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Immunology

Developmental regulation of cytokeratins in cells of the rat mammary gland studied with monoclonal antibodies

(myoepithelial cells/lumenal cells/ducts/end-buds)

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ABSTRACT We have isolated two monoclonal antibodies to cytokeratins and determined their cell specificities. They display interesting localization within the rat mammary gland. One (1A10) shows specificity for myoepithelial cells; the other (24B42) is specific for lumenal cells at various stages of development. These two monoclonal antibodies and three others to cytokeratin previously isolated were used in conjunction with antibodies to myosin and collagen IV to confirm and extend our previous findings on epithelial cell types and development within the mammary gland.

Antibodies, especially monoclonal antibodies, are very useful for identifying cell types using immunocytological methods. This approach has been used in our laboratory for studying the pathways of cell differentiation in the mammary gland (1). Antibodies to cytokeratins are especially useful in this context because there are many different kinds of cytokeratins, which display considerable cell specificity.

The cytokeratins are expressed by a multigene family that is developmentally regulated (see refs. 2 and 3 for reviews). They are expressed mostly in epithelia but also in mesothelial cells (4). Every tissue has a complex pattern of these peptides, which can be distinguished by their isoelectric point and electrophoretic mobility in gels. Many tissues share the same peptides (5, 6). The distribution of the peptides, ascertained by either extraction or by immunohistochemical methods using polyclonal antisera, differs in different organs or in different parts of the same organ—for instance in different layers of the epidermis. Similar peptides are found in different mammalian species. Using monoclonal antibodies (7), the existence of both peptide-specific and common epitopes was observed (8). Monoclonal antibodies with considerable specificity (8–10), as well as some with broad crossreactivity (11), were identified by immunohistochemistry. Using polyclonal antisera to partially purified peptides it was shown by immunohistochemistry that cytokeratins are not uniformly distributed in the mammary gland (11–13). Quantitative differences were observed between myoepithelial and epithelial cells, and in the latter cells, between ductal and secretory alveolar cells (12–14).

Cytokeratins show considerable crossreactivity among species. For instance, Lane (15) has shown that monoclonal antibodies to cytokeratins from PtK_1 cells recognize cells in the rat mammary gland. We have found that one of these antibodies (Le61) is highly selective for certain cell types (1). By taking advantage of this crossreactivity, we have prepared a set of monoclonal antibodies from mice immunized with total cow muzzle keratin. We report here on the properties of two antibodies that have considerable cell specificities. These antibodies are very useful for studying the cell types present in the rat mammary gland. They allow a further refinement of the pathway of cell differentiation in this organ. We also compare these monoclonal antibodies with three others from mice immunized with cytokeratins from PtK_1 cells (15).

MATERIALS AND METHODS

Some of the antisera used (p-myo, p-CIV, p-lam, Le61, 48B45, 57B29) have been described (1). p-myo, p-CIV, and p-lam are polyclonal antisera to myosin, collagen IV, and laminin, respectively. Le61, Le41, Le63, and Le65 were a gift from B. Lane. 24B42 and 1A10 were prepared as follows. Bovine muzzle keratin was prepared according to Franke *et al.* (16). BALB/c mice were immunized with a keratin suspension in Freund's complete adjuvant. Mice showing immune reaction toward rat mammary keratin were used for fusions using P3-NS1/1-Ag4-1 (7) or PAI cells (supplied by Theo Staehlin). Hybridoma supernatants were screened by micro ELISA using 500 ng of keratin per well. Final screening was for positive staining on cryostat-cut sections of frozen 7-week-old virgin rat mammary gland. Hybridomas were cloned repeatedly until 100% of the clones were positive.

Reaction of the monoclonal antibodies and other antisera with epithelia of different types was tested using immunoperoxidase and double-layer immunofluorescence on cryostat-cut sections of frozen tissue, without any pre- or postfixation. The antigens recognized by the two monoclonal antibodies, 1A10 and 24B42, were characterized by immunoblots (17). Bovine muzzle keratins were separated on 7.5% polyacrylamide gels in the presence of $NaDodSO_4$ and then transferred to 0.45-μm nitrocellulose paper for 3 hr at room temperature using 20% methanol/150 mM glycine/20 mM Tris·HCl, pH 8.3, as a buffer. The nitrocellulose paper was cut into strips and incubated with undiluted culture supernatants. Bound monoclonal antibody was localized using goat anti-mouse antiserum linked to horseradish peroxidase and 4-chloro-1-naphthol as the chromogenic substrate.

RESULTS

Characteristics of the Antibodies. Mice were immunized with a bovine muzzle keratin preparation containing seven peptides in the 50- to 70-kilodalton (kDa) range. Out of 288 hybridomas, 2, resulting from fusions with PAI cells, produced antibodies that strongly stained rat mammary gland keratins. Both are of the IgG1 isotype and are designated 1A10 and 24B42. Immunoblots of bovine muzzle keratin show that 1A10 binds to a keratin of 55 kDa [component VI; Franke *et al.* (18)] and 24B42 binds to keratins of 68 kDa and 50 kDa (components 1b and VII). In the mammary gland, 1A10 stains myoepithelial cells but not the luminal epithelium of the ducts, whereas 24B42 stains the luminal epithelium but not the myoepithelial cells. The antibodies show regular differences of specificity throughout the body (Table 1). 24B42 is positive and 1A10 is negative on simple cuboidal epithelium and squamous epithelium in various organs,

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Abbreviations: p-myo, p-CIV, and p-lam, polyclonal antisera to myosin, collagen IV, and laminin, respectively.

Table 1. Specificity of monoclonal antibodies 1A10 and 24B42

Tissue	1A10	24B42	Comments
Eye conjunctiva	+	−	Strat. squamous*
Skin			
Epidermis			
S. germ.	+	+	Strat. squamous†
S. granul.	+	+	
S. corneum	+/−	+/−	
Hair follicle	+	+	
Tongue	+	+	Strat. squamous†
Filiform papilae	−	−	
Salivary			
Duct epithelium	−	+	Simple cuboidal
Myoepithelium	+	−	Myoepithelial
Trachea	+	+	Pseudo strat. columnar
	−	+	Simple squamous
Esophagus	+	−	Strat. squamous*
Stomach			
Gastric mucosa	+	+	Simple columnar
Fundic glands	−	+	Cuboidal
Mesothelium	−	−	
Pancreas			
Centro-acinar cells and duct cells	−	+	Cuboidal
Liver			
Hepatocytes	−	−	
Bile epithelium	−	+	Cuboidal
Mesothelium	−	+	Simple squamous
Small intestine	+	+	Simple columnar
Large intestine			
Mucosa and crypts	+	+	Simple columnar
Goblet cells	+	+	
Ovary			
Germinal epithelial	−	+	Simple cuboidal
Fallopian tube	+	+	Simple columnar
Kidney			
Renal calyx	+	+	Transitional
Collecting tubule	−	+	Simple cuboidal
Mammary gland			
Duct	−	+	Simple cuboidal
Myoepithelium	+	−	
Bladder	+	+	Transitional
Prostate			
Squamous	+	+	Transitional
	−	+	Simple cuboidal
	−	+	Pseudo-strat. columnar
Ureter	+	+	Transitional

Strat., stratified; S., stratum; germ., germinal; granul., granulosum.
*Nonkeratinized.
†Keratinized.

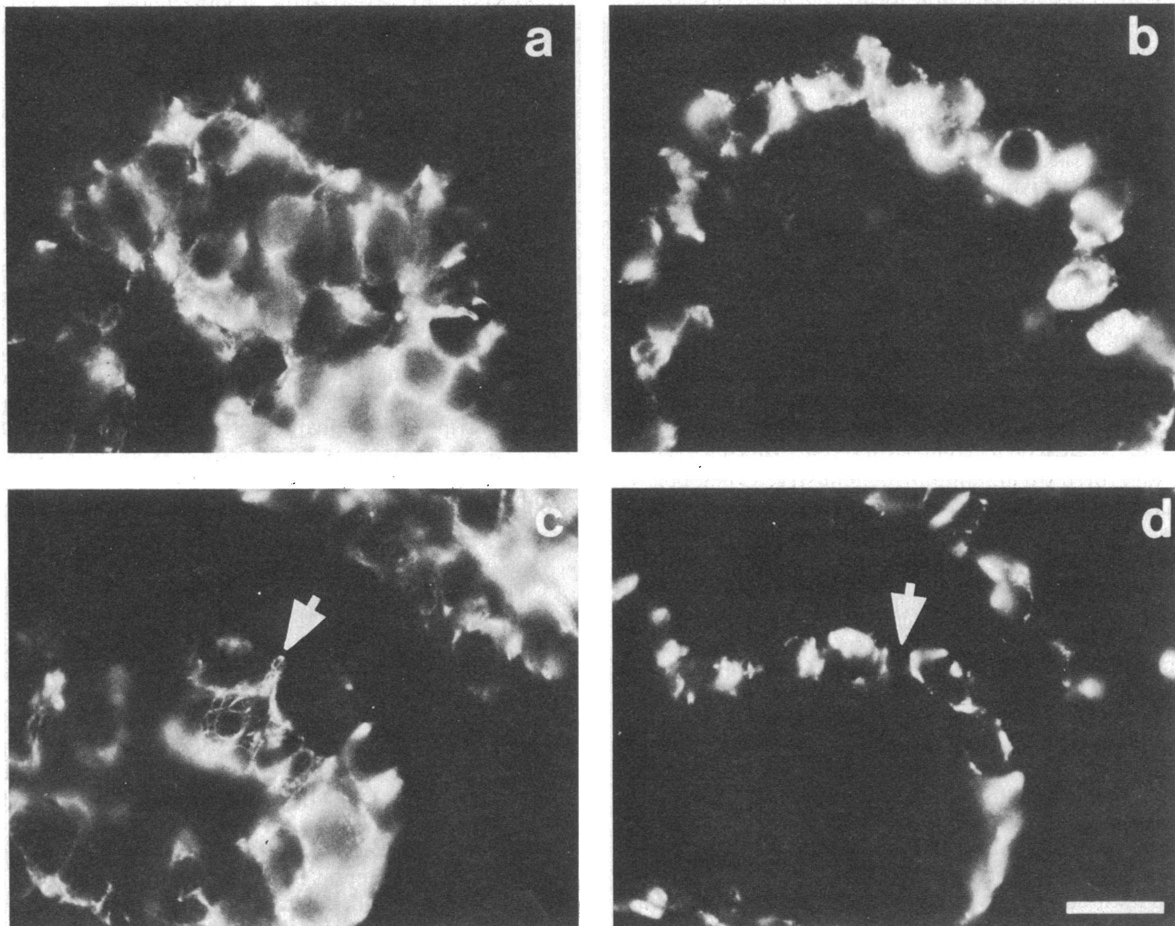

FIG. 1. Mutual exclusion between staining with 24B42 and p-myo cells in immature rat mammary gland. Cryostat-cut sections of 3-week-old rat mammary gland were stained for double immunofluorescence using 24B42 (fluorescein; *a* and *c*) and p-myo (rhodamine; *b* and *d*). *a* and *b* are the same end-bud photographed using fluorescein (*a*) and rhodamine (*b*) filters. *c* and *d* are photographs of the same section of two small ducts. Arrows in *c* and *d* show a finger of a lumenal cell stained by 24B42 (*c*) protruding between the myoepithelial cells stained by p-myo (*d*). (Bar = 20 μm.)

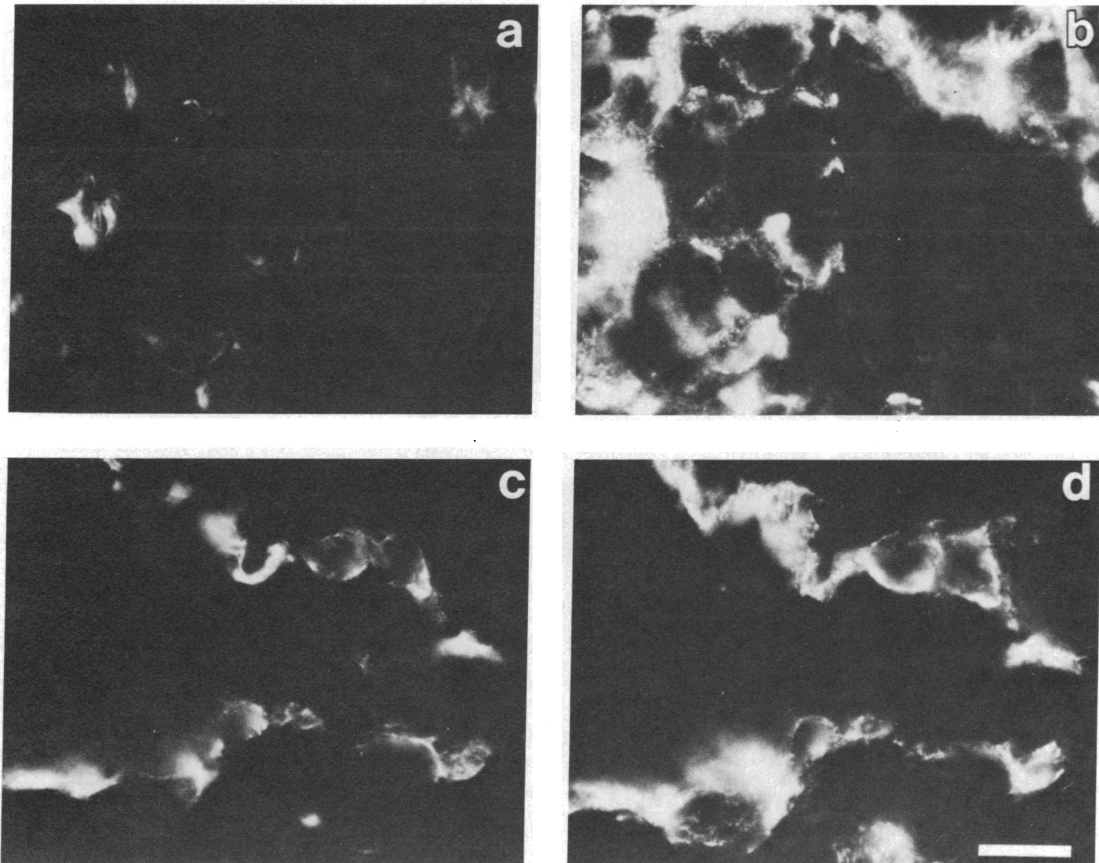

FIG. 2. Limited overlap produced by staining immature rat mammary with monoclonal antibodies 1A10 and p-myo. Two parts of the same end-bud are shown, each stained for double immunofluorescence using 1A10 (fluorescein; *a* and *c*) and p-myo (rhodamine; *b* and *d*). *a* and *b* correspond to the tip of the end-bud, most distal from the nipple, where the overlap of the two stains is limited. *c* and *d* show the base of the end-bud, where it narrows into a duct and the two stains coincide. (Bar = 20 μm.)

whereas 1A10, but not 24B42, stains myoepithelial cells also in the salivary glands, as well as cells of nonkeratinized stratified squamous epithelium. In addition, both monoclonal antibodies stain transitional epithelium, simple columnar, and keratinized stratified squamous epithelium [epithelia classified according to Bergman and Afifi (19)].

Developmental Control of Cytokeratin Expression in the Mammary Gland. In the end-bud of the 3-week-old rat, the 24B42 monoclonal antibody stains a system of filaments in most of the cells, except at the periphery of the buds (Fig. 1 *A* and *B*). Most cells that are strongly stained by p-myo are not stained by this monoclonal antibody, but rare cells display double staining. In the ducts of the mammary glands of 3- or 7-week-old virgin female rats, the antibody stains the epithelial cells bordering the lumen (Fig. 1 *C* and *D*). In the lactating gland, the epithelial cells lining the lumen show a distinct network of filaments (data not shown).

In glands of 3-week-old rats, the myoepithelial cells lining some ducts appear to be stained by both p-myo and 24B42. Although we cannot exclude that the cytokeratin stained by 24B42 is present in these cells, there is great likelihood that the double staining results from very close apposition of myoepithelial and lumenal cells. At this stage of development, lumenal cells have fingers that protrude between the myoepithelial cells toward the basement membrane (Fig. 1 *C* and *D*). Moreover, lumenal cells frequently bulge at the outer edge of the ducts, still surrounded by extension of myoepithelial cells. This can be clearly shown by staining sections with both p-myo and Le61 (15), which stains a rich network of cytoplasmic filaments, whereas 24B42 stains filaments very close to the cell membrane. The lumenal cells stained by Le61 also show fingers that penetrate between the myoepithelial cells, reaching the basement membrane (data not shown). In 7-week-old animals, the myoepithelial cells form more compact layers, so that the apparent overlap of the two markers cannot take place. At this stage, only very thin fingers of luminal cells reach the basement membrane.

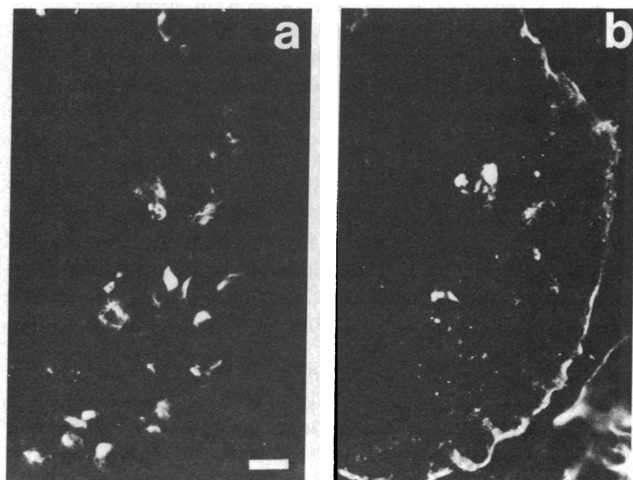

FIG. 3. Partial overlap of 1A10 and collagen IV staining in end-buds of rat mammary gland. The edge of the end-bud is outlined by staining the basement membrane with collagen IV (rhodamine; *b*). For many cells the cytokeratin stained by 1A10 (fluorescein; *a*) overlaps with granular collagen IV staining. (Bar = 20 μm.)

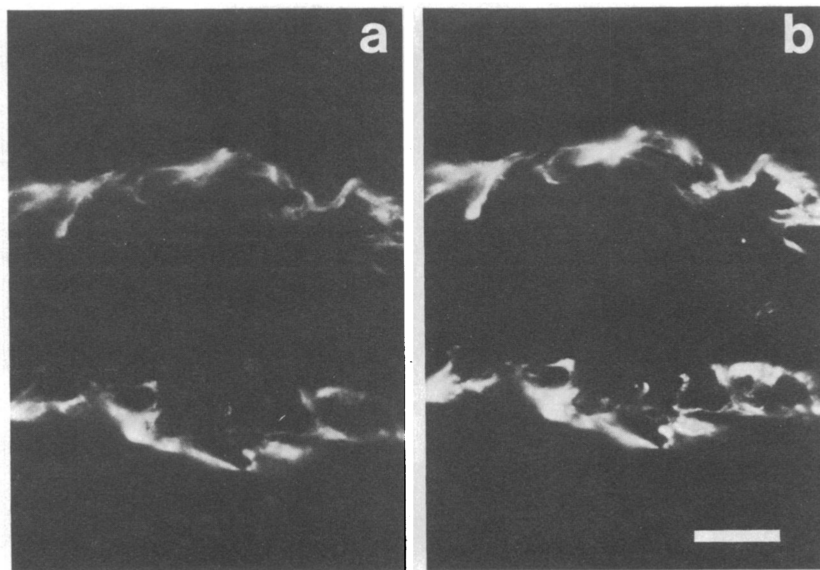

FIG. 4. Complete overlap between staining with cytokeratin monoclonal 1A10 and p-myo in a mature duct in rat mammary gland. A longitudinal section of a duct of a 7-week-old rat mammary gland was stained for 1A10 (fluorescein; *a*) and p-myo (rhodamine; *b*). (Bar = 20 μm.)

Myoepithelial cells are never stained in ducts or in alveoli.

These characteristics show that 24B42 is specific for cytokeratin filaments of cells of the lumenal lineage of the rat mammary gland, including the precursor cells in the end-buds. Cells of the myoepithelial lineage identified by the high myosin content (1) are not stained (Fig. 1 *B* and *D*). The monoclonal antibody may stain putative stem cells in the end-buds, but this is not a certainty.

In the end-buds of 3-week-old rats, 1A10 mostly stains cells in the proximity of the basement membrane (which is recognized by staining with p-CIV antiserum) (Fig. 2 *A* and *C*). These cells are usually also stained with p-myo antiserum (Fig. 2 *B* and *D*). The relationship between 1A10 and p-myo staining is as follows: (*i*) Many cells in the tip of the buds, usually in the most peripheral layers, stain strongly with p-myo but very little or not at all with 1A10. (*ii*) Some cells in the distal part of the buds, but interior to those stained by p-myo alone, are stained by both antibodies. The 1A10 stain is often confined to the tip of the cells, whereas the p-myo stain is throughout the cell. Some of the cells contain granules stained by p-CIV (Fig. 3). (*iii*) Exceptional cells in the end-bud interior (about 0.1% of all the cells) stain strongly with 1A10 but weakly or not at all with p-myo. (*iv*) Toward the base of the end-bud, cells of an outer layer adjacent to the basement membrane are stained equally by 1A10, p-myo, and p-CIV. In these cells, the 1A10 staining is more intense than in double-stained cells at the tip of the buds, and it extends through most of the cell body. (*v*) A weak but distinct 1A10 staining is also observed in central cells of the end-bud, which are not stained by p-myo.

In the ducts and ductules of 3- or 7-week-old animals, 1A10 intensely stains myoepithelial cells; the stain coincides with that of p-myo and p-CIV (Fig. 4). The epithelial cells at the lumen of the duct are stained very faintly, if at all. In the alveoli of lactating animals, 1A10 brilliantly stains the myoepithelial cells but not the epithelial cells at the lumen (data not shown).

We have also determined the cell specificity of Lane's monoclonal antibodies Le41, Le63, and Le65 (15). Le41 is a broad spectrum monoclonal antibody that stains cytokeratin filaments in all cell types of rat mammary epithelia—from the end-buds to the alveoli of lactating glands, including both lumenal and myoepithelial cells. Only rare cells, stained by a p-ker antiserum, at the tips of the end-buds stain weakly or not at all with Le41. Le63 stains mostly cells of the central areas of end-buds; it weakly stains the lumenal epithelium of ducts and ductules. The cells are rather uniformly stained. Le63 does not stain appreciably the alveolar cells in lactating glands or cells of the myoepithelial lineage at any location. Le65 stains most cells in end-buds, even some that are stained by p-myo. It does not stain cells of the myoepithelial lineage. Le65 also stains a proportion of lumenal cells in ducts and ductules with a distribution comparable to that of cells stained by Le61, which has already been described (1).

DISCUSSION

Monoclonal antibodies from mice immunized with cow muzzle or PtK_1 cytokeratins recognize a variety of cell types within the rat mammary gland. These antibodies have various ranges of specificity. Le41 has the broadest range, because it recognizes cytokeratins in both myoepithelial cells and lumenal cells at all stages of differentiation. The nature of the few cells at the tips of end-buds that remain unstained is unknown. They are clearly epithelial cells because they contain other cytokeratins. Le63, Le65, and 24B42 all stain cytokeratins in cells of the lumenal lineage but not of the myoepithelial lineage. The distribution of Le63 and 24B42 is similar in the end-buds and in the ducts. In the ducts, however, Le63 stains the lumenal cells faintly, whereas 24B42 stains them brilliantly. In lactating glands 24B42 stains the alveolar epithelium, whereas Le63 does not. The distribution of cytokeratins recognized by Le65 is similar to that already reported for Le61 (1): both antibodies stain rare p-myo$^+$ cells in end-buds (the putative stem cells) as well as selected cells in the ducts and ductules. The cell specificity of two antibodies in the mammary gland may be identical.

Both 1A10 and 24B42, like the antibodies previously described (1), are essentially lineage specific. Monoclonal antibody 1A10 is fairly specific for the myoepithelial lineage. Based on our previous work (1) the types of cells stained brilliantly by 1A10 in the end-buds can be assessed as follows. (*i*) Cells that are p-myo$^+$ 1A10$^+$: pro ME I and II (because they are all related to a continuous basement membrane stained by p-CIV). (*ii*) Cells that are p-myo$^-$ 1A10$^+$: undefined cells of the latter class are rare and are centrally located in the bud, away from other 1A10$^+$ cells. Perhaps these are lumenal precursor cells with an aberrant expression of the cytokeratin. 1A10 affords a further differentiation between pro-ME I and pro-ME II cells, because the former cells are only stained in a small apical area, whereas the latter cells contain a cytokeratin network throughout their cytoplasm. 1A10, however, weakly stains inner (lumenal) cells of end-buds and faintly stains lumenal duct cells. 24B42 appears to stain some myoepithelial cells in immature ducts,

but the image may derive from overlapping of tightly enmeshed cells.

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