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ARTICLE

Spatially correlated phenotyping reveals K5-positive luminal progenitor cells and p63-K5/14-positive stem cell-like cells in human breast epithelium

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Abstract

Understanding the mechanisms regulating human mammary epithelium requires knowledge of the cellular constituents of this tissue. Different and partially contradictory definitions and concepts describing the cellular hierarchy of mammary epithelium have been proposed, including our studies of keratins K5 and/or K14 as markers of progenitor cells. Furthermore, we and others have suggested that the p53 homolog p63 is a marker of human breast epithelial stem cells. In this investigation, we expand our previous studies by testing whether immunohistochemical staining with monospecific anti-keratin antibodies in combination with an antibody against the stem cell marker p63 might help refine the different morphologic phenotypes in normal breast epithelium. We used in situ multilabel staining for p63, different keratins, the myoepithelial marker smooth muscle actin (SMA), the estrogen receptor (ER), and Ki67 to dissect and quantify the cellular components of 16 normal pre- and postmenopausal human breast epithelial tissue samples at the single-cell level. Importantly, we confirm the existence of K5+ only cells and suggest that they, in contrast to the current view, are key luminal precursor cells from which K8/18+ progeny cells evolve. These cells are further modified by the expression of ER and Ki67. We have also identified a population of p63+K5+ cells that are only found in nipple ducts. Based on our findings, we propose a new concept of the cellular hierarchy of human breast epithelium, including K5 luminal lineage progenitors throughout the ductal-lobular axis and p63+K5+ progenitors confined to the nipple ducts.

Introduction

Knowledge of the complexity of human adult mammary gland epithelium is a prerequisite for understanding normal physiological regeneration and to develop concepts of abnormal proliferative disease. Recent studies using cell sorting (CD49f, EpCAM), Hoechst 33342 staining, cell fate mapping experiments in transgenic mice, in vitro and

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transplantation assays, multicolor immunofluorescence stainings, and molecular analyses have generated partially contradicting models of the breast epithelium [1–28] (Supplementary Figure 1). Several of these studies, have identified subpopulations of bipotent mammary cells in human and mouse breast epithelia with "stemness" features that are immunohistochemically strongly positive for basal keratins K5 and/or K14 [1, 6, 13]. Other studies have proposed a ductal stem cell zone characterized by the accumulation of K14+K19+ cells [8] or a subpopulation of ER+ cells [29, 30]. Finally, based on molecular cell tracing experiments, a model has been proposed, postulating the existence of unipotent myoepithelial and luminal stem cells in the mouse mammary epithelium [22]. More recently, the stem cell marker p63 has been suggested as a marker of human breast epithelial stem cells [3]. This is in line with our findings, that human breast epithelium contains p63+K5+ cells [31].

Here we expand on our previous studies [1, 31] to further test the hypothesis that K5+ and/or p63+K5+ progenitors play a role in the maintenance of human breast epithelium. We quantitatively evaluated the expression of p63 and basal keratins K5 and K14 as phenotypic markers of stem-/progenitor cells in the mammary cell hierarchy. We used multicolor stainings to study the stem cell markers p63 [32–38], K5, and K14 [1, 39], the differentiation markers and luminal keratins K18, K8/18, and K19 [1, 40–46], and the myoepithelial lineage markers smooth muscle actin (SMA) [47, 48], smooth muscle myosin heavy chain [49], calponin [50, 51], and CD10 [47, 52–55]. We also evaluated the functional markers estrogen receptor (ER)-alpha [5, 23, 29, 56–61] and Ki67 [62–66]. Based on our findings, we propose a modified concept of the cellular hierarchy of human breast epithelium, including K5+ luminal lineage progenitor cells in the ductal-lobular axis and p63+K5+ progenitor cells confined to nipple ducts.

Material and methods

Case selection

Histologically normal breast tissue samples were obtained from 16 women aged 27–80 years (8 premenopausal aged 27–42 years, and 8 postmenopausal aged 52–80 years) who were undergoing surgery for breast carcinoma, fibroadenoma, or reduction mammoplasty, under informed patient consent. The samples were retrieved from the archives of the Department of Pathology of the University of Muenster (WB) and the Institute of Pathology of the Friedrich Bonfoeffer Clinic in Neubrandenburg. All tissues were immediately formalin-fixed and paraffin-embedded. Only grossly, histologically, and immunohistochemically normal tissues were included. The samples were taken at a distance of at least 3 cm from the site of the tumor. Samples with any type of epithelial hyperplasia (blunt duct adenosis, columnar cell change/hyperplasia, usual ductal hyperplasia) or intraepithelial atypia (flat epithelial atypia, atypical ductal hyperplasia, ductal carcinoma in situ, lobular neoplasia), as defined by the current WHO classification of breast tumors [67], were excluded. Parity history, menstrual cycle status, use of oral contraceptives, and hormone replacement therapy were not included in this study.

Table 1 Primary antibodies used in this study

Antibody	Catalog no.	Clone	Source	Dilution
p63	CM163C	4A4	Biocare Medical	1:50
K5	305R-16	ER16014	MEDAC	1:100
K5/6	M 7237	D5/16 B4	Dako	1:50
K14	Ab7800	LL002	AbCam	1:50
K18-FITC	F4772-2ML	CY-90	Sigma	1;50
K8/18	Mob189	5D3	Zytomed	1:50
K19	MSK017	A53-B/A2.26	Zytomed	1:100
ER	RM-9101	SP1	Thermo Fisher	1:50
Ki67	RM-9106	SP6	Thermo Fisher	1:100
SMA	ab5694	Rabbit polyclonal	AbCam	1:200
Calponin	M 3556	CALP1	DAKO	1:50
CD10	NCL-L-CD10-270	56C6	Novocastra	1:10
SMM-HC[a]	MSK030	1A4	Zytomed	1:50

[a]Smooth muscle myosin heavy chain

Bright-field microscopy

The primary antibodies used are shown in Table 1. For bright-field microscopy, primary antibodies were detected using the Dako LSAB REAL Detection System (Naphthol phosphate/Fast Red, no. K5005; Dako Corporation, Hamburg, Germany) or AmpliStain™ Horseradish Peroxidase (HRP) conjugates (SDT GmbH, Baesweiler, Germany) according to the manufacturers' instructions [68]. The HRP label was visualized using a 3,3′-diaminobenzidine (DAB) substrate kit (Vector Laboratories, Burlingame, CA, USA).

In situ multicolor staining

Triple immunostaining was performed using antibodies against p63, basal keratins K5 and K14, luminal keratins K18-fluorescein isothiocyanate (FITC), K8/18, K19, SMA, calponin, and CD10, and the functional markers ER and Ki67 (compare Fig. 1). As double staining with the three

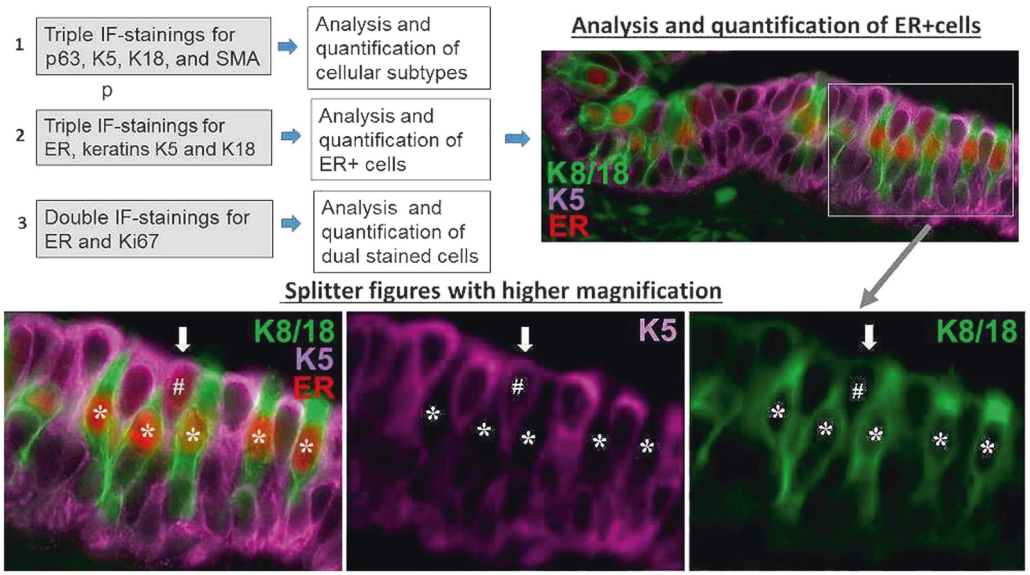

Fig. 1 Study design. Multiple immunofluorescence stainings for p63, K5, K8/18, SMA, ER, Ki67 were used to characterize the different phenotypes of cells found in normal human breast epithelium. The analysis and the quantification of the different expression patterns was performed using merge and splitter figures at higher magnification. This picture shows a triple staining for ER, K5, and K8/18. Notice that the ER+K8/18+ cells (asterisks) and the single-ER+K5+ cell (hashtag) are easily recognized

luminal keratin antibodies each showed identical results (Supplementary Figures 8-10), K8/18 was used throughout the text. Likewise, multiple immunofluorescence stainings for SMA, calponin, smooth muscle myosin heavy chain, and CD10 revealed identical results (Supplementary Figures 8-11); although, the staining intensity for these markers differed in single cells. SMA was used in all triple staining studies and is therefore used throughout the text. For all studies, secondary antibodies (Dianova, Hamburg, Germany, and Molecular Probes, Darmstadt, Germany) conjugated with Cy3, Alexa Fluor-488, Alexa Fluor-647, or biotin were used. For simultaneous visualization of the primary antibodies of the same IgG isotype, the antibodies were non-covalently labeled in vitro with a reporter molecule employing monovalent IgG Fc-specific Fab fragments [69]. The reporter molecule was either the fluorophore Cy3 or biotin. The latter was visualized using fluorophore-labeled streptavidin. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, 5 µg/ml in phosphate-buffered saline [PBS]) for 15 s, and the sections were then mounted using VectaShield (Vector Laboratories, Burlingame, USA).

Image acquisition

Immunostained sections were examined with a Zeiss microscope (Axio Imager Z1). Images were captured with an AxioCam black and white digital microscope camera. The AxioVision image processing program (Carl Zeiss Vision, Germany) allows fluorophores to be visualized in any artificial color. For example, in some figures, we chose the yellow color for the red fluorophore Cy3 because the other two labels were green and red. Far-red fluorescence (Alexa 647) was also presented in different artificial colors, although usually in pink (magenta).

Quantification of immunohistochemical and multicolor immunofluorescence staining

The different p63 and keratin epithelial phenotypes and their functional features (Ki67 and ER expression) were quantified and expressed as percentages of the total number of cells counted. Photographs were randomly acquired using a high-power lens and the appropriate filter sets in succession for the visualization of DAPI, Cy5 or Alexa Fluor 647, Cy3, and FITC or Alexa Fluor 488 to assess the presence of these antigens in single-labeled cells. Positively stained cells were determined by counting cells within at least 10 fields of view per slide using a ×40 objective (700 × 500 µm). Quantification of the cell content was performed using a counting program incorporated in the AxioVision software. Ten merged images per triple staining and case were taken using the splitter display of the AxioVision image processing software (Carl Zeiss Vision, Germany) to analyze and count cell by cell at higher magnification (Fig. 1).

Results

Immunohistology

Immunohistochemically, nipple ducts are characterized by a basal (myoepithelial) layer positive for p63, basal keratins

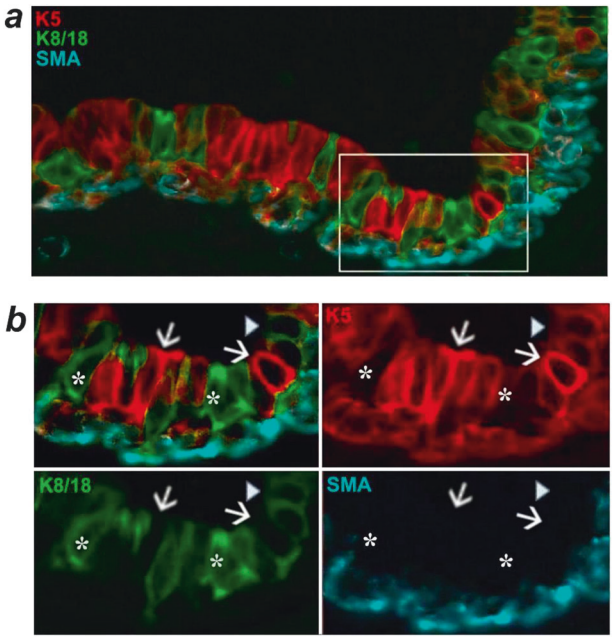

Fig. 2 Nipple duct: Triple immunofluorescence staining for K5 (red), K8/18 (green), and SMA (cyan). **a** Merge picture and **b** marge and splitter figures at higher magnification. The cells of the outer cell layer of the duct express K5 and SMA. The luminal layer contains K5-positive cells (arrows), K5+K18+ intermediary cells (arrow head) and K18+ glandular cells (asterisks)

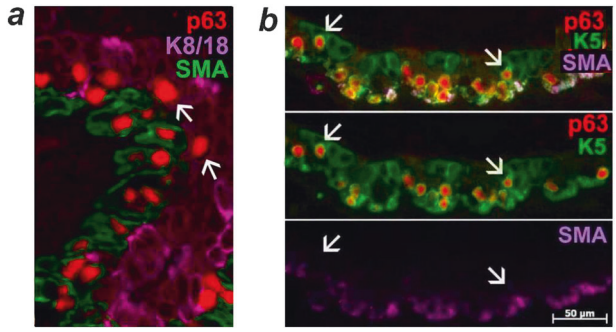

Fig. 3 Nipple duct. **a** Triple immunofluorescence staining for p63, K8/18, and SMA. p63+ cells (arrows) at the interface of the myoepithelial and luminal layer are negative not only for K8/18 but also for SMA (see also Supplementary Figure 12). **b** Triple staining for p63(red) and K5 (green), and SMA (pink). Notice that p63+ cells are tethered to the luminal cells (arrows) and show a transition of p63+K5+ cells to K5+ cells in a more luminal position

K5 (variably) and K14, and myoepithelial markers, such as SMA, calponin, and CD10. The luminal layer of the ductal epithelium showed a heterogeneous staining pattern with many luminal cells being positive for basal keratins K5 and/or glandular keratins K8/18 (Supplementary Figure 2). Based on the staining of basal keratins K5 and luminal keratins K8/18, two types of terminal ductal-lobular units (TDLU) could be distinguished (Supplementary Figure 3): one type containing mainly or even only luminal cells expressing keratins K8/18 (mature lobule) and a second type consisting of luminal cells that, in varying numbers, co-expressed luminal keratins K8/18 and the basal keratin K5 (immature lobule).

Nipple ducts

Triple immunofluorescence staining revealed a multitude of multi-colored cells within the nipple ducts (Supplementary Figure 4). In terms of keratin expression patterns, three different cell types could be robustly distinguished in the luminal layer (Fig. 2 and Supplementary Figures 5-7): K5+K14+/− cells, intermediary cells co-expressing basal keratins K5+K14+/− and luminal keratins K8/18, and finally K8/18+ glandular cells (Fig. 2b and Supplementary Figure 6). Similar findings were obtained when using K18-FITC or K19 instead of K8/18 antibodies (Supplementary Figures 8-11). K5+K14+/− luminal cells appeared to transform to K8/18+ cells through a shift in their keratin expression from basal keratins to glandular keratins. Luminal cells expressing only K14 were rarely found (Supplementary Figure 5). Surprisingly, we found a small number of p63+K5/14+ cells at the interphase of the myoepithelial/luminal layer of the nipple ducts in all 16 cases (compare Fig. 3a, b and Supplementary Figure 12. These cells expressed neither keratins K8/18 nor the myoepithelial marker SMA. Some of these p63+/K5/14+ cells seemed to be restricted to the luminal layer with a transition to K5-only-positive cells (Fig. 3), thus showing a striking similarity to the cellular constituents of salivary gland excretory ducts (Supplementary Figure 13). The quantification of the luminal epithelium of the breast nipple ducts of pre- and postmenopausal women are shown in Tables 2 and 3. Concerning the K5(K14+/−) cells and p63+K5+ progenitors in the ductal-lobular axis and the ER-expression pattern in nipple ducts and lobules, we found no differences between fibroadenoma or carcinoma bearing breasts or samples obtained from reduction mammoplasty. Importantly, quantification of a total of 10,331 cells across all cases revealed a small number of K5-only-positive cells (average 5.1%), approximately two-thirds of K5 and K8/18 co-expressing intermediary cells (average 63.47%), and one-third of K8/18-only-positive cells (average 31.4%) (Fig. 4a; Table 2). Less than an average of 1% of the cells expressed p63 and K5/14 and lacked K8/18 and SMA (Table 2).

About one-fifth (average 20.8%) of the cells expressed ER, and nearly all of these co-expressed K8/18. Only a small percentage of ER+ cells co-expressed K5 (Fig. 5a, Table 3). Analyses of the expression of Ki67 and ER revealed double staining of Ki67 and ER in 2.3% and 10.6% of Ki67+ cells in premenopausal and postmenopausal females, respectively (Fig. 5b, Supplementary Figure 14, Table 4).

Table 2 Quantification of triple-stained sections for K5, K8/18, and SMA in 8 premenopausal and 8 postmenopausal women (range in brackets)

Object	No. of cases	Total no. of luminal cells	K5+K8/18-neg. cells	K5+K8/18+ cells	K8/18+K5-neg. cells	P63+K5/14+ only cells[a]
Ducts, prem.	$n = 8$	6188	278 4.5% (1.2–13.8%)	4112 66.5% (31.8–86.7%)	1798 29.3% (10.3–54.3%)	0.92%* (0.49–1.8%)
Ducts, postm.	$n = 8$	4143	251 6.1% (1.7–10.86%)	2446 59% (30.5–86.5%)	1446 34.9% (11.3–60.2%	0.88% (0.43–1.38%)
Ducts, total	$n = 16$	10,331	529 5.1%	6558 63.47%	3244 31.40%	0.89%
TDLUs, prem.	$n = 8$	4864	77 1.06% (0.16–3.75%	1437 29.5% (6–63.4%)	3350 68.9% (35.2%–92%)	0.09 (0–0.36%)
TDLUs, postm.	$n = 6$	5498	141 2.6% (0.53–4.7%)	1767 32.1% (14.4–44.5%)	3590 65.3% (54.95–84.7%)	0.05% (0–0.17%)
TDLUs, total	$n = 16$	10,362	218 2.1%	3204 30.92%	6940 66.97%	0.07%

[a]p63+ K5/14 positive cells were calculated on the basis of triple stainings for p63, K18, and SMA

Terminal duct-lobular units (TDLUs)

Triple immunofluorescence staining of TDLUs revealed the full set of luminal cells with K5+K14+/− cells, K5+K8/18 + intermediary cells, and K8/18+ glandular cells (Fig. 6). The myoepithelial layer consisted of p63+/K5+ (variably)/K14+/SMA+ cells. The quantification of TDLUs from premenopausal and postmenopausal women are shown in Tables 2 and 3. Overall, quantification of a total of 10,362 luminal lobular cells revealed that a small number of cells expressed K5 only (average 2.1%), approximately one-third co-expressed K5 and K8/18 (average 30.92%), and nearly two-thirds expressed only the glandular keratins K8/18 (average 66.97%) (Fig. 4b and Table 2). In contrast to the nipple ducts, the p63+ cells in TDLUs were confined to the myoepithelial cell layer (Fig. 7a).

Analysis of the ER status revealed nearly one-third of ER + cells (average 29.4%), including mainly ER and K18 co-expressing cells (average 28.9%) and a minor fraction (0.5%) of ER+K5+K18+ cells (Table 3). Quantification of Ki67 and ER double stainings revealed that 3.3 and 17.03% of Ki67+ cells co-expressed Ki67 and ER in premenopausal and postmenopausal women, respectively (Fig. 7b and Table 4).

Discussion

Here we present a modified concept of the cellular organization of normal human breast epithelium (Fig. 8) compared to that envisioned in most current concepts (Supplementary Figure 1). Importantly, we have identified a discrete population of K5+(K14+/−) progenitor cells which, in contrast to the current view, are regarded as part of a larger contiguous, tightly linked cell population in the luminal layer of both nipple ducts and lobules. Glandular differentiation involves the sequential modulation of these cells with a shift from K5+(K14+/−) cells via K5+K8/18+ intermediary cells to K8/18+ glandular cells, as shown in our working model in Fig. 8. Our findings are in line with the observations of Lim et al. [3] who identified a $CD49f^{pos}$ (alpha-6 integrin)/$EpCAM^{pos}$ subpopulation of luminal-restricted progenitor cells characterized immunohistochemically by the expression of K5/6 (49.9%), K8/18 (91%), MUC1 (80%), and ER (28%), but lacking p63 (Fig. 9). In vitro studies, showed that these cells generated only homogeneous glandular structures. This study is notable because it emphasizes our observations that lineage identity cannot be based solely on the presence or absence of basal keratins K5 and/or K14. For example, K5+ (and/or K14+) cells co-expressing p63 and SMA clearly belong to the myoepithelial/basal lineage [70, 71], whereas K5+(K14 +/−) only cells and K5+(K14+/−)K8/18+ cells, according to our observations, belong to the luminal lineage.

We have also identified a small population of p63+K5/14+ precursor cells residing at the interface between the myoepithelial and luminal cell layers of the human nipple duct epithelium. These cells seem to be the least differentiated cells expressing neither the luminal keratins K8/18 nor the myoepithelial marker SMA. Based on previous data, we suggest that these cells may undergo a transition from their original p63+K5/14+ precursor state to a K5+K14 +/− luminal precursor state, and that they also may generate the p63+ SMA+ myoepithelial cells [3]. For example,

Table 3 Quantification of triple stainings for ER, K5, and K8/18 in premenopausal and postmenopausal women (range in brackets)

Objects	No. of cases	Total no. of luminal cells	ER+ cells	ER+K5+ cells	ER+K5+K8/18+ cells	ER+K8/18+ cells
Ducts, prem.	$n = 8$	9817	1565 15.94% (11.36%–24.5%)	3 0.03% (0–0.08%)	88 0.89% (0–3.3%)	1473 15.00% (10.08–21.2%)
Ducts, postm.	$n = 5$	7197	1978 27.5% (21.47–36.4%)	4 0.06% (0–0.2%)	61 0.85% (0.4–2.56%	1913 26.6% (19.92–33.7%)
Ducts, total	$n = 13$	17,014	3543 20.8%	7 0.04%	149 0.87%	3386 19.9%
TDLUs, prem.	$n = 8$	6518	1314 19.91% (12.91–29.51%)	0	13 0.2% (0–0.44%)	1301 19.71% 12.47–29.32%)
TDLUs, postm.	$n = 5$	6651	2559 38.4% (29.45–46.74%)	0	53 0.79% (0.12–18%)	2506 37.67% (24.9%–37.1%)
TDLUs, total	$n = 13$	13,169	3873 29.4%	0	66 0.5%	3807 28.9%

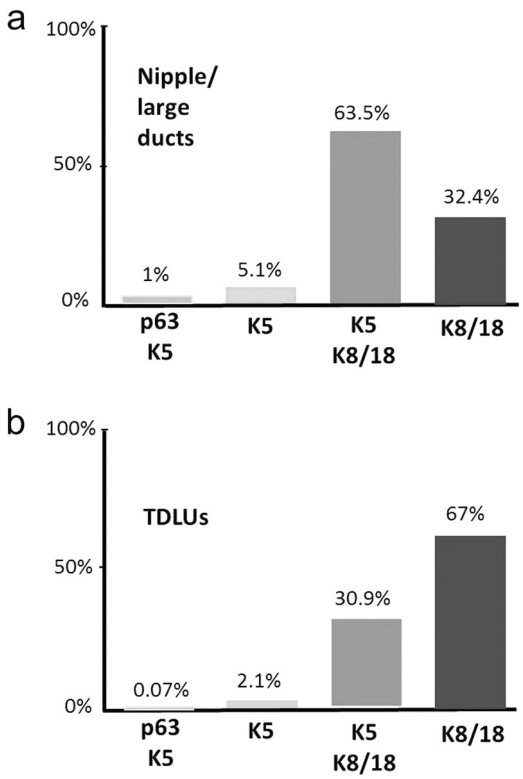

Fig. 4 Diagrams showing the average frequency of different cells types in normal breast epithelium of ducts (**a**) and lobules (**b**)

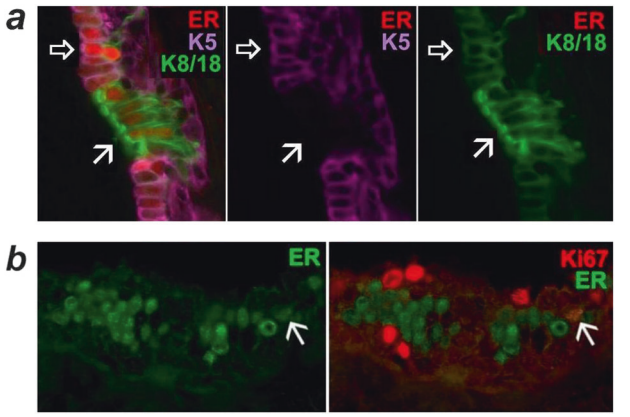

Fig. 5 Nipple duct. **a** Triple immunofluorescence staining for ER (red), K5 (pink), and K8/18 (green). The picture shows that ER is here is expressed in K18+ luminal cells (arrows) and in K5+K8/18+ intermediary cells (open arrows). **b** Double staining for Ki67 and ER, which shows mutually exclusive ER or Ki67-positivity with only one exception (arrow)

Table 4 Quantification of double stainings for Ki67 and ER

Object	Ki67 (total)	Ki67+ only	Ki67+/ER+
Ducts, premenopausal ($n = 6$)	260	254 (97.69%)	6 (2.30%)
Ducts, postmenopausal ($n = 5$)	302	270 (89.4%)	32 (10.6%)
Ducts, total ($n = 11$)	562	524 (93.24%)	38 (6.76%)
TDLUs, premenopausal ($n = 6$)	302	292 (96.7%)	10 (3.3%)
TDLUs, postmenopausal ($n = 5$)	270	244 (90.04%)	46 (17.03%)
TDLUs, total ($n = 11$)	592	536 (90.5%)	56 (9.5%)

Lim et al. observed a CD49f^{high} EpCAM^{neg} subpopulation of human breast epithelial progenitors characterized immunohistochemically by the expression of p63 (76%), K14 (70%), and K5/6 (52%), and lacking K8/18 (2.3%) and ER (0.2%). In transplantation assays, only this subpopulation of human epithelial breast cells showed bilinear differentiation potential [3] (cf. Fig. 8). Furthermore, the view

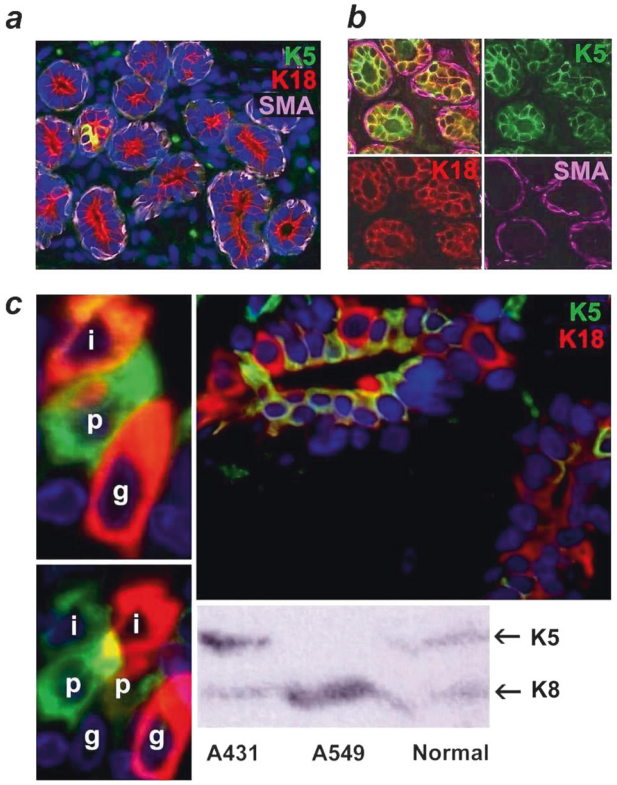

Fig. 6 Breast lobules. **a**, **b** Triple stainings for K5, K18, and SMA. **a** This picture highlights a mature lobule with expression of only K8/18 in nearly all acinar cells. Only one acinus contains cells with a hybrid color, indicating the co-expression of K5 and K8/18. The myoepithelial cells stain for SMA. **b** This lobule contains many luminal cells co-expressing the basal keratin K5 and the luminal keratin K8/18, indicating intermediary cells. **c** Double staining for K5 (green) and K18 (red) showing the heterogeneity of luminal cells with a K5+ luminal progenitor (p), K5+K8/18+ intermediary cell (i) and a K8/18 + glandular cell (g). The western blotting immunoreaction with K5 and K18 antibodies of total cellular proteins from micro-dissected lobular cells and of two well-known cell lines (A413 and A449) shows, as expected, 2 bands in normal epithelial cells (**c** from ref. [46])

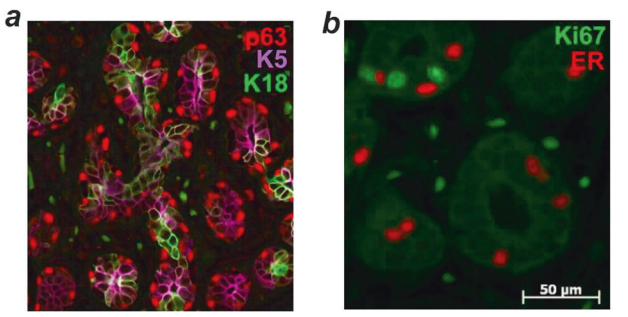

Fig. 7 Breast lobule **a** Triple immunofluorescence staining for p63, K5, and K8/18. Notice that p63-positive cells reside only in the basal position. **b** Double staining for ER and Ki67 shows mutually exclusive ER or Ki67-positivity

that p63 may play a role in breast epithelium is supported by recent studies showing that Notch signaling downregulates p63 expression prior to luminal lineage commitment [72]. Similarly, Notch inactivation in mouse mammary glands leads to accumulation of immature p63+K5+ cells (even in luminal position) and K14+K18+ cells [73]. Finally, the crucial role of p63 in the maintenance of epithelium has also been demonstrated at several other anatomical sites, including, for example, the thymus, epidermis, upper airways, and prostate [36, 37, 74–80].

In the present study, the average ER expression level of lobular cells was higher than that in nipple ducts. In agreement with the literature, the level of ER expression in the lobular epithelium of postmenopausal women (38.4%) was much higher than in premenopausal women (19.9%). Among the different luminal phenotypes, we found ER-alpha expression in K8/18+ differentiated cells, whereas only a limited number of K5+K8/18+ progenitor cells showed ER-positivity. The significance of ER expression in these progenitors remains unclear. Similarly, and in agreement with the literature, increased levels of Ki67+ER+ cells were observed in TDLUs of postmenopausal as compared to premenopausal women [59, 81].

The present observations have several important implications. Thus, our study indicates that the current view of K8/18+ luminal cells vs. K5/14+ basal/myoepithelial cells as basic biological constituents of normal human breast epithelium and their tumors [82, 83] needs to be modified. The finding of K5+K14+/− progenitors within the luminal layer has important conceptual implications not only for our understanding of normal regeneration. For example, a subset of basal-type breast carcinomas may, as suggested by Lim et al.[3], be better classified as the luminal progenitor subtype. Furthermore, the finding of rare p63+K5+ tumors, such as, for example adenoid cystic carcinoma and syringomatous tumors/low-grade adenosquamous carcinoma of the breast, suggests the existence of a corresponding p63 +K5+ normal cell type as previously discussed [84–86]. With this conceptual knowledge, we may now approach the question of developmental relationships between the cell types in normal breast epithelium and their counterparts in proliferative breast diseases [87]. It is plausible that the diversity of phenotypic and functional characteristics of breast tumors may emerge from a combination of cell of origin features and specific acquired genetic/epigenetic changes of these cells during tumorigenesis [87–91].

Our model does not fully comply with some of the previously published data. We and others have proposed that K5+ and/or K14+ cells are bipotent progenitors in the mammary epithelium [1, 6, 92]. This concept has been based on K5+ and/or K14+ cells as the least differentiated cells that differentiate toward glandular epithelial or myoepithelial end cells. However, as discussed above, the introduction of p63 as an important biomarker in breast epithelium questions the view of K5+K14+/− cells as

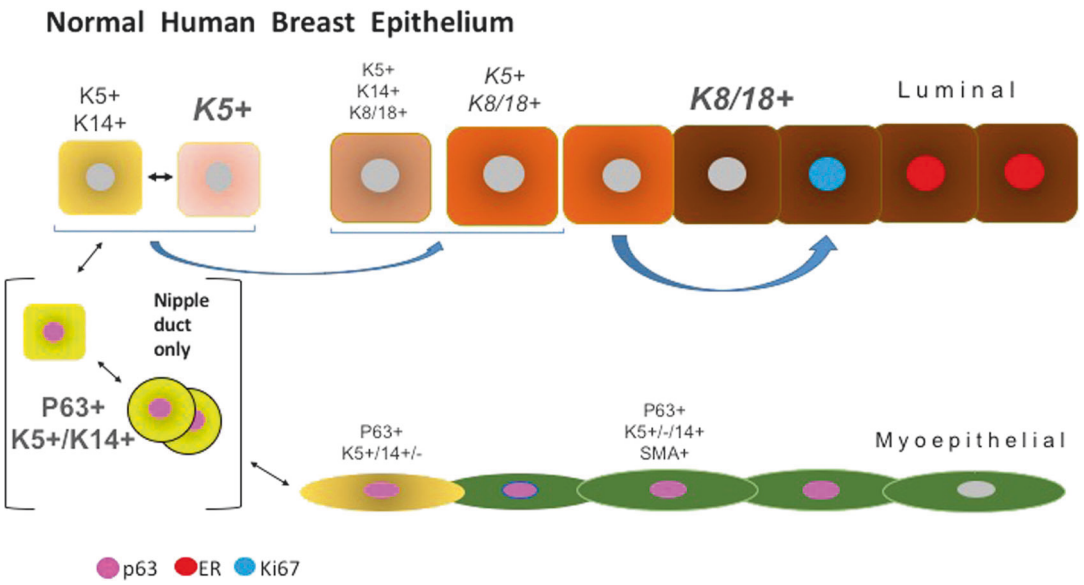

Fig. 8 Tentative working model of the cellular composition of the human breast epithelium, based on p63− and keratin expression pattern. We observed K5+K14+/− progenitor cells and their progeny only in luminal cells. Notably, only the nipple ducts contain p63+K5/14+ progenitor cells

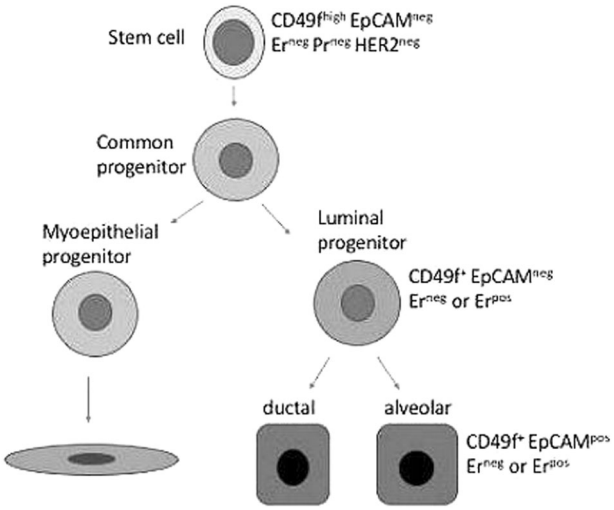

Fig. 9 Hypothetical model of the human breast epithelium, proposed by Lim et al.[3], based on fluorescence-activated cell sorting of human breast epithelial cells with antibodies to surface markers CD49f (alpha-6 integrin) and CD326 (EpCAM)

early bipotent progenitors. A different concept of human breast epithelium has also been proposed by Petersen and coworkers [7, 8], who described a ductal stem cell zone characterized by accumulation of K14+K19+ stem cells. Although, we can confirm the existence of K14+K19+ cells in the luminal epithelium (cf. Supplementary Figure 7), we interpret them as intermediary glandular cells identical to K5+K8/18+ cells. Finally, one study using a genetic lineage-tracing approach in adult mice [6] suggested the existence of unipotent K14+ basal and K8/18+ luminal progenitors and noted that K14+ cells do not contribute to the luminal lineage [22]. In the present study, we clearly show that K5+K14+/− cells are constituents of the human luminal breast epithelium and should therefore be interpreted in the context of luminal cell differentiation [3].

A limitation of this study is that the number of cases studied is relatively small. This is due to the fact that the study and methods used are laborious and time consuming since the quantification is done at the single-cell level on merged and single-channel images and there are no high-throughput techniques available. However, further developments in this area are needed since recent studies have indicated that the cellular "makeup" of the breast epithelium may have an important impact on breast cancer development [3].

In summary, we provide new evidence for the existence of K5+K14+/− luminal progenitors from which glandular progeny cells evolve. We also demonstrate the presence of a small subpopulation of p63+K5/14+ bipotent progenitors confined to the nipple ducts. Moreover, we show that ER expression is associated with differentiated glandular cells and is only rarely observed in progenitors. Based on these findings, we propose a new, modified model of the hierarchical organization of breast ducts and lobules. Future refinements of molecular breast epithelial signatures may provide new important insights into the definition and regulation of stem/progenitor cells in normal and diseased breast tissues.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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