

Cytokeratin expression during mouse embryonic and early postnatal mammary gland development

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Abstract Cytokeratins are intermediate filament proteins found in most epithelial cells including the mammary epithelium. Specific cytokeratin expression has been found to mark different epithelial cell lineages and also to associate with putative mammary stem/progenitor cells. However, a comparative analysis of the expression of cytokeratins during embryonic and postnatal mammary development is currently lacking. Moreover, it is not clear whether the different classes of putative mammary stem/progenitor cells exist during embryonic development. Here, we use double/triple-label immunofluorescence and immunohistochemistry to systematically compare the expression of cytokeratin 5 (K5), cytokeratin 6 (K6), cytokeratin 8 (K8), cytokeratin 14 (K14) and cytokeratin 19 (K19) in embryonic and early postnatal mouse mammary glands. We show that K6⁺ and K8⁺/K14⁺ putative mammary progenitor cells arise during embryogenesis with distinct temporal and spatial distributions. Moreover, we describe a transient disconnection of the expression of K5 and K14, two cytokeratins that are often co-expressed, during the first postnatal weeks of

mammary development. Finally, we report that cytokeratin expression in cultured primary mammary epithelial cells mimics that during the early stages of postnatal mammary development. These studies demonstrate an embryonic origin of putative mammary stem/progenitor cells. Moreover, they provide additional insights into the use of specific cytokeratins as markers of mammary epithelial differentiation, or the use of their promoters to direct gene overexpression or ablation in genetic studies of mouse mammary development.

Keywords Cytokeratin · K5 · K6 · K8 · K14 · K19 · Stem and progenitor cells · Mammary gland development

Introduction

Mammary gland arises from the multipotent surface ectoderm during embryogenesis (Veltmaat et al. 2003), relying on reciprocal epithelial-mesenchymal interactions for morphogenesis. In mice, mammary placode forms between E10.5 and E11.5, and progresses through bud (E12.5–E15.5) and sprout (E16.5) stages to give rise to a rudimentary mammary tree before birth. Following birth, the mammary gland grows isometrically with the body, but expands rapidly via elongation and branching during puberty at around 3–7 weeks of age, culminating in a mature gland by approximately 10–12 weeks of age. Proliferation of mammary progenitor cells that reside in tips, or terminal end buds (TEB), of the elongating ducts facilitates such expansion (Ball 1998; Kenney et al. 2001; Woodward et al. 2005). During pregnancy, small structures bud off the ductal compartment and differentiate to form an expanded lobulo-alveolar compartment composed of basal myoepithelium and secretory luminal epithelium (Hennighausen

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and Robinson 2001). These lobulo-alveolar structures involute after lactation, to be rebuilt with each subsequent pregnancy. The recognition of this regenerative capacity led to the recent isolation of stem/progenitor cell-enriched populations from the mouse mammary epithelium (Shackleton et al. 2006; Stingl et al. 2006).

Cytokeratins are intermediate filament proteins found in most epithelial cells. Two types of cytokeratins exist and are often found as heterodimers between acidic type I cytokeratin (K9–K20) and basic or neutral type II cytokeratin (K1–K8) (Moll et al. 1982, 2008). Several cytokeratins are lineage markers within the mammary epithelium. Among these, both K5 and K14 are found to mark basal/myoepithelial cells, whereas K8/K18 expression marks luminal cells of mouse mammary gland (Mikaelian et al. 2006). There is also limited knowledge on the association of certain cytokeratin expression with a mammary stem/progenitor cell state. Of particular note, K6 is regarded as a putative multipotent/bipotent mammary epithelial progenitor cell marker in mice due to its strong expression in the body cells of TEB, its transient expansion during early pregnancy, and its persistent although rare expression during pregnancy, lactation, and involution (Grimm et al. 2006; Sapino et al. 1993; Smith et al. 1990). K14⁺/K8⁺ suprabasal/luminal cells have been infrequently detected in adult mouse mammary glands and are proposed to be luminally restricted progenitor cells (Li et al. 2007). K19 is considered a neutral switch cytokeratin the expression of which is compatible with cell type flexibility (Stasiak et al. 1989), and has been shown to be a luminal marker in human mammary glands (Bartek et al. 1990). Moreover, K14/K19-double positive transitional cells have been detected in adult human mammary glands and also proposed to be multipotent progenitor cells (Villadsen et al. 2007). However, whether such K14⁺/K19⁺ cells also exist in mouse mammary glands has not been addressed. To date, a comprehensive knowledge of the expression of these cytokeratins during embryonic and early postnatal development is still lacking. Such information will facilitate our understanding of how lineage specification occurs during mammary development. Moreover, it will shed light into the embryonic origin of adult mammary stem/progenitor cells, a notion important not only for our fundamental understanding of epithelial stem cell biology, but also for studies of breast cancers, particularly those of a stem/progenitor cell origin.

In this work, we use double- and triple-label immunofluorescence and immunohistochemistry to compare the expression of K5, K6, K8, K14 and K19 during mammary gland development. We show that K6⁺, and K8⁺/K14⁺ or K14⁺/K19⁺ double positive cells arise during embryogenesis with distinct temporal and spatial distributions. Moreover, we report a transient separation of K5 and K14

expression during the first two postnatal weeks of development, with abundant K14⁺ but K5⁻ cells present in the suprabasal/luminal compartment. Finally, we analyze the expression of K5, K6, and K14 during growth and differentiation of primary mammary epithelial cells.

Materials and methods

Mice

Mice in a 129svXC57Bl/6 (129XB6) mixed genetic background were used for the study. Embryonic stages were determined according to the copulation plug date (E0.5 was counted as the first day a copulation plug was observed). For each experiment, multiple samples from at least two different animals were examined. All experiments have been approved by, and conform to the regulatory guidelines of the IACUC committee of University of California, Irvine.

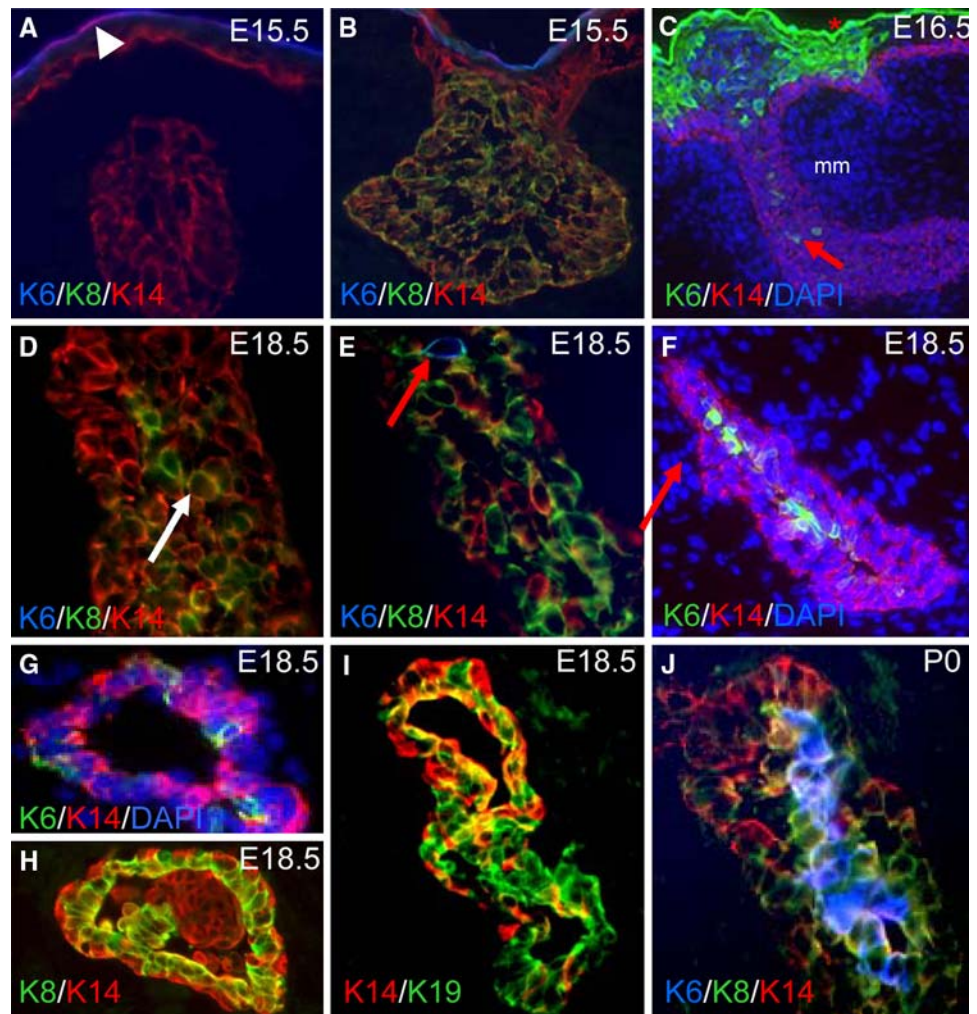
Isolation and culturing of primary mammary epithelial cells

Primary mammary epithelial cells were isolated from 129XB6 hybrid mice as described (Gu et al. 2009) and cultured at 37°C and 5% CO₂ in DME/F12 medium containing 10% FBS, 5 µg/ml insulin, 10 ng/ml EGF, 1 µg/ml hydrocortisone, Pen/Strep (100 U, 100 µg/ml). For Matrigel culture, cells from 8 to 10-week-old virgin females were plated on Matrigel (BD Pharmingen, catalog #354230) per well in Lab-Tek II chamber slides (catalog #154534), cultured for 14 days, and fixed in 2% paraformaldehyde for 20 min at room temperature. Colonies were then permeabilized with PBS containing 0.5% Triton X-100 for 10 min at 4°C, followed by three 10–15 min rinses with PBS/glycine (130 mM NaCl, 7 mM Na₂HPO₄, 3.5 mM NaH₂PO₄; 100 mM glycine) at room temperature. Non-specific interactions were blocked by incubating with 200 µl/well of IF buffer (130 mM NaCl, 7 mM Na₂HPO₄, 3.5 mM NaH₂PO₄; 7.7 mM NaN₃, 0.1% BSA, 0.2% Triton X-100, 0.05% Tween-20) containing 10% goat serum for 45–60 min at room temperature, after which indirect immunofluorescence was performed as described below except that images were acquired on a Zeiss LSM510-META confocal microscope.

Indirect immunofluorescence and immunohistochemistry

For embryonic studies, freshly dissected embryos were frozen in O.C.T. embedding medium (Tissue-Tek #4583) and stored at –80°C until use. Cross-sections (6–10 µm) of the embryos containing the rudimentary mammary glands (#4 except for Fig. 1a) were cut using a cryostat (Microm

Fig. 1 K6, K8, K14 and K19 expression in embryonic mammary glands. Shown are results of indirect immunofluorescence at the indicated ages using the indicated antibodies (color-coded to match the fluorophore). DAPI stains the nuclei. *Arrowhead* in **a** points to the K6-stained periderm (*blue*). *Asterisk* in **c**, nipple sheath. *mm*, mammary mesenchyme. *Red arrows* in **c**, **e** and **f** point to K6⁺ mammary cells, whereas *white arrow* in **d** points to K14⁺/K8⁺ cells. **e**, **j** Longitudinal sections through the inner core layers. **h**, **i**, **j** confocal images



#HM505E). For postnatal studies, fat pads containing mammary epithelia (#3 or #4) were dissected, and either frozen in OCT for the preparation of frozen sections, or for better morphology fixed in 4% paraformaldehyde for 6 h at room temperature (see below). Frozen sections were subjected to indirect immunofluorescence as described (Dai et al. 1998). Briefly, the samples were fixed in 4% paraformaldehyde for 10 min at room temperature, followed by 3 washes in PBS containing 0.1% Triton (PBT), 5 min each. The samples were then blocked in 20% normal goat serum in PBT containing 0.005–0.01% gelatin (NGS/gelatin). Primary antibody incubation was performed in 2% NGS/gelatin for 1 h to overnight, followed by a wash with 2% NGS/gelatin. The following primary antibodies were used: K5 (rabbit, 1:500), K6 (rabbit, 1:500) and K14 (chicken, 1:4000), generous gifts of Julie Segre, NHGRI/NIH (Gu et al. 2009); K8 (Troma-I) and K19 (Troma-III) (rat, 1:50; Developmental Studies Hybridoma Bank). Secondary antibody incubation was in 2% NGS at room temperature for 1 h, followed by three PBS washes. FITC-conjugated goat anti-rat, Rhodamine-conjugated goat anti-chicken and

AMCA-conjugated goat anti-rabbit secondary antibodies (Jackson ImmunoResearch) were used. The samples were then incubated with DAPI (2 µg/ml) for 10 min at room temperature, followed by three PBS washes. The slides were mounted in Antifade medium (Vectashield H-1000, Vector Laboratories), and images were taken at room temperature using a compound fluorescence microscope (Eclipse E600, Nikon) with Plan Apo 20x/0.75 NA or Plan Fluor 40x/0.75 NA objectives (Nikon), and a RT SLIDER camera equipped with SPOT 4.0.9 software (Diagnostic Instruments, Inc.). Acquired images were processed by Photoshop CS 8.0 (Adobe).

For immunohistochemistry, fixed postnatal mammary gland samples from above were washed once in PBS for 5 min, once in 30% ethanol for 15 min, and twice in 70% ethanol overnight. Following further washes in 95 and 100% ethanol for half an hour each, the samples were cleared with Xylene for half an hour, and then incubated and embedded in paraffin. Sections (5 µm) were cut using a microtome, cleared with HistoClear (Fisher Scientific) twice for 15 min each, then rehydrated with washes of 100%

Table 1 Summary of keratin expression during mammary gland development

Marker	Age						
	E15.5	E18.5	P14	P18	3 W	8 W	12 W
K5	+++	+++	ba++	ba++	ba++	ba++	ND
K6	+/-	su+	su+	su++	su++	su+	-
K8	+/-	su++	ND	ND	ND	ND	ND
K14	+++	+++	ba++/su+	ba++/su+	ba++/su+	ba++	ba++

ba basal, *su* suprabasal/luminal, +/- scattered positivity, + to +++ indicating relative abundance of positive cells, *ND* not determined

(2 × 5 min), 95% (2 × 5 min), and 70% (1 × 5 min) ethanol, followed by washes with water (1 × 5 min) and PBT (1 × 5 min). The slides were then heated for 20 min in 10 mM citrate buffer (pH 6.0) in a microwave oven for antigen retrieval. Rabbit anti-K5 or K6 antibodies (primary) and biotinylated anti-rabbit IgG (H + L) (Vector Laboratories, Cat: BA-1000) (secondary) were used, and signal detection was performed using the VECTASTAIN elite ABC Kit (Vector, Cat: PK-6100) and AEC (RED) single solution (Zymed, Cat: 00-1111) according to instructions from manufacturers. All immunofluorescence and immunohistochemistry experiments were performed with negative controls where no primary antibody was added.

Results and discussion

Expression of lineage-specific cytokeratins during embryonic mammary development

We first examined the expression of lineage-specific and/or putative progenitor-associated cytokeratins, including K6, K8, K14 and K19, in embryonic mammary glands (Table 1). At E15.5 and in less developed mammary buds, only K14 expression was observed (Fig. 1a), whereas in more advanced mammary buds, most K14⁺ cells began to co-express K8 (Fig. 1b). K6 expression at this stage was seen in skin periderm as expected, but was rarely detectable in mammary buds (Fig. 1a, b). At E16.5, strong K6 expression was observed in nipple sheath—in sharp contrast to the neighboring epidermal cells that normally do not express K6 protein unless upon injury (Eichner et al. 1984; Moll et al. 1982), and scattered K6⁺ cells were also found in nipple skin between the sheath as well as in the upper portion of the mammary sprout (Fig. 1c). Moreover, the distal border of K6 positivity coincided with the boundary of the mammary mesenchyme. By E18.5 to newborn stage, K8⁺ cells adopted a luminal-like location and were physically separated from K14⁺ cells, which were now mostly occupying the outer layers (Fig. 1d shows a longitudinal section through the outer layers of the primary mammary duct, whereas Fig. 1h shows a cross section).

This said, many K14⁺ cells were also found in the inner layers and some of them co-expressed K8 (Figs. 1e–j). At these ages (i.e., E18.5–newborn), K6⁺ cells became more abundant, and their distribution showed regional variation but an enrichment in the inner layers (Fig. 1e–g, j). Moreover, the K6⁺ cells appeared to be largely distinct from the K14⁺ cells. When double stained for K14 and K19, three populations were seen, including K14⁺K19⁺, K14⁺K19⁻, K14⁻K19⁺ (Fig. 1i). Several conclusions can be drawn from these studies. First, single-lineage cells, such as those expressing only K14 or K8 or K19, are already specified during embryonic mammary development. Second, K6⁺, K14⁺/K8⁺ and K14⁺/K19⁺ cells all exist in embryonic mammary glands, indicating an embryonic origin for these putative multipotent/bipotent or luminally restricted progenitor cells. Last, although emerging at a similar developmental stage, K6⁺ and K14⁺/K8⁺ cells are overall two distinct populations of cells that likely arise independently during embryogenesis. This said, occasionally cells that are double positive for K6 and K14 or for K6 and K8 are found (data not shown).

The apparently continuous presence of K6⁺ cells from the overlying nipple skin to the opening as well as the top portion of the mammary sprout is interesting. Previously, it has been suggested based on morphological criteria that epidermal cells ingress into the mammary sprout (Veltmaat et al. 2003). The molecular similarity between the nipple skin and the upper sprout, based on the observation that they both express transcription factor Lef1 (Foley et al. 2001), is also consistent with this proposal. Our work now provides additional support for the molecular similarities between these regions. More importantly, since K6 protein expression is a hallmark of hyperproliferative keratinocytes upon wounding, our results now suggest that the formation of the nipple and the top part of mammary sprout likely involves the activation of epidermal keratinocytes. The close association of the K6-expressing epithelial territories with the underlying mammary mesenchyme suggests that the latter may secrete signals that regulate keratinocyte activation. Clearly, lineage-tracing experiments in the future will be informative in establishing a definitive relationship between the mammary sprout and its overlying epidermis.

Moreover, additional studies are needed to elucidate the fate(s) of the putative ingressing epidermal cells and whether the K6⁺ cells within the mammary sprout contribute to the subsequent morphogenesis of the gland.

K6 expression during postnatal mammary development—largely distinct from, but occasionally overlapping with K14 expression

We next examined the expression of K6 during postnatal mammary development and compared it with K14 expression (Table 1). Between P11 and P14, scattered K6⁺ cells were found, mostly in the inner core of the ductal termini, and some co-stained for K14 (Fig. 2a–c and data not shown). At P18, more prominent K6 expression was seen, particularly in inner cells of the ductal termini, and again a few cells were double positive for K6 and K14 (Fig. 2d). At 3 weeks, K6 expression was readily observed in wild-type TEB body cells and in scattered ductal epithelial cells, and K6⁺/K14⁺ cells still existed (Fig. 2e and data not shown).

That said, most K6⁺ cells that arise between 2 and 3 weeks after birth appeared to be K14[−] (Figure 2d, e). A similar pattern of expression persisted during later virgin mammary development, as abundant K6⁺ cells were still seen in both ductal termini and ducts of 8-week mammary glands, whether assayed by immunofluorescence on frozen sections or immunohistochemistry on paraffin sections (Fig. 2g, h and data not shown). Upon maturation, however, K6 expression became virtually undetectable (Fig. 2f), a finding that is consistent with previous reports (Gu et al. 2009; Li et al. 2003; Smith et al. 1990). These results indicate that, as during embryonic development, postnatal mammary K6⁺ cells constitute an overall distinct population from K14⁺ cells. However, the developing mammary glands in virgin females also contain a small number of K6⁺/K14⁺ cells. These double positive cells may share the same developmental potential as the suprabasal/luminal K6/K14-coexpressing cells that are detected in pregnant and lactating glands and proposed to be mammary stem cells (Smith et al. 1990). Whether K6⁺/K14⁺ and K6⁺/K14[−]

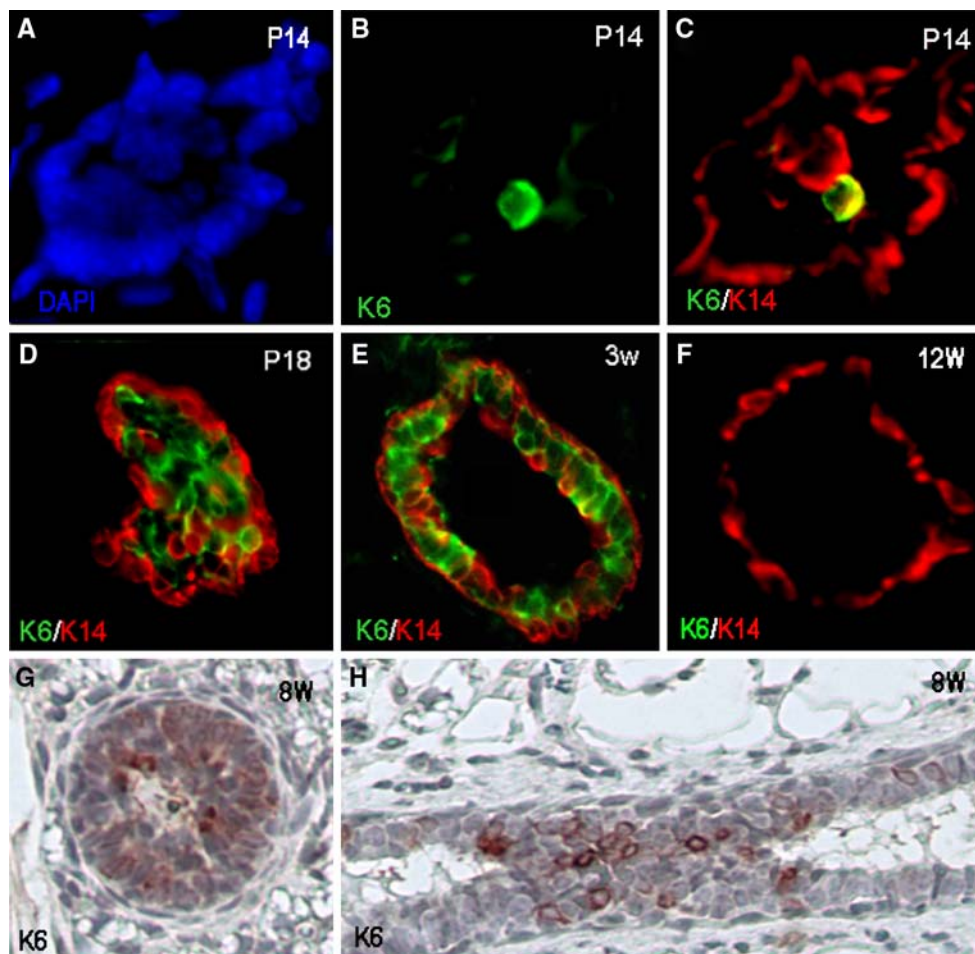


Fig. 2 K6 expression in postnatal virgin mammary glands. Shown are results of indirect immunofluorescence on frozen sections using K6 (green) and K14 (red) antibodies (a–f) or immunohistochemistry on

paraffin sections using only K6 antibody (g, h) at the indicated ages. For P14 analysis, DAPI staining (a) visualizes the nuclei; (b) shows K6 staining while (c) is the corresponding merged image

cells are progenitor populations that differ in developmental potency is an interesting question for future investigation.

Previously, Mikaelian et al. were not able to detect any K6 expression at any stage of the mammary gland development, and concluded that K6 is not a marker of the mammary gland stem cell (Mikaelian et al. 2006). However, our study of multiple stages of development clearly revealed the presence of K6-expressing cells. This said, we observed only scattered K6⁺ cells in most regions of embryonic and early postnatal mammary glands, whereas Grimm et al. reported that nearly all cells in their cross sections of embryonic mammary gland stained positive for K6 (Grimm et al. 2006). As the same antibody is used in both studies, we surmise that our data looking at multiple regions and multiple stages present a more accurate and comprehensive picture of K6 expression in developing mammary glands. Alternatively, the difference may be due to methods in antigen retrieval or mouse strains.

Expression of K5 and K14 overlaps in basal/myoepithelial cells throughout mammary development, with additional K14 expression detected in many suprabasal/luminal cells between 2 and 3 weeks after birth

Although K5 and K14 are both lineage markers of mammary basal/myoepithelial cells, the promoters of the corresponding genes when used to drive Cre recombinase-directed overexpression of the same protein resulted in different mammary phenotypes (Gu et al. 2009; Teuliere et al. 2005). This prompted us to carefully compare their expression throughout embryonic and postnatal mammary gland development using double immunofluorescence (Table 1). At E15.5 and E18.5, nearly all epithelial cells expressed both K5 and K14, with some cells displaying stronger K14 while others stronger K5 signals (Fig. 3a, b). The same was true at P11 (Fig. 3c). At P14 and P18, the outer basal/myoepithelial cells expressed both K5 and K14, whereas some suprabasal/luminal cells expressed K14 but not K5 (Fig. 3d, e). Suprabasal/luminal K5⁻K14⁺ cells persisted until 3 weeks, with the abundance of such cells varying from section to section depending on distance from the TEB (Fig. 3f and data not shown). The number of suprabasal/luminal K14⁺ cells dramatically decreased after 3 weeks, such that in adult mammary glands (8–12 weeks), few such cells were found (Fig. 3g). From the ages of P14 to adult and for all sections analyzed with either immunofluorescence on frozen sections or immunohistochemistry on paraffin sections, K5 expression was near-completely confined to the basal compartment (Fig. 3d–h). Together, these results show that in addition to the basal compartment, K14, but not K5, was transiently expressed in the suprabasal/luminal compartment of pre-pubertal mammary epithelium.

Our finding of K5 expression in embryonic and prepubertal mammary glands contrasts a previous study reporting that the first stage of K5 expression in mammary glands is postnatal day 21 while K14 is expressed as earlier as E13.5 (Mikaelian et al. 2006). The use of different antibodies and different efficiency of staining methods likely underlie this discrepancy. A temporal and spatial overlap of K5 and K14 expression during mammary development is more consistent with previous findings that K5 is the main partner of K14 and that the two genes largely co-express in the epidermis (Byrne et al. 1994; Purkis et al. 1990).

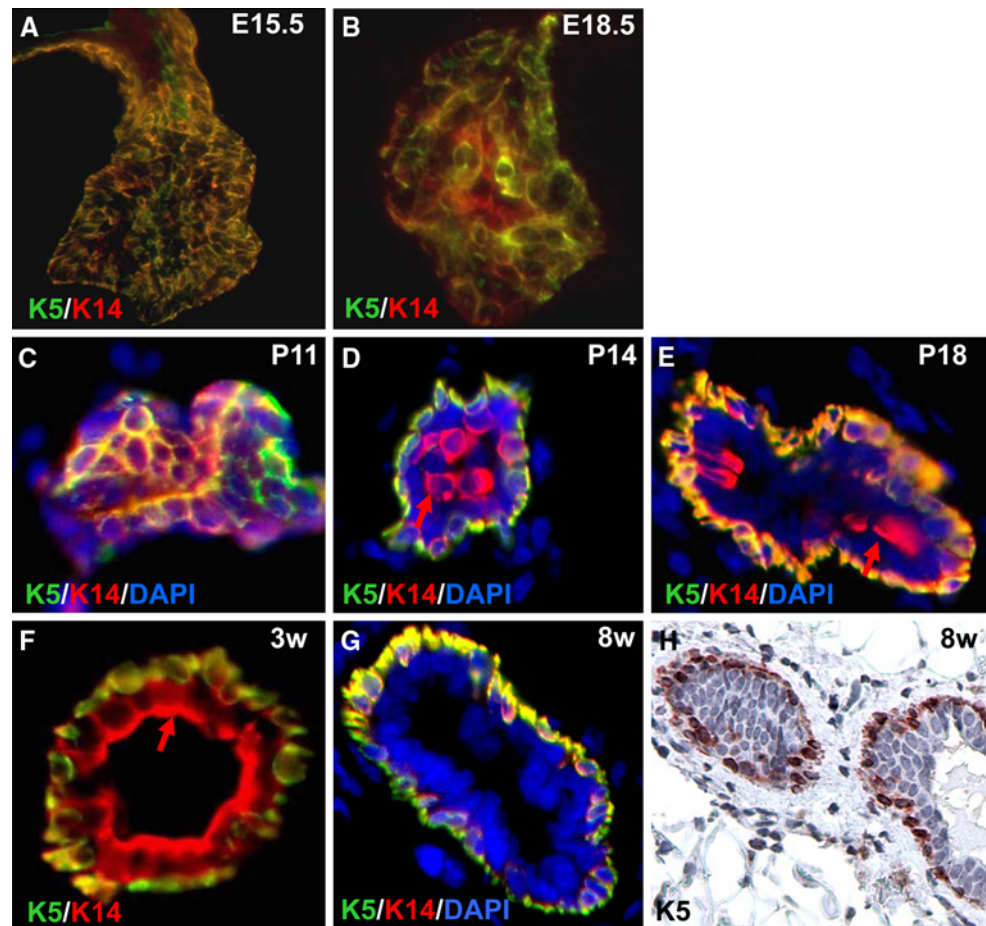
The transient expression of K14 in the suprabasal/luminal compartment is consistent with previous observation of K14⁺/K8⁺ suprabasal/luminal cells that are found infrequently in adult mammary glands and proposed to be stem/progenitor cells (Li et al. 2007). Our comparative expression analysis shows that suprabasal/luminal K14⁺ cells disappear earlier during postnatal mammary epithelial development than the putative K6⁺ progenitor cells. If K6⁺ and suprabasal/luminal K14⁺ cells are indeed progenitor cells as previously proposed, then our finding would suggest that different pools of progenitor cells might be used at different developmental stages to contribute to distinct morphogenetic processes.

Our detailed analysis of K5/K14 expression in the developing mammary gland now provides a possible explanation for the aforementioned phenotypic differences between mouse models using K5 or K14 promoter to drive genetic alterations in mammary glands. Although past mammary studies focused heavily on the use of hormonally responsive promoters, such as MMTV and WAP, to direct gene expression or ablation, the use of K5 and K14 promoters has become increasingly popular as this allows targeting of genetic changes to developmental mammary stem/progenitor cells (Berton et al. 2003; Choi et al. 2009; Gu et al. 2009; Taddei et al. 2008). In this context, our expression data offer a useful guide on exactly when and where the desired genetic changes would occur—information needed for predicting and interpreting phenotypic outcomes.

K5/K6/K14 expression in cultured primary mammary epithelial cells mimics the expression pattern in vivo

Primary mammary epithelial cells (MEC) can be cultured from mammary glands and serve as a useful in vitro model for mechanistic studies. We therefore cultured MEC cells and analyzed their expression of K5, K6 and K14. K5⁺, K6⁺, or K14⁺ cells were readily detected in MEC culture, even in those derived from mature mammary glands that normally do not contain an appreciable number of K6⁺ cells in vivo (Fig. 4a–c and data not shown). In a representative experiment, approximately half (51 ± 7%) of K14⁺ cells also expressed K5, whereas all K5⁺ cells expressed K14. Therefore, K5 and K14 expression during in vitro passaging

Fig. 3 Double immunofluorescence analysis of K5 and K14 expression in embryonic and postnatal mammary glands. Shown are results of indirect immunofluorescence on frozen sections using K5 (green) and K14 (red) antibodies (a–g) or immunohistochemistry on paraffin sections using only K5 antibody (h). DAPI (blue) stains the nuclei. Arrows point to suprabasal/luminal K14⁺ (d–f) cells



resembles the situation during prepubertal development rather than in mature, adult mammary glands. A relatively small fraction ($16 \pm 1\%$) of K14⁺ cells was positive for K6. In contrast, a vast majority ($93 \pm 3\%$) of K6⁺ cells were also K14⁺, whereas only less than 10% of them were K14⁻. The large overlap between K6 and K14 expression in culture contrasts the observation *in vivo*, where K6⁺ and K14⁺ cells appear to be overall distinct populations. However, overlapping expression does occur *in vivo* in a small population of double positive cells, especially during the first 2 weeks after birth. Therefore, K6⁺K14⁺ cells are likely younger than K6⁺K14⁻ or K6⁻K14⁺ cells during lineage evolution. Taken together, these findings suggest that *in vitro* culturing conditions may allow MEC cells to manifest and/or regain a “progenitor” cell status. Perhaps the relatively dormant stem cells *in vivo* are activated upon culturing and become K6⁺. Alternatively, this could simply be due to adaptation to culture conditions. Regardless of the underlying mechanism, cultured primary MEC cells are reminiscent of a primitive developmental status and might serve as a good *in vitro* model of mammary development to dissect the lineage relationships between K5⁺, K6⁺ and K14⁺ cells. Finally, the cellular dynamics that we observed *in vivo* and *in culture* raises the possibility that an abundance

of K5⁻K6⁺K14⁺ cells associates with a phase of active cell proliferation and tissue growth. Previously, Tyner and Fuchs showed that K6 and K14 expression is higher in cultured keratinocytes than in skin epidermis (Tyner and Fuchs 1986). Therefore, increased K6 expression upon *in vitro* culture seems to be a common feature of at least two different epithelial cell lineages.

We also examined K6 expression in colonies formed on Matrigel. Primary MEC cells produced different types of colonies: some were large and composed of both solid and irregular lumen-containing structures (Fig. 4d, e), while others were either solid (Fig. 4f, g) or showing a round acinar morphology reminiscent of those formed by more differentiated luminal progenitor cells (Fig. 4h, i) (Stingl et al. 2006). The large, mixed-type colonies are likely formed by stem and/or early progenitor cells (Stingl et al. 2006). Altogether, we detected K6 expression in 59–71% of the total colonies examined. In the mixed-type colonies, cells with strong K6 expression were predominantly found clustered in the solid portion, showing a preference for outside locations (Fig. 4d, e). Scattered strong K6⁺ cells or weakly stained cells were also found in the epithelia lining the irregular lumen in these colonies (Fig. 4d). K6 expression was also detected in standing-alone solid colonies (Fig. 4f, g),

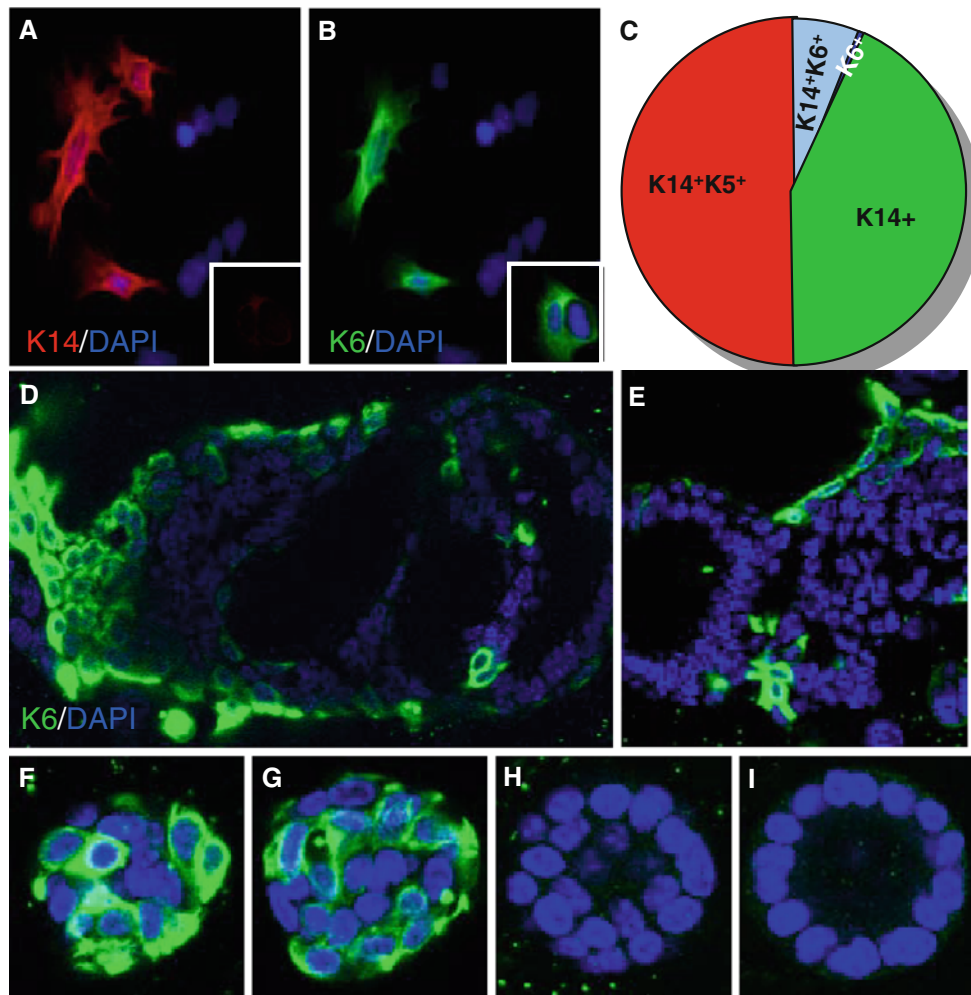


Fig. 4 K5/K6/K14 expression in cultured primary MEC cells. **a, b** Double immunofluorescence of cells from a 13-month-old female using K14 (**a**, red) and K6 (**b**, green) antibodies. *Insets* show a dividing cell that is K6⁺ but K14⁻. **c** Pie diagram showing the percent distribution of K5, K6, and K14 expressing cells. The area of the circle equals the total number of cells that express either K5, K6, or K14, or a com-

bination of them. Data shown are taken from cultures fixed at day 9 after the initial plating. **d–i** Confocal images of K6 (green) and DAPI (blue) immunofluorescence on Matrigel colonies formed by MEC cells from 8 to 10-week-old females. Shown in **f** and **g** or **h** and **i** are *z*-sections at two different planes of the same colony

but rarely seen in the spherical acinar structures (Fig. 4h, i). Collectively, our results reveal K6⁺ cells in two different cell culture models, namely monolayer and Matrigel, and demonstrate an association of K6 expression with the *in vitro* differentiation activity of mammary stem/progenitor cells.

In summary, we present a comparative analysis of cytokeratin expression during embryonic and postnatal mammary development. Our study provides evidence for an embryonic origin of putative mammary stem/progenitor cells and informs the use of specific cytokeratins as markers of mammary epithelial differentiation, as well as the use of their promoters to direct desired genetic alterations.

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