of MCF10A, generated by culturing stem-like cells under non-adherent conditions, also generated a barrier-forming epithelial membrane with cells expressing markers of both basal and lumenal differentiation (CD10 and MUC1, respectively). Progressive changes associated with differentiation, including wholesale inhibition of cell-cycle genes and stimulation of cell and tissue morphogenic genes, were observed by gene expression profiling. Clustering and gene ontology categorization of significantly altered genes revealed a pattern of lumenal epithelial-cell-specific differentiation.

Keywords Breast · Transepithelial electrical resistance · Tight junctions · Milk protein · MCF10A · Gene expression profile · Human

Introduction

Postnatal mammary gland development is controlled by interactions between endocrine hormones and factors that act locally to drive mammary development and maintain homeostasis (Srivastava et al. 2003; Wood et al. 2000; Stull et al. 2007; Neville et al. 2002). Rodent models, i.e., rats and mice, have been the predominant experimental context in which to study mammary physiology and development. These animal models share many features with the human breast and are experimentally tractable, but rodent models are not ideal for studying the influence of local factors on human mammary gland development. Obvious differences between rodent mammary gland and human breast are structural. The rodent mammary epithelium is associated with an extensive adipose stroma, whereas the human mammary epithelium is surrounded by fibrous connective tissue, and the human breast adipose tissue is largely remote from the epithelium. These morphologic features manifest themselves as differences in the physiological

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interactions between stroma and epithelium in the human and mouse (Parmar and Cunha 2004). On a practical level, it has not been possible to grow and differentiate normal human mammary epithelial cells in the mouse mammary fat pad (Sheffield 1988). Recent studies have attempted to circumvent this problem by co-transplanting human stroma and epithelium in mice (Kuperwasser et al. 2004; Proia and Kuperwasser 2006). This human-in-mouse model is useful but has some practical limitations as a platform for studying many aspects of development, homeostasis, and tumorigenesis. Important hormonal differences also exist between human and rodent, including the lack of a functional luteal phase in rodents (Cardiff and Wellings 1999) and poor binding of rodent prolactin to human prolactin receptors (Utama et al. 2006). Overall, there continues to be a need for studies of differentiation and signaling in well-characterized human mammary epithelial systems, including investigations of local signaling events, epithelial transport ion fluxes, and cell-cell interactions.

Tight junctions in the mammary epithelium have been studied primarily in the context of pregnancy, parturition, lactation, and involution because of their integral role in these events. Although the resting gland (non-pregnant, non-lactating) has not been extensively studied, tight junction proteins are expressed in the human ductal network at this stage; however, the permeability of the membrane is not known (Tokes et al. 2005). At the time of parturition, high-resistance tight junctions are established in the human mammary epithelium (Nguyen et al. 2001). Compared with true milk, the first-milk (colostrum) contains higher concentrations of serum proteins, sodium, and chloride, and lower concentrations of potassium and lactose. The changes that accompany the transition to true milk are attributable to barrier formation, which results from the closure of the tight junctions (Nguyen and Neville 1998). Conversely, involution is accompanied by the opening of the tight junctions and the breaching of the epithelial barrier resulting in leakage of milk components into the interstitium and plasma (Nguyen and Neville 1998; Shamay et al. 2003). In the context of breast cancer, changes in the expression of tight junction proteins correlate with tumor progression, and tight junction dysregulation is necessary for tumor cell migration and invasion (Kominsky et al. 2003; Martin et al. 2004).

Given the central role of barrier formation in mammary epithelial development and pathogenesis, any useful model for studying local regulation in the mammary epithelium must be able to establish a patent tight junction barrier in a predictable manner. Advances in understanding the microenvironment of the human mammary gland are currently restricted because few studies have involved human cells that display the characteristics of a differentiated mammary epithelium (Rudland and Barraclough 1990; DiRenzo et al. 2002).

Here, we report physiological changes and gene expression profiles of an in vitro model by using a multipotent human mammary epithelial cell line, MCF10A (Soule et al. 1990). This model has enabled our laboratory to characterize a novel signaling system involved in the homeostatic control of milk synthesis and secretion (Stull et al. 2007).

Materials and methods

Cell culture and fluorescein-isothiocyanate-inulin flux An immortalized human mammary epithelial cell line, MCF10A, was used. The normal growth medium for MCF10As was DMEM:F12 (1:1; Cellgro) with 2 mM glutamine, containing 5% horse serum, insulin (10 μg ml^{-1}; Gibco), hydrocortisone (0.5 μg ml^{-1}; Sigma), epithelial growth factor (EGF; 20 ng ml^{-1}; Upstate), 1 IU ml^{-1} penicillin, 0.1 μg ml^{-1} streptomycin, 0.25 μg ml^{-1} amphotericin B (Cellgro). Cells were grown in monolayer to 90%–95% confluency, trypsinized, and counted for seeding onto permeable supports (Transwell, Corning; 0.4 μm pores, polyester) in normal growth medium. MCF10A cells were seeded on 12-well Transwells at 10^5 cells/cm^2. Both chambers of media were changed strictly on a 24-h schedule, unless otherwise noted. Where indicated, 9-cis-retinoic acid (9-cis RA; Sigma) was administered in both chambers. Transepithelial electrical resistance (TEER) was measured daily with an Epithelial Volt-Ohm Meter (EVOM; World Precision Instruments), prior to media change. For inulin flux experiments, 0.5 mg/ml of fluorescein-isothiocyanate-inulin (FITC-inulin) was included in the bottom chamber medium and allowed to equilibrate for 20–22 h. After incubation, 100 μl of both the top and bottom chamber media was transferred to a 96-well plate for fluorescence quantification on a plate reader (Labsystems Fluoroskan II).

Unlike anchorage-dependent cells that express lineage-specific phenotypes, cells with progenitor/stem cell properties survive and proliferate when cultured in the presence of a low-adherence substrate. The resulting colonies are capable of generating self-renewing lineage-restricted daughter cell populations (Liu et al. 2005). In the case of mammary epithelium, these stem cell colonies have been referred to as non-adherent mammospheres (Dontu et al. 2003a). A trypsinized suspension of MCF10As was filtered through a 40-μm sieve and then counted. The single cell suspension was transferred to a 6-well ultra-low-adherence plate (Corning) at a density of 2,500 cells/well. Non-adherent culture medium was composed of the following: MEBM (Cambrex), B-27 supplement (Invitrogen), human EGF (20 ng/ml), insulin (5 μg/ml), and hydrocortisone

(0.5 μg/ml). After 5 days in culture, the surviving non-adherent mammosphere colonies were serially diluted onto normal tissue culture plastic multiwell dishes. A well containing a single colony was expanded, passaged onto T75 culture flasks (designated MCF10A-UC1), and then cultured on Transwell permeable membranes for analyses.

Immunofluorescence and imaging Cells were grown on permeable supports to peak TEER, fixed by a brief incubation in 4% paraformaldehyde (in phosphate-buffered saline), and immunostained as floating sections of membranes. The floating sections were permeabilized in 0.1% Triton X-100 and incubated in borate buffer pH 8.5 (80 mM boric acid, 20 mM sodium borate) overnight at 75°C for antigen retrieval. The following antibodies and fluorescent stains were used: rabbit polyclonal anti-occludin (5 μg ml^{-1}; Zymed), mouse monoclonal anti-E-cadherin (0.5 μg ml^{-1}; BD Bioscience), mouse anti-mucin 1 (0.8 μg ml^{-1}; Santa Cruz), rabbit anti-CD10 (0.8 μg ml^{-1}; Santa Cruz), rabbit anti-human milk proteins serum (1:500; Nordic Immunology), goat anti-rabbit Alexa Fluor 488 (2 μg ml^{-1}; Molecular Probes), goat anti-rabbit Alexa Fluor 546 (2 μg ml^{-1}; Molecular Probes), goat anti-mouse Alexa Fluor 488 (2 μg ml^{-1}; Molecular Probes), goat anti-mouse Alexa Fluor 546 (2 μg ml^{-1}; Molecular Probes), LipidTOX (1:500; Invitrogen), and TO-PRO-3 iodide dye (1 μM; 642/661; Molecular Probes). Images were collected by using a Zeiss LSM510 Confocal Microscope. The excitation sources were argon lasers at 488 nm and 543 nm and a helium/neon laser at 633 nm, with emission collection at 505–530 nm, 560–615 nm, and 650–720 nm, respectively.

Matrigel culture MCF10A cells were cultured in Matrigel (BD Bioscience) as previously described with some modifications (Debnath et al. 2003). The medium supplied during differentiation in Matrigel contained 2% horse serum, insulin (10 μg ml^{-1}; Gibco), hydrocortisone (0.5 μg ml^{-1}; Sigma), EGF (5 ng ml^{-1}; Upstate), 1 IU ml^{-1} penicillin, 0.1 μg ml^{-1} streptomycin, 0.25 μg ml^{-1} amphotericin B (Cellgro) but did not contain cholera toxin; 9-cis-RA (Sigma) was added at the first medium change (day 3) at 10 nM.

RNA extraction Total RNA from cells grown in monolayer and Transwell cultures was extracted by using Tri reagent (MRC, Cincinnati, Ohio) and cleaned by using DNaseI (Promega, Madison, WI) followed by standard phenol/chloroform precipitation and extraction. The RNA extracted representing Monolayer MCF10A was extracted 5 days after the cells reached 100% confluence. Total RNA from Transwell cultures was extracted at the following trans-epithelial resistances: 200–300 Ω*cm^2 (Base), 1400–1600 Ω*cm^2 (Midpoint), and 3000–3200 Ω*cm^2 (Plateau).

Microarray hybridization and analysis Gene expression experiments were carried out on the Affymetrix Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, Calif.). The quality of total RNA was first analyzed by an Agilent Bioanalyzer 2100, and RNA samples with an RNA integrity number greater than 7.0 were considered acceptable for microarray analysis. Total RNA samples were amplified for one round, and the amplified RNA (aRNA) was biotinylated by using the Ambion Biotin-Enhanced Message Amp II kit (#1791, Ambion, Austin, Texas), following the manufacturer's protocols. Hybridization, staining, and washing were carried out with the Affymetrix GeneChip Hybridization Wash and Stain Kit following the manufacturer's protocols, and the arrays were hybridized for 16 h at 45°C. The data were analyzed to identify differentially expressed genes among the Monolayer, Base, Midpoint, and Plateau conditions, as defined as above. Analysis was performed by using R statistical software and the *limma* Bioconductor package (Smyth 2004). All steps of data preprocessing, including background correction, normalization, and expression set summaries were performed by using RMA (Robust Multi-chip Average). Estimated fold changes at each time point were calculated by an analysis of variance (ANOVA), and resulting t-statistics from each comparison were modified by using an intensity-based empirical Bayes method (Sartor et al. 2006) to obtain accurate significance levels. *P*-values were adjusted by using the False Discovery Rate (*FDR*) method (Benjamini and Hochberg 1995), and genes with $FDR<0.05$ and a fold change of >50% were considered significant for differential expression and were used in the creation of the Venn diagram and expression profile patterns, and for gene ontology (GO) enrichment analysis. The latter was performed with David 2007 (Dennis et al. 2003) and used to test separately genes up- and down-regulated through the differentiation process. For assignment of genes to specific expression profile patterns, a fold change of >50% alone was employed. Microarray data for the three independent replicates of Monolayer, Base, Midpoint, and Plateau are available from the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/) with accession number GSE10070.

Results

Polarization and barrier establishment of MCF10A Transwell model

Formations of adherens junctions and tight junctions are hallmarks of the polarization and barrier function of any epithelium. The cells in this model formed a stratified

epithelium consisting of multiple cell layers (Fig. 1c; see also Stull et al. 2007). Each part of Fig. 1 displays an X-Y plane image and a reconstructed X-Z-plane image. The Z-plane images (top) result from bisecting the X-Y plane at the indicated green line. Cells in the bottom layer (underlying cells) were tightly packed, with irregularly situated nuclei and relatively small cytoplasmic volumes. In contrast, the cells in the uppermost layer (superficial cells) had much larger cytoplasmic volumes and morphologies that varied from narrow columnar to broad squamous cells. Immunostaining for E-cadherin and occludin was used to visualize adherens and tight junctions, respectively (Fig. 1a,b). The staining of tight junction proteins, and by implication the barrier function, was exclusively in the superficial layer cells and was concentrated in the lateral membranes. E-cadherin generally co-localized with occludin, both being primarily membrane-associated. Numerous cells possessed E-cadherin in cytoplasmic organelles (Fig. 1 arrowheads) that appeared to occupy a particular niche in a suprabasal zone, making contact with both the most basal and the most superficial cells.

The integrity of epithelial tight junctions can be quantified by measuring the TEER or flux of a metabolically inert molecule such as inulin. FITC-inulin flux represents the paracellular movement of a 3.5-kDa molecule, whereas TEER represents trans- and paracelluar ionic impedance. Under control conditions (see Materials and methods), the plateau resistance and time to plateau inflection in this cell line was 3200–3700 $\Omega*cm^2$ and 11–13 days, respectively (Fig. 2). The flux of FITC-inulin (3.5 kDa) significantly declined to <5% by day 8 (Fig. 2). Furthermore, the decline in inulin flux preceded the rapid rise in TEER.

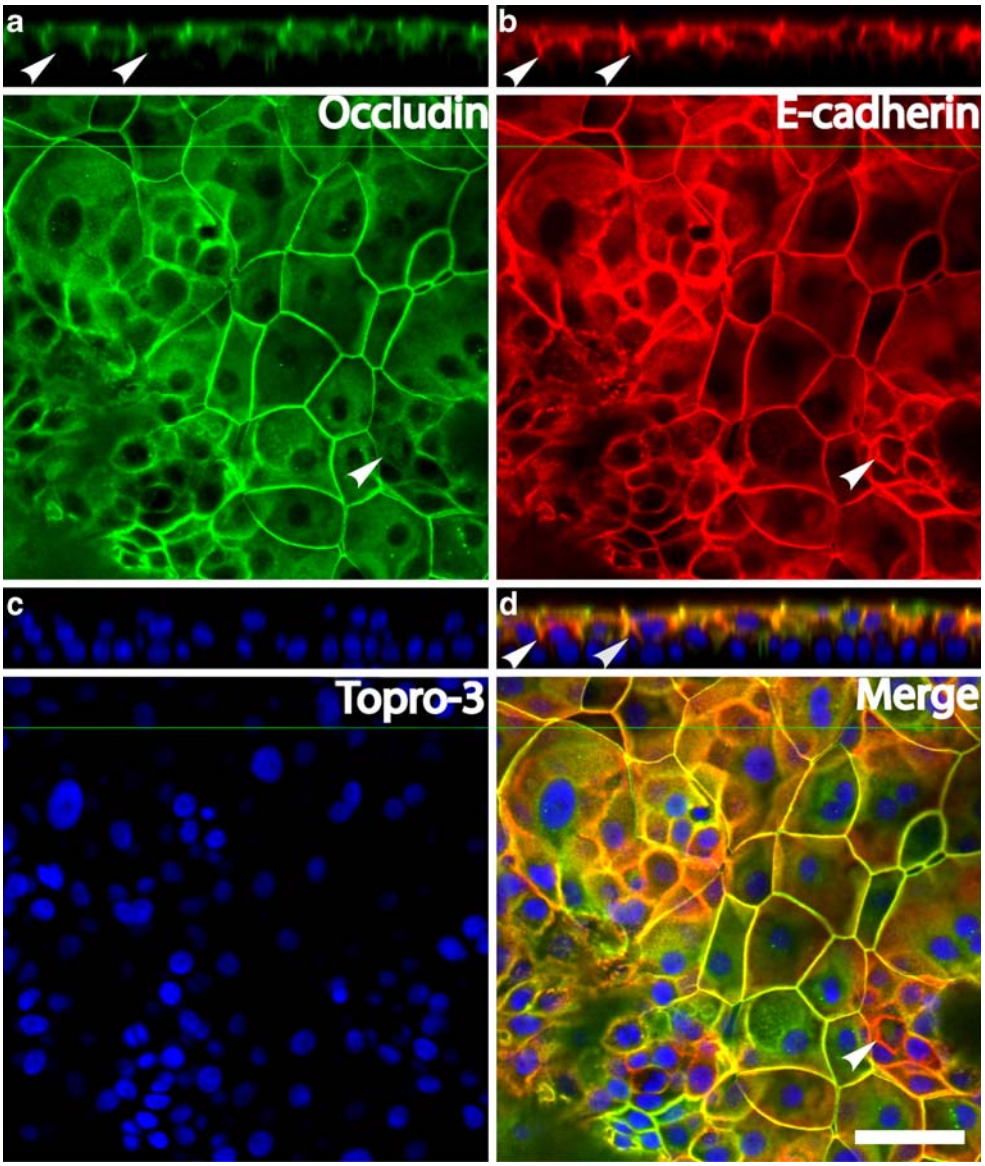

Fig. 1 Localization of tight junctions and adherens junctions. Junction localization is shown by immunostaining for occludin (**a**) representing tight junctions and for E-cadherin (**b**) representing adherens junctions. Nuclei were counterstained (**c**), and the merged image (**d**) shows the apical cell layer forming the barrier. *Arrowheads* highlight a probable third cell type identified by cytoplasmic E-cadherin and occupying a suprabasal niche. *Bottom* XY plane image, *top* Z plane image cut along the displayed *green* line (**a**–**d**). *Bars* 50 μm

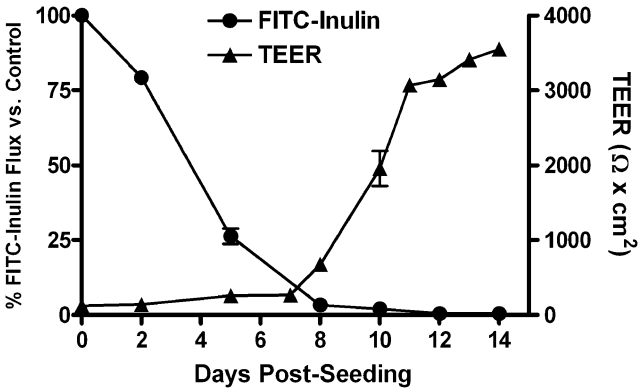

Fig. 2 Barrier formation in MCF10A Transwell cultures. *Left Y-axis* FITC-inulin flux (*filled circles*) as a percentage of flux in an empty Transwell (no cells). *Right Y-axis* TEER (*filled triangles*) over the same time period on the *X-axis*. Note the decrease in inulin (3.5 kDa) flux precedes the rapid rise in TEER

These observations demonstrate the capacity of MCF10A cells to form a high-resistance functional barrier in vitro, in contrast to previous studies in which no barrier formation was observed (Sourisseau et al. 2006; Underwood et al. 2006; Fogg et al. 2005).

Differentiated cells in the MCF10A Transwell model

Various proteins have been identified as markers of mammary epithelial cell differentiation (Li and Rosen 2005; Stingl et al. 2005). Among these, mucin-1 (MUC1) has proven to be a useful lumenal cell marker. MUC1 is a membrane glycoprotein that is expressed on lumenal cells in the breast and other glandular organs and is often dysregulated in cancers (Mather et al. 2001; Taylor-Papadimitriou et al. 2002). MUC1 was stained exclusively in the superficial cells (Fig. 3b). An established basal cell marker is the common acute lymphoblastic leukemia antigen (CALLA), also known as cluster of differentiation-10 (CD10; Gusterson et al. 1986). CD10 is expressed within the basal layer of the breast epithelium in both contractile (myoepithelial) and non-contractile cells (Gusterson et al. 1986). CD10 in MCF10A Transwell cultures showed intense staining in the underlying cell layer, with the greatest staining intensity in a perinuclear pattern that suggested high protein concentrations in the endoplasmic reticulum and/or Golgi apparatus (Fig. 3a).

The differentiation of MCF10A cells into MUC1-expressing lumenal cells provided a rationale for determining whether the cells synthesized components of milk. LipidTOX, a fluorescent neutral lipid stain, showed the accumulation of neutral lipid droplets in Transwell cultures of MCF10A cells (Fig. 3d). No significant accumulation of lipid droplets occurred in cells cultured as conventional monolayers (not shown). In addition, using a multivalent polyclonal antibody mixture made against human milk proteins, we observed staining for milk components in the MUC1-positive superficial layer of cells (Fig. 3c). Formation of milk-protein-containing vesicles was also evident (Fig. 3c, arrowheads). We could not definitively attach the phenotype of these differentiated MCF10A cells with their equivalent basal and lumenal homologs in vivo. Whereas these cells generically express milk proteins, they do not express caseins or aldolase C (Matsuda et al. 2003), nor do they express either the prolactin or progesterone receptors, all of which are characteristic of the most specialized lumenal alveolar cells in the pregnant and lactating mammary gland. The lack of these markers, combined with the clear basal-lumenal differentiation of the membranes suggests that the MCF10A cells may differentiate only to a stage similar to ductal epithelium.

The development of two cell phenotypes from a long-term established cell line implies that MCF10A cells retain some level of pluripotency. Furthermore, these cell differentiation markers validate that the model replicates several features that are reflective of mammary epithelial

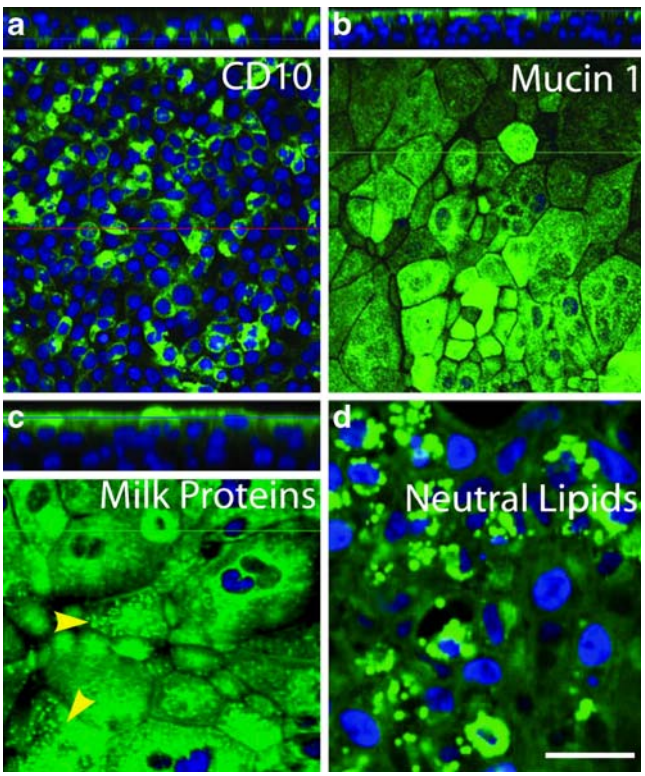

Fig. 3 Differentiation of MCF10A cells into multiple phenotypes. The different cell phenotypes are evidenced by CD10 (**a**) staining of the underlying layer of cells and MUC1 (**b**) staining in the superficial layer of cells. The superficial layer of cells synthesizes milk proteins (**c**) and lipid droplets (**d**). *Arrowheads* in **c** highlight milk protein vesicles. *Bottom* XY plane image, *top* Z plane image cut along the displayed *green* line (*a–c*). *Bars* 50 μm

development in vivo. However, one obvious feature that we have not observed in this model system is the myoepithelial cell. The basal layer did not produce cells with the myoepithelial morphology, and we did not detect smooth muscle actin, a marker for myoepithelium (not shown).

To validate that MCF10A cell cultures retain cells with a multipotent differentiation capability, a clonal subpopulation was generated from a single progenitor cell by using a method previously described for mammary epithelial stem cells (see Materials and methods; Dontu et al. 2003a). Briefly, a single cell suspension of MCF10A cells was plated into ultra-low-adherence plates and grown in a stem-cell-supportive medium. Approximately 1%–2% of the cells survived and proliferated, forming non-adherent mammospheres (Fig. 4a; Dontu et al. 2003a; Reynolds and Weiss 1996), which are anatomically and physiological distinct from three-dimensional mammospheres grown in a semisolid matrix (Debnath et al. 2002). A single non-adherent mammosphere was isolated and allowed to reattach and proliferate on normal plastic. This clonal subline (MCF10A-UC1) was cultured in Transwells and assayed by morphological and physiological criteria. Similar to membranes derived from the original MCF10A cells, MCF10A-UC1 cell membranes were able to generate a barrier with a comparable plateau TEER (~2800 Ω*cm^2) and both lumenal (MUC1$^+$ CD10$^-$) and basal (MUC1$^-$ CD10$^+$) cell phenotypes (Fig. 4b). These results imply that MCF10A cultures retain a population of self-renewing stem-like cells, in addition to non-stem cells. We have not determined the proliferative potential of each variety of cell under normal culture conditions, but since the stem-like cells represent only 1%–2% of the population, other cells probably contribute to the normal growth of the cells in culture.

Effects of medium conditioning and of RA

In its normal microenvironment, the mammary epithelium receives nutrients and distributes metabolites to the circulation through its basal side. We tested the consequence of withholding fresh medium for a period of several days while monitoring TEER. If the medium in both the upper and lower chambers was replenished every 24 h, the TEER was maintained above 3500 Ω*cm^2 and gradually drifted upward over several days (Fig. 5a). In contrast, if the medium in neither chamber was changed, the TEER fell precipitously on the 3rd day. The TEER also fell on day 3 if only the medium in the upper chamber was changed; however, if only the medium in the lower chamber was changed daily, TEER remained at the plateau level and did not increase (Fig. 5a). Therefore, the composition of the lower chamber has a major impact on maintaining the membrane barrier function, possibly because of medium depletion or the accumulation of one or more inhibitory substances.

Retinoid signaling inhibits growth and promotes differentiation in many tissues, including the mammary gland (Moon et al. 1985), and the various isoforms of the retinoic acid receptors (RAR, RXR) are differentially expressed during the stages of mammary gland development (Wang et al. 2005). We first verified the differentiation-promoting activity of 9-cis RA by monitoring lumen formation of MCF10A mammospheres in growth-factor-replete Matrigel. This control experiment was performed because the effect of RA with regard to promoting lumen formation has been demonstrated previously in mouse epithelial cells (Montesano and Soulié 2002). Under control conditions in growth-factor-replete Matrigel, the cells grew as solid colonies rather than spheroid cysts, presumably because of excess growth factors (Fig. 5b). Incubation in the presence of 9-cis RA (10 nM) resulted in cystic mammospheres, each with a more obvious lumen, indicating proper polarization and differentiation of the cells (Fig. 5b). The differentiation activity of RA has led to its testing as a breast cancer chemopreventative agent (Patel et al. 2007). Having confirmed that 9-cis RA promotes morphological differentiation (lumen formation) in MCF10A cells, we tested its effect on barrier formation in the Transwell model (Fig. 5c). Incubation with 9-cis RA caused a concentration-dependent increase in maximum TEER. Additionally, treatment with 9-cis RA accelerated differentiation, with the plateau inflection of TEER occurring by day 8, compared with the typical time course, which reached the plateau at 12 days (Fig. 5c).

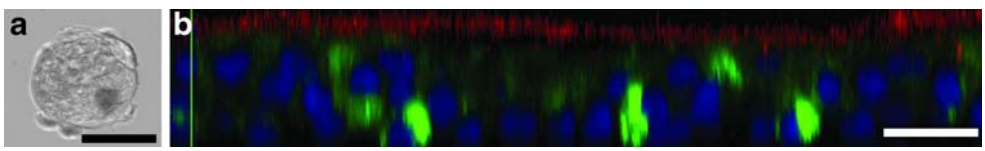

Fig. 4 Development of MCF10A epithelial membranes from a clonal subpopulation. **a** Phase-contrast image of a 7-day-old non-adherent mammosphere generated from a single cell suspension of MCF10A. *Bar* 75 μm. A single colony was subsequently proliferated on normal plastic (MCF10-UC1) and assayed for barrier formation in Transwells. **b** Immunostaining of MCF10A-UC1 for MUC1 (*red*), CD10 (*green*), and nuclei (*blue*) in one of the Transwells fixed at plateau TEER. *Bar* 25 μm

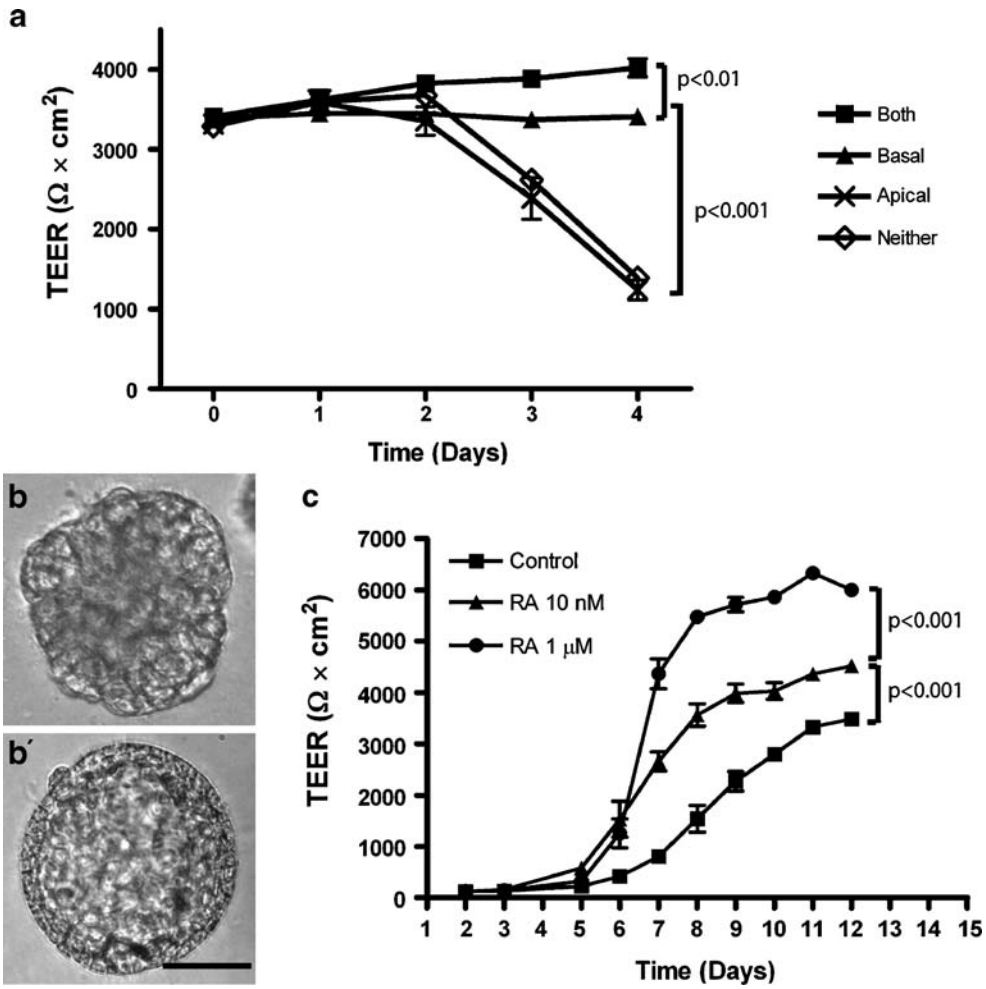

Fig. 5 Effect of medium conditioning and retinoic acid (*RA*) on MCF10A polarization. **a** Maintenance of the barrier only when the basal chamber is replenished. After peak TEER was achieved, only the listed chambers of medium were replenished daily. The polarized epithelium was dependent on the basal chamber being replenished. **b**, **b'** RA, a powerful differentiation agent, caused lumen formation in MCF10A mammospheres. **c** In the context of the MCF10A Transwell model, RA caused a dose-dependent increase in peak TEER achieved and shortened the time to plateau inflection. Means on the final day of the experiment were analyzed by one-way ANOVA (n=6) followed by the Holm-Sidak post-hoc test (GraphPad InStat software). Data are presented as means±SEM and are considered significant when the chance of type I error was <0.05

Gene expression analysis of differentiating MCF10A cells on Transwells

To improve our understanding of the differences occurring in MCF10A cells as they polarized and differentiated in the Transwell model, we performed gene expression profiling with Affymetrix Human Genome U133 Plus 2.0 Arrays. Four experimental time points, each performed in triplicate, were sampled, viz., conventional cultures of MCF10A cells cultured 5 days after reaching confluence on plastic (Monolayer), and MCF10A cells plated on Transwells sampled at three TEER values: 200–300 Ω*cm^2 (Base), 1400–1600 Ω*cm^2 (Midpoint), and 3000–3200 Ω*cm^2 (Plateau). Consistent with the immunostaining results in Fig. 3, the microarray assay showed MUC1 and CD10 to be significantly up-regulated in the Transwell cultures (by approximately 13-fold and five-fold, respectively). The majority of gene expression changes observed were between confluent monolayer MCF10A cells and any of the Transwell time points assayed. Figure 6 depicts a Venn diagram for two of those comparisons (Base versus Monolayer and Plateau versus Monolayer). For example, of the 1133 genes that were significantly down-regulated in Base versus Monolayer, 888 (78.4%) were also down-regulated in the Plateau versus Monolayer comparison (Fig. 6). Of the 1219 genes that were up-regulated in Base versus Monolayer, 894 (73.3%) were also up-regulated in Plateau versus Monolayer comparison. Five genes were oppositely regulated on comparing Base versus Plateau; three were up-regulated in Plateau and down-regulated in Base, while the other two were *vice versa*.

Gene clustering organizes gene expression patterns into coordinate regulatory profiles. Figure 7 depicts six functionally informative clustering patterns. Large numbers of genes were downwardly or upwardly regulated comparing monolayer cultures with the Transwell format and did not change significantly thereafter (Fig. 7a,b). Genes that gradually changed as the TEER increased, ultimately reaching significant differences, are illustrated by clusters C and D, respectively (Fig. 7c,d). Clusters E and F represent genes that changed significantly in the transition from monolayer to base and again changed significantly

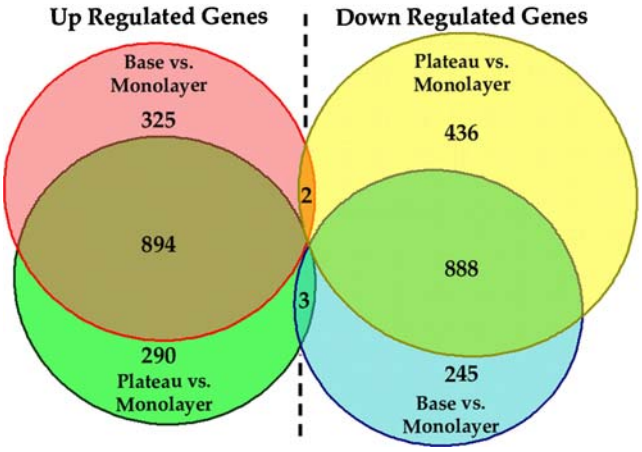

Fig. 6 Venn diagram representing overall gene expression changes in MCF10A cells. Two separate comparisons; Base versus Monolayer (*red* and *blue circles*) and Plateau versus Monolayer (*green* and *yellow circles*) are depicted. Downwardly regulated genes (*Down Regulated Genes*) are represented *right* side (*blue* and *yellow circles*), whereas upwardly regulated genes (*Up Regulated Genes*) are represented *left* (*red* and *green circles*). Criteria for inclusion were *FDR*<0.05 and ≥1.5-fold difference

back toward their initial expression level (Fig. 7e,f). Not all genes returned to the monolayer expression level; however, the expression at plateau was significantly different from base. The hatched areas between curves in clusters C–F represent variability in the rates of change among these genes. Groups of genes within clusters generally correlated well with GO categories (Ashburner et al. 2000; http://www.geneontology.org/).

Using GO categorization, a pattern of differentiation during the Transwell phase of culture became evident (Table 1). For example, genes regulating the progression through the cell cycle ($P=2.3\cdot10^{-6}$, $FDR=9.5\cdot10^{-5}$) and cell division ($P=5.3\cdot10^{-6}$, $FDR=2.0\cdot10^{-4}$), including p53, cyclin D1, and CDK2, were downwardly regulated as the MCF10A cells differentiated onto Transwells. Notably, the highly significant changes in cell-cycle genes occurred in the context of comparing contact-inhibited monolayer cultures with the Transwell cultures; hence, the changes do not simply reflect a difference between exponentially proliferating and static cultures.

Genes associated with extracellular matrix components such as matrix metalloproteases, mucins, and collagens were upwardly regulated ($P=6.6\cdot10^{-7}$, $FDR=1.6\cdot10^{-4}$; Table 1). Several pregnancy-associated gene products were changed during differentiation, including parathyroid hormone-related protein (PTHrP), steroid sulfatase (an enzyme that catalyzes the conversion of sulfated estrogen metabolites to estrogens), and pregnancy-specific glycoprotein. The tight junction proteins occludin and claudins 1, 4, 7, and 8 were also up-regulated in the Transwell format. This subset of claudins has previously been identified in the mammary epithelium of mice (Blackman et al. 2005; Hewitt et al. 2006; Blanchard et al. 2006). Further indicative of the lumenal phenotype, the expression of the gamma aminobutyric acid $(GABA)_A$ receptor subunit pi and the transcription factor GATA3 was stimulated by 231-fold and 1.9-fold, respectively. Although $GABA_A$ receptors have no well-characterized function in mammary tissue, $GABA_A\pi$ is expressed primarily in lumenal mammary epithelial cells and is commonly dysregulated in breast cancers (Zafrakas et al. 2006). Expression of $GABA_A\pi$ was verified by semi-quantitative reverse transcription with the polymerase chain reaction in a comparison of monolayer to Transwell (data not shown). GATA3 is an essential transcription factor involved in the differentiation and maintenance of a lumenal epithelial cell population (Kouros-Mehr et al. 2006). Conditional deletion of GATA-3 results in epithelial progenitor expansion and a reduction of differentiated lumenal cells (Asselin-Labat et al. 2007).

Discussion

Advances in mammary gland and breast cancer biology sometimes are occasioned by the development or refinement of useful experimental models, such as has been the case with the widespread use of genetically manipulated mice (Matthaei 2007; Dunbar and Wysolmerski 2001). In the case of human breast biology, advances depend largely on in vitro methods and associated xenografting techniques (Kuperwasser et al. 2004; Proia and Kuperwasser 2006; Debnath et al. 2003). Many human cell lines have been derived from breast cancers, but only a few from normal (i.e., untransformed) human mammary epithelium. Among these, MCF10A cells and derivatives of this line have been the most widely used (Debnath et al. 2002; Overholtzer et al. 2007). Here, we provide evidence of the multipotent epithelial differentiation of these cells at the molecular and cellular levels. These characteristics are prerequisites for studying the mechanisms of normal differentiation, physiological regulation, and homeostasis in the human mammary epithelium. Salient features of this model include reliable differentiation of multipotent cultures into basal and lumenal phenotypes, formation of a high-resistance epithelial barrier, responsiveness to differentiating signals, and rational changes in the expression of genes involved in cell growth, differentiation, and reproductive function.

Previous to our recent paper (Stull et al. 2007), no reports had demonstrated tight junction formation by human breast epithelial cells, including MCF10A. On the contrary, an absence of tight junctions in MCF10A cells was detailed in two separate papers (Underwood et al. 2006; Fogg et al. 2005). In one case, ultrastructure criteria

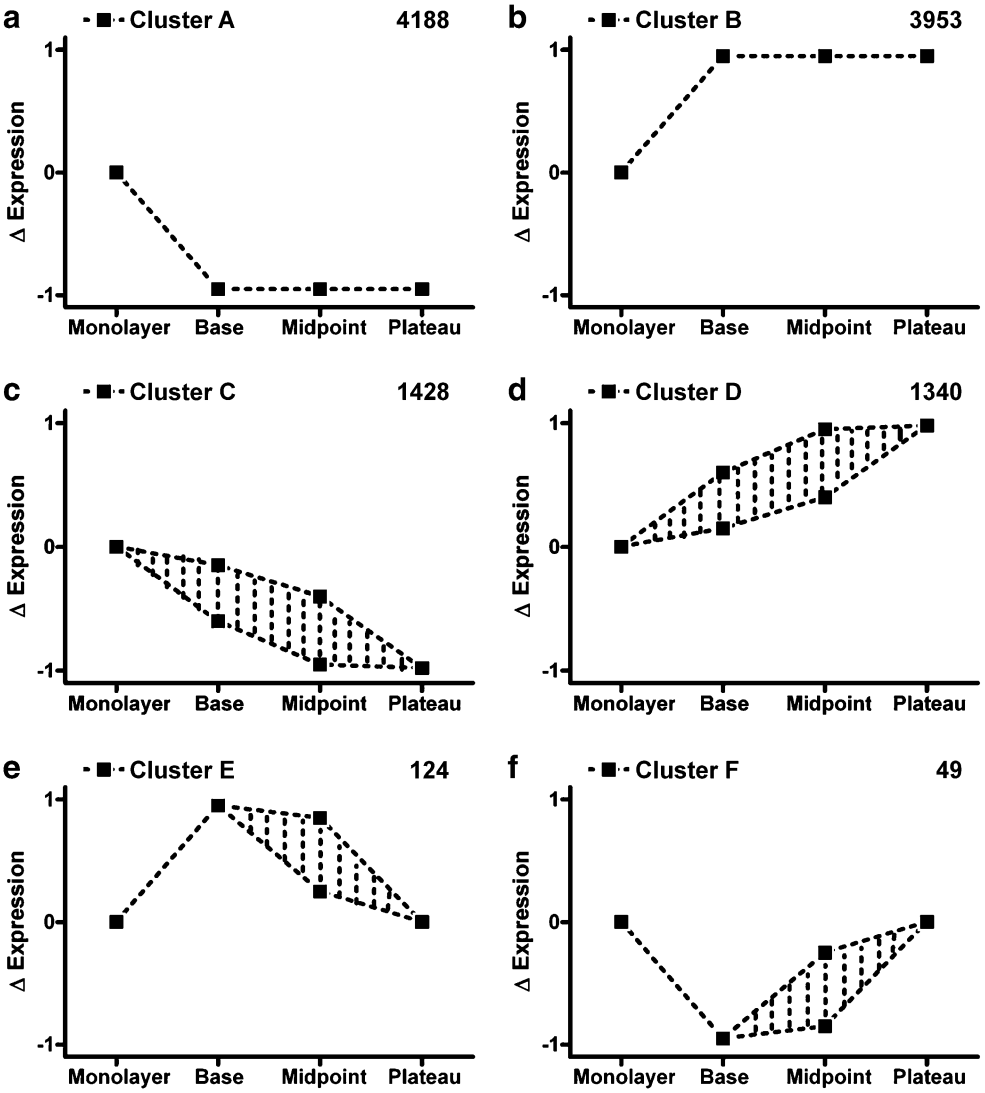

Fig. 7 Clustered expression profiles over MCF10A Transwell differentiation. All four time points, viz., *Monolayer*, *Base*, *Midpoint*, and *Plateau* are shown on the *X-axis*, and total number of genes in each cluster is listed. Criteria for obtaining the total number of significantly changed genes was ≥1.5-fold difference and *FDR*<0.05. Criterion for stepwise comparisons between two connected *X-axis* time points was ≥1.5-fold change

were used for three-dimensional mammosphere cultures, showing that MCF10A cells formed desmosome and adherens junction contacts between adjacent cells, but no tight junctions (Underwood et al. 2006). In a second report, transepithelial resistance and ZO-1 immunostaining were measured in Transwell cultures and showed a lack of tight junction development (Fogg et al. 2005). Our system with MCF10A cells not only shows that this cell line can indeed form tight junctions, but also that tight junction functionality is highly regulated (present studies; see also Stull et al. 2007). The difference between our studies and those published by other laboratories raises the obvious question: why? In general, the culture methods that we used in three-dimensional mammospheres on Transwells were similar to the others, with the single exception that our culture conditions omitted cholera toxin. Cholera toxin was identified early in our studies as being incompatible with developing tight junctions in MCF10A Transwell cultures. Among other features of the system that were tested, the choice of substrate (in this case uncoated polyester), inclusion of EGF and hydrocortisone, and the quality of lot-tested horse serum were found to be important.

The mammary gland parenchyma is composed of two highly differentiated epithelial cell types (lumenal and myoepithelial) and less-specialized cells in the basal compartment including one or more types of progenitor cells (Li and Rosen 2005; Smith and Chepko 2001; Vaillant et al. 2007; Wicha 2006). Lumenal cells include multiple subtypes, comprising cells that line both the ducts and the alveoli. Although both ductal and alveolar cells express milk proteins, they can be distinguished by the expression of aldolase C (Matsuda et al. 2003) and by the greater lipid secretory capacity in alveolar cells. Lumenal epithelial cells also vary in their expression of hormone receptor genes (Clarke 2006; Anderson and Clarke 2004; Harris et al. 2004; Naylor et al. 2003; Binart et al. 2000). The apparent

Table 1 Gene ontology (*GO*) categories of significantly changed genes. Criteria for inclusion were *FDR*<0.05 and ≥1.5-fold difference. Individual genes listed are not a complete list of all genes within each GO category but are representative. Cluster denotations refer to Fig. 7. Complete data series of microarray chips are available at the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/) with accession number GSE10070

GO category/*gene name*	Cluster	Maximum fold difference from monolayer
Cell cycle		
p53	C	−2.0
CDK2	C	−2.3
cyclin E2	A	−3.1
CDC25A	A	−4.2
cyclin D1	A	−1.6
Extracellular matrix		
Mucin 4	B	3.5
MMP1,3,9,10	D	60.2, 2.6, 8.0, 78.5
Structural constituent of the cytoskeleton		
Keratins 4, 6A, 6B, 13, 14, 16, 17, 23	B	72.4, 2.9, 33.7, 76.9, 12.0, 45.0, 8.8, 12.7
Keratin 18	A	−3.2
Cytoskeleton		
MUC1	B	13.3
Involucrin	B	11.8
Integrin β8	B	2.0
Adducin3	B	1.7
Tissue development		
Collagen Type 1α1	B	10.3
Collagen Type 7α1	B	6.4
Cellular retinoic acid binding protein 2	B	8.1
Desmoplakin	B	3.0
Reproductive physiological process		
Inhibin, beta A	F	−1.8
Parathyroid hormone-related protein	D	7.8
Pappalysin 1	A	−2.3
Steroid sulfatase	D	2.3
Pregnancy-specific beta-1-glycoprotein 4	D	1.6
Morphogenesis		
Wnt5A	F	−2.2
Transforming growth factor-β2	F	−4.1
Insulin-like growth factor binding protein-3	D	25.2
Gap junction protein, alpha 1, (connexin 43)	D	114.4
Miscellaneous		
GATA-3	D	1.9
Forkhead Box Q1	F	−2.5
Forkhead box O1A	B	3.3
Gamma aminobutyric acid A receptor π	B	231.0
Crystallin, alpha B	D	27.8
CD10 (MME, CALLA)	B	4.8
E-cadherin	B	2.3
Occludin	B	3.5
Claudin 1, 4, 7, 8	B	2.3, 3.8, 2.9, 20.5

ductal phenotype of the lumenal cells in this model is evidenced by their lack of prolactin receptor expression and the down-regulation of keratin 18 (unpublished results; Table 1; Rudland and Hughes 1989). Myoepithelial cells are specialized contractile cells found between the lumenal cells and the stroma. They express smooth muscle α-actin and CD10 and are stimulated to contract by oxytocin. Within the basal compartment, stem and/progenitor cells can be found, which various authors have proposed to number from <1% to nearly 10%. Many studies have attempted to characterize the mammary gland stem cells either by functional assays (self-renewal, long-term label retention) or by the presence and absence of particular cell markers such as MUC1, CD10, keratins, smooth muscle α-actin, and epithelial-specific antigen (Liu et al. 2005; Dontu et al. 2003a; Smith and Chepko 2001; Gudjonsson et al. 2005; Dontu et al. 2003b). As multifaceted and vast as this body of literature is, the nature and complexity of the apparent hierarchy of mammary stem/progenitor cells is still unresolved. Our model shows that the MCF10A cells possess properties of multipotent progenitor cells, as evidenced by differentiation into basal ($CD10^+$, $MUC1^-$) and lumenal ($CD10^-$, $MUC1^+$) phenotypes. Consequently, this model should be a useful tool for testing pathways involved in the proliferation and differentiation of these populations.

Broad-based gene profiling by microarray hybridization has been highly valuable as a means of validating the kind of changes that one might expect during the differentiation of MCF10A cultures. One issue that this method resolves is the nature of the multiple cell layering that occurs on the Transwell substrate. Highly proliferative, substrate-independent cancer cells avoid "contact inhibition" and pile up in multiple layers based on unrestrained proliferation. The suppression of cell-cycle genes and the induction of epithelial development and differentiation genes (Table 1), together with the morphological evidence (Fig. 1), demonstrate that the multiple cell layers in Transwell-cultured MCF10A represent an organized developmental process that is similar to the multiple cell layers observed in vivo, as opposed to a disorganized pathology. One source of ambiguity in gene profiling is the presence of multiple cell types. Whereas this is less of a problem when using an established cell line, clearly the differentiated MCF10A cell samples that we have profiled include at least two (superficial and underlying) cell types, and probably a third (suprabasal cells with cytoplamic E-cadherin; Fig. 1d arrowheads). Although this limits the information that we could extract from this initial profiling study, the relative simplicity of producing differentiated MCF10A cell populations, compared with purifying single cell population from human specimens, should make it possible to perform extensive gene profiling on well-defined cell populations

that can be sorted from the MCF10A cultures based on the markers that we have identified here. One interesting question to address would be the relationship of the underlying population of MCF10A cells to basal cells in vivo, particularly the myoepithelium.

Among the gene expression changes in the Transwell differentiation process, particularly interesting genes were identified, in addition to the general suppression of the cell cycle and shifts in the expression of cytoskeleton and extracellular matrix genes. For example, PTHrP expression was induced approximately 10-fold during differentiation. This hormone is a master signal for the development and maintenance of the mammary epithelial phenotype (Dunbar and Wysolmerski 1999; Foley et al. 2001). PTHrP receptors were also expressed in the MCF10A cultures, but the receptor levels did not change under the various conditions. The presence of the receptors together with the differential expression of the ligand leads one to speculate that PTHrP functions as an autocrine/paracrine factor in the MCF10A cultures. Among the small number of genes that fell into Cluster F (declined significantly after plating on Transwells, then reinduced as TEER increased), insulin-like growth factor binding protein-3, transforming growth factor-β, and FOXQ1 are associated in a growth inhibitory pathway downstream of Akt/protein kinase B (Roarty and Serra 2007; Samatar et al. 2002). The induction of these genes implies that a second Akt-dependent growth inhibitory pathway is induced during the final differentiation of the MCF10A cultures.

In summary, we have demonstrated the multipotent differentiation of human mammary epithelial cells that predictably assemble tight junctions and thus develop a low-conductance barrier. Tight junction patency is dependent on the composition of the bottom chamber medium, and RA accelerates and amplifies the TEER of MCF10A membranes. Overall, the MCF10A Transwell model recapitulates several important characteristics of the in vivo environment of the mammary gland (barrier formation, lumenal vs basal cell phenotypes with appropriate expression of markers for each). This model is highly useful for studying endocrine and autocrine-paracrine developmental regulation and physiological changes of human mammary epithelium. This system can probably be further refined.

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References

Anderson E, Clarke RB (2004) Steroid receptors and cell cycle in normal mammary epithelium. J Mammary Gland Biol Neoplasia 9:3–13

Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, Harris MA, Hill DP, Issel-Tarver L, Kasarskis A, Lewis S, Matese JC, Richardson JE, Ringwald M, Rubin GM, Sherlock G (2000) Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat Genet 25:25–29

Asselin-Labat ML, Sutherland KD, Barker H, Thomas R, Shackleton M, Forrest NC, Hartley L, Robb L, Grosveld FG, van der Wees J, Lindeman GJ, Visvader JE (2007) Gata-3 is an essential regulator of mammary-gland morphogenesis and luminal-cell differentiation. Nat Cell Biol 9:201–209

Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc [B] 57:289–300

Binart N, Ormandy CJ, Kelly PA (2000) Mammary gland development and the prolactin receptor. Adv Exp Med Biol 480:85–92

Blackman B, Russell T, Nordeen SK, Medina D, Neville MC (2005) Claudin 7 expression and localization in the normal murine mammary gland and murine mammary tumors. Breast Cancer Res 7:R248–R255

Blanchard AA, Watson PH, Shiu RP, Leygue E, Nistor A, Wong P, Myal Y (2006) Differential expression of claudin 1, 3, and 4 during normal mammary gland development in the mouse. DNA Cell Biol 25:79–86

Cardiff RD, Wellings SR (1999) The comparative pathology of human and mouse mammary glands. J Mammary Gland Biol Neoplasia 4:105–122

Clarke RB (2006) Ovarian steroids and the human breast: regulation of stem cells and cell proliferation. Maturitas 54:327–334

Debnath J, Mills KR, Collins NL, Reginato MJ, Muthuswamy SK, Brugge JS (2002) The role of apoptosis in creating and maintaining luminal space within normal and oncogene-expressing mammary acini. Cell 111:29–40

Debnath J, Muthuswamy SK, Brugge JS (2003) Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures. Methods 30:256–268

Dennis G Jr, Sherman BT, Hosack DA, Yang J, Gao W, Lane HC, Lempicki RA (2003) DAVID: Database for Annotation, Visualization, and Integrated Discovery. Genome Biol 4:P3

DiRenzo J, Signoretti S, Nakamura N, Rivera-Gonzalez R, Sellers W, Loda M, Brown M (2002) Growth factor requirements and basal phenotype of an immortalized mammary epithelial cell line. Cancer Res 62:89–98

Dontu G, Abdallah WM, Foley JM, Jackson KW, Clarke MF, Kawamura MJ, Wicha MS (2003a) In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. Genes Dev 17:1253–1270

Dontu G, Al-Hajj M, Abdallah WM, Clarke MF, Wicha MS (2003b) Stem cells in normal breast development and breast cancer. Cell Prolif 36 (Suppl 1):59–72

Dunbar ME, Wysolmerski JJ (1999) Parathyroid hormone-related protein: a developmental regulatory molecule necessary for mammary gland development. J Mammary Gland Biol Neoplasia 4:21–34

Dunbar ME, Wysolmerski JJ (2001) Mammary ductal and alveolar development: lesson learned from genetically manipulated mice. Microsc Res Tech 52:163–170

Fogg VC, Liu CJ, Margolis B (2005) Multiple regions of Crumbs3 are required for tight junction formation in MCF10A cells. J Cell Sci 118:2859–2869

Foley J, Dann P, Hong J, Cosgrove J, Dreyer B, Rimm D, Dunbar M, Philbrick W, Wysolmerski J (2001) Parathyroid hormone-related protein maintains mammary epithelial fate and triggers nipple skin differentiation during embryonic breast development. Development 128:513–525

Gudjonsson T, Adriance MC, Sternlicht MD, Petersen OW, Bissell MJ (2005) Myoepithelial cells: their origin and function in breast

morphogenesis and neoplasia. J Mammary Gland Biol Neoplasia 10:261–272
Gusterson BA, Monaghan P, Mahendran R (1986) Identification of myoepithelial cells in human and rat breasts by anti-common acute lymphoblastic leukemia antigen antibody A12. J Natl Cancer Inst 77:343–349
Harris J, Stanford PM, Oakes SR, Ormandy CJ (2004) Prolactin and the prolactin receptor: new targets of an old hormone. Ann Med 36:414–425
Hewitt KJ, Agarwal R, Morin PJ (2006) The claudin gene family: expression in normal and neoplastic tissues. BMC Cancer 6:186
Kominsky SL, Argani P, Korz D, Evron E, Raman V, Garrett E, Rein A, Sauter G, Kallioniemi O-, Sukumar S (2003) Loss of the tight junction protein claudin-7 correlates with histological grade in both ductal carcinoma in situ and invasive ductal carcinoma of the breast. Oncogene 22:2021–2033
Kouros-Mehr H, Slorach EM, Sternlicht MD, Werb Z (2006) GATA-3 maintains the differentiation of the luminal cell fate in the mammary gland. Cell 127:1041–1055
Kuperwasser C, Chavarria T, Wu M, Magrane G, Gray JW, Carey L, Richardson A, Weinberg RA (2004) Reconstruction of functionally normal and malignant human breast tissues in mice. Proc Natl Acad Sci USA 101:4966–4971
Li Y, Rosen JM (2005) Stem/progenitor cells in mouse mammary gland development and breast cancer. J Mammary Gland Biol Neoplasia 10:17–24
Liu S, Dontu G, Wicha MS (2005) Mammary stem cells, self-renewal pathways, and carcinogenesis. Breast Cancer Res 7:86–95
Martin TA, Watkins G, Mansel RE, Jiang WG (2004) Loss of tight junction plaque molecules in breast cancer tissues is associated with a poor prognosis in patients with breast cancer. Eur J Cancer 40:2717–2725
Mather IH, Jack LJ, Madara PJ, Johnson VG (2001) The distribution of MUC1, an apical membrane glycoprotein, in mammary epithelial cells at the resolution of the electron microscope: implications for the mechanism of milk secretion. Cell Tissue Res 304:91–101
Matsuda M, Lockefeer JA, Horseman ND (2003) Aldolase C/zebrin gene regulation by prolactin during pregnancy and lactation. Endocrine 20:91–100
Matthaei KI (2007) Genetically manipulated mice: a powerful tool with unsuspected caveats. J Physiol (Lond) 582:481–488
Montesano R, Soulié P (2002) Retinoids induce lumen morphogenesis in mammary epithelial cells. J Cell Sci 115:4419–4431
Moon RC, Mehta RG, McCormick DL (1985) Retinoids and mammary gland differentiation. Ciba Found Symp 113:156–167
Naylor MJ, Lockefeer JA, Horseman ND, Ormandy CJ (2003) Prolactin regulates mammary epithelial cell proliferation via autocrine/paracrine mechanism. Endocrine 20:111–114
Neville MC, McFadden TB, Forsyth I (2002) Hormonal regulation of mammary differentiation and milk secretion. J Mammary Gland Biol Neoplasia 7:49–66
Nguyen DA, Neville MC (1998) Tight junction regulation in the mammary gland. J Mammary Gland Biol Neoplasia 3:233–246
Nguyen DA, Parlow AF, Neville MC (2001) Hormonal regulation of tight junction closure in the mouse mammary epithelium during the transition from pregnancy to lactation. J Endocrinol 170:347–356
Overholtzer M, Mailleux AA, Mouneimne G, Normand G, Schnitt SJ, King RW, Cibas ES, Brugge JS (2007) A nonapoptotic cell death process, entosis, that occurs by cell-in-cell invasion. Cell 131:966–979
Parmar H, Cunha GR (2004) Epithelial-stromal interactions in the mouse and human mammary gland in vivo. Endocr Relat Cancer 11:437–458
Patel JB, Mehta J, Belosay A, Sabnis G, Khandelwal A, Brodie AMH, Soprano DR, Njar VCO (2007) Novel retinoic acid metabolism blocking agents have potent inhibitory activities on human breast cancer cells and tumour growth. Br J Cancer 96:1204–1215
Proia DA, Kuperwasser C (2006) Reconstruction of human mammary tissues in a mouse model. Nat Protoc 1:206–214
Reynolds BA, Weiss S (1996) Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. Dev Biol 175:1–13
Roarty K, Serra R (2007) Wnt5a is required for proper mammary gland development and TGF-beta-mediated inhibition of ductal growth. Development 134:3929–3939
Rudland PS, Barraclough R (1990) Differentiation of simian virus 40 transformed human mammary epithelial stem cell lines to myoepithelial-like cells is associated with increased expression of viral large T antigen. J Cell Physiol 142:657–665
Rudland PS, Hughes CM (1989) Immunocytochemical identification of cell types in human mammary gland: variations in cellular markers are dependent on glandular topography and differentiation. J Histochem Cytochem 37:1087–1100
Samatar AA, Wang L, Mirza A, Koseoglu S, Liu S, Kumar CC (2002) Transforming growth factor-beta 2 is a transcriptional target for Akt/protein kinase B via forkhead transcription factor. J Biol Chem 277:28118–28126
Sartor MA, Tomlinson CR, Wesselkamper SC, Sivaganesan S, Leikauf GD, Medvedovic M (2006) Intensity-based hierarchical Bayes method improves testing for differentially expressed genes in microarray experiments. BMC Bioinformatics 7:538
Shamay A, Shapiro F, Leitner G, Silanikove N (2003) Infusions of casein hydrolyzates into the mammary gland disrupt tight junction integrity and induce involution in cows. J Dairy Sci 86:1250–1258
Sheffield LG (1988) Organization and growth of mammary epithelia in the mammary gland fat pad. J Dairy Sci 71:2855–2874
Smith GH, Chepko G (2001) Mammary epithelial stem cells. Microsc Res Tech 52:190–203
Smyth GK (2004) Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol 3:article 3
Soule HD, Maloney TM, Wolman SR, Peterson WD Jr, Brenz R, McGrath CM, Russo J, Pauley RJ, Jones RF, Brooks SC (1990) Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10. Cancer Res 50:6075–6086
Sourisseau T, Georgiadis A, Tsapara A, Ali RR, Pestell R, Matter K, Balda MS (2006) Regulation of PCNA and cyclin D1 expression and epithelial morphogenesis by the ZO-1-regulated transcription factor ZONAB/DbpA. Mol Cell Biol 26:2387–2398
Srivastava S, Matsuda M, Hou Z, Bailey JP, Kitazawa R, Herbst MP, Horseman ND (2003) Receptor activator of NF-kappaB ligand induction via Jak2 and Stat5a in mammary epithelial cells. J Biol Chem 278:46171–46178
Stingl J, Raouf A, Emerman JT, Eaves CJ (2005) Epithelial progenitors in the normal human mammary gland. J Mammary Gland Biol Neoplasia 10:49–59
Stull MA, Pai V, Vomachka AJ, Marshall AM, Jacob GA, Horseman ND (2007) Mammary gland homeostasis employs serotonergic regulation of epithelial tight junctions. Proc Natl Acad Sci USA 104:16708–16713
Taylor-Papadimitriou J, Burchell JM, Plunkett T, Graham R, Correa I, Miles D, Smith M (2002) MUC1 and the immunobiology of cancer. J Mammary Gland Biol Neoplasia 7:209–221
Tokes AM, Kulka J, Paku S, Szik A, Paska C, Novak PK, Szilak L, Kiss A, Bogi K, Schaff Z (2005) Claudin-1, -3 and -4 proteins and mRNA expression in benign and malignant breast lesions: a research study. Breast Cancer Res 7:R296–R305
Underwood JM, Imbalzano KM, Weaver VM, Fischer AH, Imbalzano AN, Nickerson JA (2006) The ultrastructure of MCF-10A acini. J Cell Physiol 208:141–148

Utama FE, LeBaron MJ, Neilson LM, Sultan AS, Parlow AF, Wagner KU, Rui H (2006) Human prolactin receptors are insensitive to mouse prolactin: implications for xenotransplant modeling of human breast cancer in mice. J Endocrinol 188: 589–601

Vaillant F, Asselin-Labat ML, Shackleton M, Lindeman GJ, Visvader JE (2007) The emerging picture of the mouse mammary stem cell. Stem Cell Rev 3:114–123

Wang YA, Shen K, Wang Y, Brooks SC (2005) Retinoic acid signaling is required for proper morphogenesis of mammary gland. Dev Dyn 234:892–899

Wicha MS (2006) Identification of murine mammary stem cells: implications for studies of mammary development and carcinogenesis. Breast Cancer Res 8:109

Wood TL, Richert MM, Stull MA, Allar MA (2000) The insulin-like growth factors (IGFs) and IGF binding proteins in postnatal development of murine mammary glands. J Mammary Gland Biol Neoplasia 5:31–42

Zafrakas M, Chorovicer M, Klaman I, Kristiansen G, Wild PJ, Heindrichs U, Knuchel R, Dahl E (2006) Systematic characterisation of GABRP expression in sporadic breast cancer and normal breast tissue. Int J Cancer 118:1453–1459