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Coexpression Patterns of Vimentin and Glial Filament Protein with Cytokeratins in the Normal, Hyperplastic, and Neoplastic Breast

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The authors studied by immunohistochemistry the intermediate filament (IF) protein profile of 66 frozen samples of breast tissue, including normal parenchyma, all variants of fibrocystic disease (FCD), fibroadenomas, cystosarcoma phylloides, and ductal and lobular carcinomas. Monoclonal antibodies (MAbs) to cytokeratins included MAb KA 1, which binds to polypeptide 5 in a complex with polypeptide 14 and recognizes preferentially myoepithelial cells; MAb KA4, which binds to polypeptides 14, 15, 16 and 19; individual MAbs to polypeptides 7, 13, and 16, 17, 18, and 19, and the MAb mixture AE1/AE3. The authors also applied three MAbs to vimentin (Vim), and three MAbs to glial filament protein (GFP). Selected samples were studied by double-label immunofluorescence microscopy and by staining sequential sections with some of the said MAbs, an MAb to alpha-smooth muscle actin, and well-characterized polyclonal antibodies for the possible coexpression of diverse types of cytoskeletal proteins. Gel electrophoresis and immunoblot analysis also were performed. All samples reacted for cytokeratins with MAbs AE1/AE3, although the reaction did not involve all cells. Monoclonal antibody KA4 stained preferentially the luminal-secretory cells in the normal breast and in FCD, whereas it stained the vast majority of cells in all carcinomas. Monoclonal antibody KA1 stained preferentially the basal-myoepithelial cells of the normal breast and FCD while staining tumor cell subpopulations in 4 of 31 carcinomas. Vimentin-positive cells were found in 8 of 12 normal breasts and in 12 of 20 FCD; in most cases, Vim-reactive cells appeared to be myoepithelial, but occasional luminal cells were also stained. Variable subpopulations of Vim-positive cells were noted in 9 of 20 ductal and in 1 of 7 lobular carcinomas. Glial filament protein-reactive cells were found in normal breast lobules and ducts and in 15 of 20 cases of FCD; with rare exceptions, GFP-reactivity was restricted to basally located, myoepithelial-appearing cells. Occasional GFP-reactive cells were found in 3 of 31 carcinomas. Evaluation of sequential sections and double-label immunofluorescence microscopy showed the coexpression of certain cytokeratins (possibly including polypeptides 14 and 17) with vimentin and alpha-smooth muscle actin together with GFP in some myoepithelial cells. The presence of GFP in myoepithelial cells was confirmed by gel electrophoresis and immunoblotting. Our results indicate that coexpression of cytokeratin with vimentin and/or GFP is comparatively frequent in normal basal-myoepithelial cells of the breast. This IF profile is retained in various forms of FCD and benign breast neoplasms, often with an increase in GFP expression. In breast carcinomas, cytokeratin-vimentin coexpression is rather frequent but, with rare exceptions, restricted to a rather small subpopulation, whereas cytokeratin-GFP coexpression is distinctly uncommon. The biologic significance of cells in the normal, hyperplastic, and neoplastic breast that coexpress cytokeratins with vimentin and GFP remains unclear. Future studies may clarify also whether the subsets of FCD and breast carcinomas that coexpress cytokeratins with vimentin and/or GFP differ clinically from those that only express cytokeratins. (Am J Pathol 1990; 137:1143–1155)

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Table 1. *Immunohistochemical Identification of Intermediate Filament Proteins in the Normal, Hyperplastic, and Neoplastic Breast*

Diagnosis	Intermediate CKs (a)	Filament Vim (b)	Proteins GFP (c)
Normal breast	12/12	8/12	9/12
Fibrocystic disease, all variants	20/20	12/20*	15/20*
Fibroadenomas	2/2	1/2	2/2
Cystosarcoma phylloides	1/1†	1/1†	1/1†
Ductal carcinomas			
Not otherwise specified	20/20‡	9/20*	1/20*
Mucinous carcinoma	1/1	1/1§	0/1
Adenoid cystic carcinoma	1/1 (d)	0/1	0/1
Medullary carcinoma	1/1	0/1	0/1
Papillary carcinoma	1/1	1/1§	1/1*
Lobular carcinomas	7/7‡	1/7*	1/7*

(a) CK's = cytokeratins, variably detected with MAbs AE1/AE3, KA1, and KA4

(b) Vim = vimentin as detected with 2 different MAbs (see Materials and Methods)

(c) GFP = glial filament protein as detected with 3 different MAbs (see Materials and Methods)

* Only scattered cells stained (see text).

† Only vimentin was positive in both stromal and epithelial components; the other intermediate filament proteins were expressed only in the epithelium.

‡ Only 4/20 ductal and 0/7 lobular carcinomas were immunostained with MAb KA1.

§ Most of the neoplastic cells positive for vimentin.

(d) MAb KA1 did not immunostain tumor cells.

The intermediate filament (IF) complement of the normal, hyperplastic, and neoplastic breast has been analyzed with immunohistochemical and biochemical methods; and it would appear that the dominant, if not exclusive, IF proteins consist of cytokeratin polypeptides. Nagle et al[1] and Jarasch et al[2] reported on the variable distribution of cytokeratins using antibodies recognizing different polypeptides and also capable of discriminating basal-myoepithelial cells from luminal-secretory cells. They and other investigators[3-8] outlined findings of possible significance in the differential diagnosis between dysplasias and carcinomas. Breast tissue samples also were studied with vimentin antibodies; with the exception of the exceedingly rare spindle cell tumors that often stained positively,[9,10] the findings have been contradictory. Some observers reported negative results[2,11-15] (for bovine and rat breast, see Franke et al[12]), while others found variable numbers of vimentin-reactive cells in neoplastic and nontransformed breast epithelium.[5,16-19]

In the course of recent, unrelated investigations,[20,21] we found that samples of breast hyperplasias and neoplasms studied as presumably 'negative' controls showed variable parenchymal cell subpopulations that reacted convicingly with antibodies to vimentin and glial filament protein (GFP). We were encouraged to pursue the issue, given certain histologic similarities between breast and salivary glands, and the known coexpression of vimentin and GFP with cytokeratins in the normal and neoplastic salivary glands.[22-24] Thus we undertook a systematic immunohistochemical analysis of breast lesions with a broad panel of antibodies to cytokeratins, vimentin, and GFP. Selected samples also were investigated by double-label immunofluorescence, and two-dimensional gel electrophoresis and immunoblot analysis.

We studied 66 frozen samples of breast tissue, including normal ducts and lobules, all variants of fibrocystic disease (FCD), *in situ* and infiltrating variants of ductal and lobular carcinomas, fibroadenomas, and one cystosarcoma phylloides. Not surprisingly, we confirmed the presence of cytokeratins in all samples, although the reactivity patterns varied considerably with the different antibodies. Notably, we found distinct vimentin and GFP reactivity in cell subpopulations in many cases of FCD, in fibroadenomas, and also in the normal breast. In breast carcinomas, vimentin-immunoreactive cells were found in several ductal and lobular carcinomas, whereas rare GFP-positive cells were found in only some cases. Double-label immunofluorescence and evaluation of sequential sections disclosed double and triple expression of certain cytokeratins together with vimentin and GFP in some myoepithelial cells; furthermore the presence of GFP was confirmed by gel electrophoresis and immunoblotting.

Materials and Methods

Light Microscopic Immunohistochemistry

Tissues were obtained immediately after surgical removal; the specimens were derived from biopsies, 'lumpectomies,' and complete mastectomies (total n = 54), and plastic procedures (n = 12) in young women without parenchymal breast disease. Samples were immersed in precooled isopentane and snap-frozen in liquid nitrogen; they were stored at −80°C until used. In addition to the normal breast specimens, we studied samples of fibrocystic disease (n = 20), including representatives of cysts, apocrine metaplasia, florid and sclerosing adenosis, ductal and lobular hyperplasias, and papillomas; most of these samples had areas displaying more than one of those changes. Benign tumors included fibroadenomas (n = 2), and a presumably benign cystosarcoma phylloides. Malignant tumors included 20 ductal carcinomas not otherwise specified (NOS), and seven lobular carcinomas. Foci of *in situ* carcinoma were included in several of these neoplasms. Also, we studied several histologic subtypes of ductal carcinoma, including one mucinous, one adenoid cystic, one medullary, and one papillary carcinoma (Table 1). In all instances, slides from conventionally fixed,

embedded, and stained adjacent samples were critically reviewed; the diagnoses were based on broadly prevalent criteria.[25]

Four-micron–thick serial cryostat sections were cut, air dried, and briefly fixed in cold acetone. Additional sections were fixed in 10% formalin and used for routine staining with hematoxylin and eosin (H&E). Immunostaining was performed by the avidin-biotin-peroxidase complex (ABP) method as described by Hsu et al,[26] and by the indirect immunoperoxidase method. Commercial reagents were used (Vector Laboratories, Burlingame, CA). For visualization of antigen–antibody complexes, we used 3,3′ diaminobenzidine (Aldrich Chemical Co, Danvers, MA). Contrast was increased by briefly counterstaining the slides with hematoxylin. As positive controls we used well-characterized tumors known to express the pertinent antigens; negative controls included omission of the primary antibody or substitution for purified, irrelevant immunoglobulins. Details of the immunostaining protocol pertinent to the antibodies used have been published.[20,21] The following antibodies were used:

1) Cytokeratin murine MAb 'cocktail' AE1/AE3 reactive with a broad cytokeratin polypeptide spectrum was purchased from Hybritech, (La Jolla, CA). Monoclonal antibody AE1 reacts with (type I) acidic keratins 10, 14, 15, 16 and 19, whereas MAb AE3 reacts with all eight (type II) basic keratins as outlined in the catalog of Moll et al.[27]
2) Cytokeratin murine MAb K_s 1-8.136 (Progen, Heidelberg, FRG) reacting with all type II polypeptides 1-8[28]
3) Cytokeratin murine MAb KA4 reactive with cytokeratins 14, 15, 16, and 19 but not with the basic cytokeratins 1, 4, 5, and the acidic cytokeratins 10 and 11.[1]
4) Cytokeratin murine MAb KA1 noted by one- and two-dimensional gel electrophoresis to bind to cytokeratin 5 in a complex with cytokeratin 14.[1]
5) Cytokeratin MAb K_s 8.12 against polypeptides 13 and 16.[29]
6) Cytokeratin MAbs CK-7 (Boehringer, Mannheim, FRG), K_s 18.174 and K_s 19.2 (Progen, Heidelberg, FRG) recognizing polypeptides 7, 18, and 19, respectively.[30,31]
7) Cytokeratin MAb E3 specific for polypeptide 17[32] provided by Dr. S.M. Troyanovsky, Moscow, USSR.
8) Vimentin murine MAbs V9 (Boehringer Mannheim, FRG), VIM (Labsystems Oy, Helsinki, Finland), and VIM-9 (Viramed, Martinsried, FRG).
9) GFP murine MAbs included GF 12.23 and GF 12.24 (Achstaetter et al, obtainable from Progen, Heidelberg, FRG), and the GFP antibodies from Boehringer (Indianapolis IN),[33] and Labsystems Oy, (Helsinki, Finland); for comparison, a guinea pig antiserum to bovine GFP was used (provided by Dr. G. Bruder, Heidelberg, FRG).
10) MAb alpha-sm-1 specific for alpha-smooth muscle actin[34] was provided by Dr. G. Gabbiani, Geneva, Switzerland (available from Progen, Heidelberg, FRG).

Immunofluorescence Microscopy and Biochemical Analyses

Details of the indirect immunofluorescence microscopy method and the double-label technique have been extensively described[27,35,36]; fluorescein- and tetramethylrhodamine-isothiocyanate were commercially obtained (Medac and Dianova, Hamburg, FRG. Tissue preparation by microdissection of frozen sections and methods for gel electrophoresis with subsequent immunoblot analysis have been described in detail.[27,37,38] In the immunoblot experiments, cytoskeletal protein preparations from cultured human cells of the mammary carcinoma line MCF-7 and the glioma line U333CG/343MG were used for comparison.[24,27]

Results

Our immunohistochemical findings are summarized in Table 1.

Normal Breast

All cytokeratin antibodies used reacted with all samples, but the distribution of the reaction and patterns of staining varied considerably. Monoclonal antibodies AE1/AE3 immunostained primarily luminal cells, with less consistent immunoreactivity seen in basally located cells; in some samples clearly defined myoepithelial cells did not stain. Monoclonal antibody KA4 consistently did not stain the basal-myoepithelial cells, whereas it stained strongly the luminal epithelium. Monoclonal antibody KA1 generally reacted strongly with myoepithelial and basally located epithelial cells (Figure 1a); however, very occasionally MAb KA1 also reacted with the luminal cells, especially in terminal ducts and acini (not shown).

Vimentin MAbs delineated recognizable myoepithelial cells in large and small ducts and acini in 8 of 12 cases; the reaction was similar with all MAbs used. Vimentin staining was for the most part focal and faint, but occasionally it was rather strong and involved the majority, if not all, myoepithelial elements (Figure 1b). With only rare exceptions, luminal cells displayed no staining.

Glial filament protein MAbs reacted with a subpopulation of basal, ie, myoepithelial cells surrounding terminal

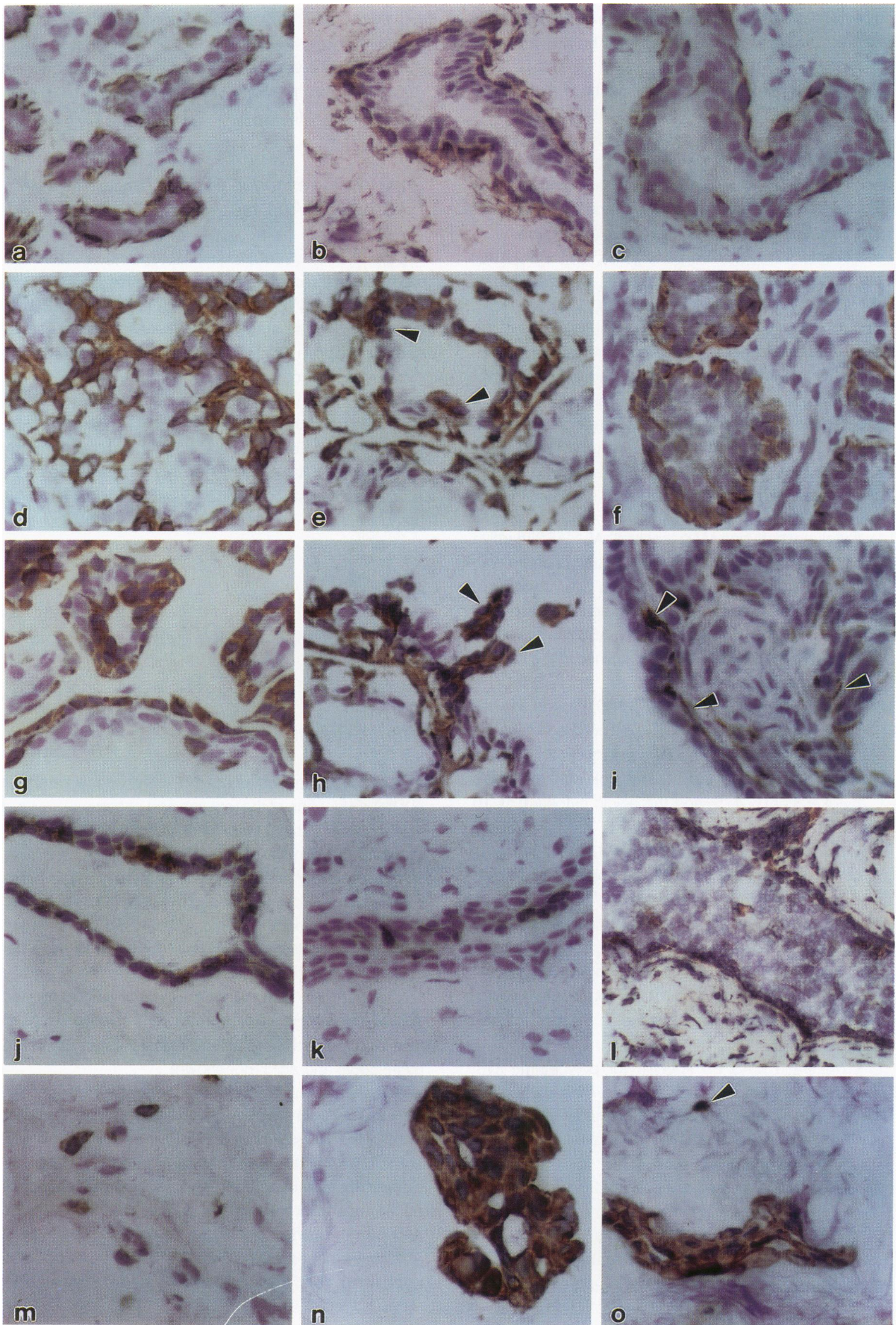
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Figure 1. a: *Normal breast; part of terminal duct and acini immunostained with cytokeratin MAb KA1. Myoepithelial cells are clearly and extensively stained (×420).* b: *Normal breast; segment of duct immunostained with vimentim MAb V9. Strong reaction in myoepithelial and stromal elements is evident (×420).* c: *Normal breast; portions of ducts immunostained with MAb G-A-5 to GFP. Note strong reaction in most but not all myoepithelial cells; stromal cells remain undecorated (×420).* d: *FCD; portion of hyperplastic duct immunostained with cytokeratin MAb KA1. A number of cells are stained while others are nonreactive (×420).* e: *FCD; portions of adjacent hyperplastic ducts immunostained with MAb V9 to vimentin. Many myoepithelial cells are stained as are some luminal elements (arrow heads) (×420).* f: *FCD; portion of hyperplastic ducts with almost complete luminal obliteration immunostained with MAb G-A-5 to GFP. Distinct reaction is restricted to myoepithelial cells (×420).* g: *Multiple papillomas; segments of small papillae immunostained with cytokeratin MAb KA1. Note variably distributed staining of luminal and basal cells (×420).* h: *Multiple papillomas; portion of large, single papilla immunostained with MAb V9 to vimentin. Note reaction of many myoepithelial cells; a number of secretory cells projecting into lumen are also stained (arrowheads) (×420).* i: *Multiple papillomas; portion of large papilla with glandular invagination immunostained with MAb G-A-5 to GFP. A number of delicate processes of myoepithelial cells are distinctly reactive (arrowheads) (×420).* j: *Fibroadenoma; portion of duct immunostained with MAb G-A-5 to GFP. Note several myoepithelial and luminal cells stained (×420).* k: *Cystosarcoma phylloides; portion of duct immunostained with MAb G-A-5 to GFP. Note several basal and luminal cells clearly immunostained while stromal elements remain unreactive (×420).* l: *Intraductal carcinoma; portion of carcinomatous duct immunostained with MAb V9 to vimentin. Note strong reaction of probably non-neoplastic myoepithelial cells; occasional neoplastic cells within duct are also reactive. Stromal cells are strongy stained (×420).* m: *Infiltrating ductal carcioma NOS immunostained with MAb G-A-5 to GFP; scattered neoplastic cells in adipose tissue. Several immunostained tumor cells are evident (×420).* n: *Colloid carcinoma immunostained with cytokeratin MAb KA4. Note strong staining of neoplastic cluster in the nonreactive mucinous pool (×420).* o: *Colloid carcinoma (same case depicted in Figure 1n) immunostained with MAb V9 to vimentin. Neoplastic cluster and isolated tumor cell (arrowhead) are clearly stained amid the nonreactive mucinous pool (×420).*

ductules and acini in 9 of 12 samples. There was notable staining heterogeneity: in certain ducts many myoepithelial cells reacted strongly while other cells in the vicinity reacted weakly or not at all (Figure 1c). No reaction was noted in luminal cells. As a rule, the subpopulation of GFP-immunoreactive myoepithelial cells was smaller than the Vim- and KA1-reactive counterparts. In most instances, no significant staining differences were observed among the various GFP Mabs used; however, in a few fields, MAb GFP 12.24 reacted with fewer cells and the reaction was weaker than with the other GFP MAbs. The GFP staining pattern was often distinctly linear involving delicate and at times branching processes; this observation held true for the normal, hyperplastic, and neoplastic breast. Rarely, round GFP-positive cytoplasmic profiles were noted (*vide infra*).

Fibrocystic Disease

All 20 FCD samples immunoreacted with the MAbs to cytokeratins. However, the intensity and extent of the reactions differed considerably, depending on the MAb used and the variants of FCD. Immunoreactions with MAbs to vimentin and GFP involved 12 of 20 and 15 of 20 cases, respectively; the extent of the staining varied considerably in relation to the variants of FCD.

In cystically dilated ducts, MAb AE1/AE3 stained most cells, but more luminal than basal cells reacted, whereas with MAb KA4 only luminal cells were consistently stained. With MAb KA1 the reverse pattern was noted, eg, the majority of reactive cells were myoepithelial as previously described.[1] Monoclonal antibodies to vimentin reacted frequently with subpopulations of basal as well as luminal cells, although the former clearly predominated. Monoclonal antibodies to GFP stained myoepithelial cell subpopulations. The luminal cells of cystic ducts with apocrine metaplasia showed strong immunoreaction with MAbs KA4 and AE1/AE3, while no staining was noted with MAb KA1 and MAbs against vimentin and GFP. Most basal-myoepithelial cells in these cysts immunostained strongly with MAb KA1, while only occasional cells reacted focally and faintly with MAbs to vimentin and GFP.

Hyperplastic ducts, with and without atypia, showed extensive reactions with MAbs AE1/AE3 and KA4, which stained the vast majority but not all cells. Monoclonal antibody KA1 stained a significant proportion of the hyperplastic cells, but rather large cellular aggregates did not react (Figure 1d). Significant subpopulations of cells in the basal regions reacted with MAbs to vimentin and GFP; with the former, we also noted immunostaining of some luminal cells, while with the latter, delicate, dendritelike processes that wrapped around neighboring cells were observed (Figures 1e, f).

In most samples of adenosis, significant albeit variable subpopulations of basal-myoepithelial cells reacted with MAbs to vimentin and GFP, and with MAb KA1. Scant immunoreaction of those cells was seen with MAb AE1/AE3, while they did not stain with MAb KA4. Notably, in occasional acini of sclerosing adenosis, most cells reacted intensely with MAb KA1. In similar ductules, most basal-myoepithelial cells reacted strongly with MAbs to GFP, whereas vimentin MAbs stained but a minority of them. In one focus of sclerosin adenosis with hyperplastic myoepithelial cells discernible by conventional stains, MAbs to GFP revealed fusiform cells around slightly dilated ducts arranged concentrically in a targetlike pattern; notably, these cells did not react with any of our MAbs to cytokeratin or to vimentin.

Papillomas showed a characteristic immunostaining pattern; with MAb AE1/AE3, most cells were stained but significant subpopulations did not react (not shown).

Monoclonal antibody KA4 stained most luminal cells, whereas MAb KA1 decorated preferentially, but not exclusively, the basal-myoepithelial cells (Figure 1g). Vimentin MAbs reacted with a few unremarkable luminal cells, but the strongest reaction was with the basal-myoepithelial cells, and with some delicate cellular tufts protruding into the lumina (Figure 1h). All the MAbs to GFP stained, and at times strongly, subpopulations of basal-myoepithelial cells, showing clear delineation of their thin, tapering cytoplasmic processes (Figure 1i).

Within the spectrum of FCD, Vim- and GFP-reactive cells were most frequently demonstrable in hyperplastic ducts, papillomas, and adenotic acini; however, we should stress that not every representative of those lesions included such cells.

Fibroadenomas and Cystosarcoma Phylloides

In the two fibroadenomas studied, most basal and luminal cells reacted with MAbs AE1/AE3 and KA4; MAb KA1 stained predominantly the basal cells, although a minority of luminal cells also were stained. Vimentin-positive cells were distributed similarly to those immunostained with MAb KA4, but were found in only ½ of the fibroadenomas. Monoclonal antibodies to GFP decorated a subset of the epithelial cells, mostly in a basal location; sometimes luminal cells were also stained (Figure 1j). The 'stromal' component stained only with vimentin MAbs.

In the single cystosarcoma phylloides studied, the 'epithelial' component reacted extensively with MAbs AE1/AE3 and KA4. Focally, Mab KA1 stained intensely many luminal cells, whereas the basal cells stained, but faintly. Vimentin MAbs stained convincingly luminal and basal cells as well as the 'stromal' elements. In adjacent sections, including profiles of the same gland, GFP MAbs stained distinctly some basal cells and possibly some luminal cells (Figure 1k), whereas 'stromal' cells remained undecorated.

Carcinomas

All 20 ductal carcinomas not otherwise specified (NOS) reacted with MAbs to cytokeratins, 9 of 20 with MAbs to vimentin, and 1 of 20 with MAbs to GFP.

Of the cytokeratin MAbs used, AE1/AE3 and KA4 reacted with the vast majority of infiltrating tumor cells in all samples; the in-situ components reacted strongly. Monoclonal antibody KA1 stained subpopulations in 4 of 20 NOS ductal carcinomas. The *in situ* components of these tumors stained, but focally and weakly, whereas a strong reaction was noted in the peripheral, presumably non-neoplastic, myoepithelial cells.

Monoclonal antibodies to vimentin reacted with scattered infiltrating elements in 9 of 20 cases; the staining pattern ranged from diffuse to that of paranuclear globular aggregates. Neither increased mitotic activity nor localized tumor necrosis was noted in association with vimentin immunoreactivity. In *in situ* foci, some vimentin-reactive cells were noted; in addition, the myoepithelial cells reacted strongly (Figure 1l) and similarly as with MAb KA1 (*vide supra*).

Convincing GFP-reactive cells were noted in a single case of infiltrating ductal carcinoma NOS; the reaction was strong in small clusters and in isolated carcinoma cells invading adipose tissue (Figure 1m). Notably, the extensive *in situ* component of the same case showed no detectable GFP reactivity, whereas MAb AE1/AE3 stained both *in situ* and infiltrating elements. Glial filament protein MAbs stained strongly scattered myoepithelial cells encircling *in situ* foci in several cases. Thus, the possibility that some GFP-positive cells noted in the aforementioned carcinoma may represent dissociated and secondarily trapped non-neoplastic cells cannot be excluded.

The mucinous carcinoma reacted strongly with MAbs AE1/AE3 and KA4 (Figure 1n), while showing no reaction with MAb KA1. Vimentin MAbs stained convincingly the majority of the same cells (Figure 1o); no reaction was obtained with any GFP MAb. One case of adenoid cystic carcinoma and one case of medullary carcinoma showed staining only with MAbs AE1/AE3 and KA4. One case of papillary carcinoma showed strong reaction with AE1/AE3 and KA4, but no staining with KA1; scattered cells reacted weakly with Vim MAbs, while a smaller subpopulation stained with GFP MAbs.

All infiltrating lobular carcinomas immunostained strongly with MAbs AE1/AE3 and KA4; occasional cells reacted with MAb KA1. One case stained convincingly with vimentin MAbs. Rare GFP-positive cells were noted in a single case of infiltrating lobular carcinoma; again, it could not be ruled out that individual positive cells may not be neoplastic.

GFP-expressing Myoepithelial Cells: Patterns of Coexpression of Cytoskeletal Proteins and Molecular Demonstration of GFP

Based on the findings outlined, samples of FCD, fibroadenomas, and intraductal carcinomas were selected for detailed analysis of coexpression patterns in GFP-reactive myoepithelial cells employing double-label immunofluorescence microscopy, and conventional immunostaining of sequential sections.

Coexpression of GFP and vimentin in a subpopulation of myoepithelial cells was shown by double-immunofluorescence microscopy (Figures 2a, b). Variable patterns of coexpression of cytokeratin with other cytoskeletal proteins were noted in the serial sections (Figures 3a to f). Essentially uniform staining of most myoepithelial cells was observed with MAb KA1 (Figure 3c), MAb E3 against cytokeratin 17 (Figure 3f), MAb VIM-9 against vimentin (Figure 3b), as well as with MAb α-sm-1 against smooth muscle actin (Figure 3d). Monoclonal antibodies KA1 and E3 also decorated some luminal cells. In contrast, antibodies against GFP stained only subpopulations of myoepithelial cells (Figures 3a, e). Because these patterns were noted in adjacent serial sections (Figures 3a to d and Figures 3e to f), it seems safe to assume true coexpression of cytokeratins 14 and 17, vimentin, and smooth muscle-type actin in the GFP-expressing cells.

Staining of further serial sections with MAbs K_s 18.174, K_s 19.2, CK-7, and K_s 8.12 directed against cytokeratins 18, 19, 7 and 13/16, respectively, showed no reaction by myoepithelial cells. A weakly positive to negative reaction of myoepithelial cells was observed, with MAb K_s pan 1-8 recognizing all type II cytokeratins.

Cytoskeletal proteins of samples of multiple intraductal papillomas were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) or two-dimensional gel electrophoresis, transferred to nitrocellulose membranes, and subjected to immunoblotting analysis (Figure 4a to d). Glial filament protein-specific antibodies showed, on SDS-PAGE, a strong reaction with a band of the same electrophoretic mobility as authentic GFP (Figure 4b). Total protein staining demonstrated the presence of a complex pattern of cytokeratins comprising stratification-related polypeptides 5, 6, 14, 15, and 17 and simple epithelia-type polypeptides 7, 8, 18, and 19, as well as vimentin and actin (Figure 4c). The GFP reaction was then confirmed by immunoblotting after two-dimensional gel electrophoresis, in which a weak but significant reaction was noted, with a single polypeptide spot displaying the exact gel-electrophoretic coordinates of GFP (Figure 4d). These results indicate the presence of GFP in these samples, and most probably reflect the immunoreactive protein in a subset of myoepithelial cells.

Discussion

This immunohistochemical and biochemical study of the normal, hyperplastic, and neoplastic breast has generally confirmed the variable expression of different cytokeratin polypeptides. We have also shown that cell subpopulations in the normal breast, the spectrum of FCD, benign, and malignant neoplasms may coexpress cytokeratins with vimentin and with GFP. The identity of the latter IF protein, which was unexpected, was confirmed by immunoblot analyses after gel electrophoresis.

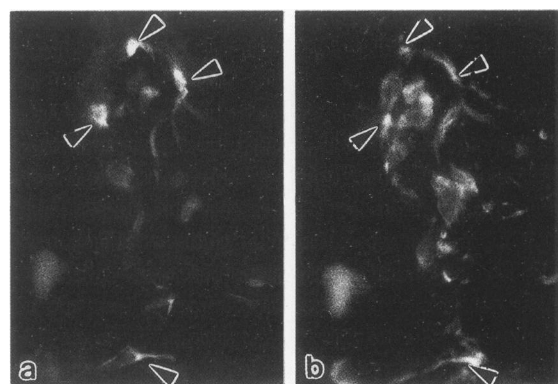

Figure 2. *Multiple intraductal papillomas (same case as Figures 1g, h, and i). Double-label immunofluorescence with GFP guinea pig antibody* (a) *and vimentin VIM-9 antibody* (b). *Note extensive coexpression in a number of cells (arrowheads), some of which display conspicuous cytoplasmic processes (×450).*

Our findings with the cytokeratin MAbs KA1 and KA4 that preferentially recognize basal-myoepithelial and luminal-secretory cells, respectively, generally agreed with previous reports.[1,2] However, we found that in the case of *in situ* and infiltrating ductal carcinomas, the number of cases and the relative proportion of cells that express a 'myoepithelial' cytokeratin profile as defined by their reactivity with MAb KA1 is somewhat higher than previously reported. Interestingly, earlier electron microscopic studies had suggested that myoepithelial differentiation in breast carcinomas was comparatively frequent.[39,40] Our findings also differed from previous descriptions with regard to the topographic distribution of the KA1-reactive, ie, phenotypically myoepithelial cells, as we noted that while those cells are indeed predominantly basally located, they also may be found in a juxtaluminal position in occasional foci of sclerosing adenosis, fibroadenomas, and cystosarcoma phylloides. Thus one may speculate that in certain proliferative breast lesions, and in some benign and low-grade malignant neoplasms, the normal pattern of basal distribution of myoepithelial cells may change. Alternatively one might argue that in certain conditions, luminal cells may acquire cytokeratin polypeptides that are 'normally' part of the myoepithelial repertory. We may add that the positive reaction with MAb KA1 in cells of a quasiluminal position does not necessarily indicate myoepithelial differentiation, as cytokeratin 14 recognized by this MAb also is expressed in most stratified squamous epithelia,[1,27] and may thus reflect a sort of 'squamous metaplasia.'

Initial reports indicated that in the normal breast parenchyma, in FCD and in benign and malignant epithelial breast tumors, the IF cytoskeletal proteins consisted of cytokeratin polypeptides, and that vimentin was not ex-

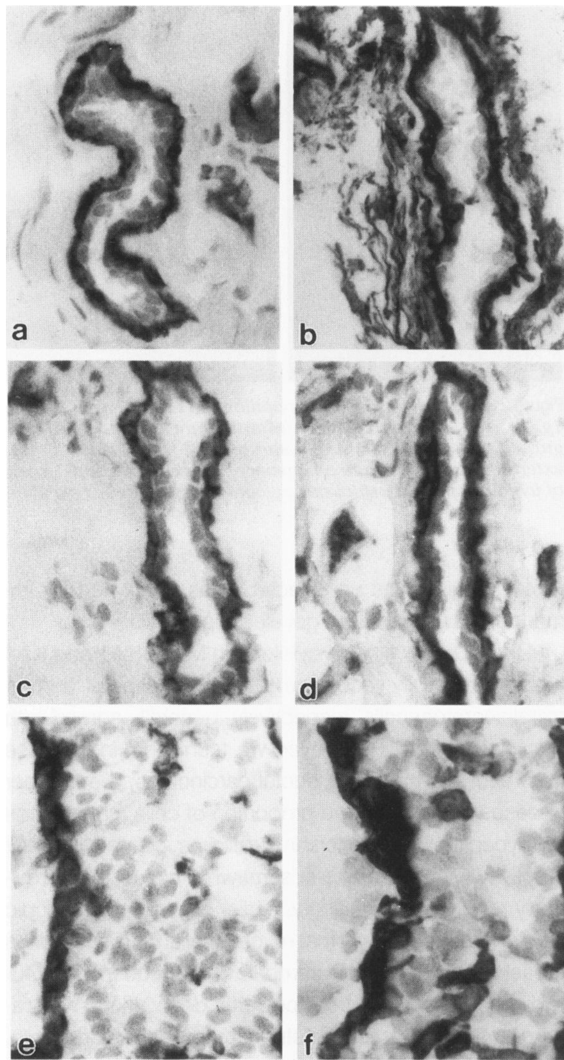

Figure 3. *Step sections of non-neoplastic duct immunostained with GFP GA5 antibody* (a) *and vimentin VIM-9 antibody* (b). *Note virtually uninterrupted staining of myoepithelial cells in both sections; the stromal elements are stained with vimentin* (b) *while remaining undecorated with GFP* (a) *(×450).* c, d: *Further steps from the same duct depicted in* a *and* b *immunostained with cytokeratin MAb KA1* (c) *and alpha-smooth muscle actin* (d) *antibodies. Myoepithelial elements are stained in both sections, but the reaction appears stronger and more extensive in 3d (×450).* e, f: *Step sections of intraductal carcinoma depicted in* I. *Immunostained with GFP GF-5 antibody and with antibody E3 to cytokeratin polypeptide 17. Myoepithelial cells are clearly reactive with GFP antibody* (e); *note similar, albeit stronger, reaction with cytokeratin antibody* (f) *(×450).*

pressed; some but not all of these studies were performed in conventionally fixed and paraffin-embedded samples.[11-15] However, in 1987, Azumi and Battifora[17] described the presence of vimentin-positive cells in 5 of 43 ethanol-fixed samples of breast carcinomas, while no vimentin-reactive cells were found in formalin-fixed tissues. Another report described vimentin-reactive cells in 1 of 38 samples of breast carcinomas.[41] In subsequent studies, Raymond and Leong[18] detected some vimentin-reactive cells in 7 of 20 cases of FCD and benign breast tumors, and in 38 of 63 breast carcinomas; in a subsequent report, the same observers noted that 43 of 84 breast carcinomas had variable numbers of vimentin-positive cells.[19] While the relative percentages of Vim-positive cells varied considerably from case to case, our overall results on the presence of vimentin-reactive cells in FCD, benign breast tumors, and breast carcinomas generally agree with the latter reports. Interestingly Raymond and Leong[19] suggested that vimentin expression may be a potential predictor of aggressive behavior in breast carcinomas. Yet we detected some vimentin-reactive basal cells in ducts and acini of several samples of unquestionably normal breasts. Thus, regardless of the possible validity of that prognostic suggestion, it cannot be argued that vimentin expression in hyperplastic and neoplastic breast represents a pathologic phenomenon. Yet the relative frequency of vimentin-reactive cells was much higher in the hyperplastic and transformed breast epithelium than in the normal counterpart. In this context, recent data suggested again that rich vimentin expression in breast carcinomas *in vivo* and *in vitro* was correlated with estrogen receptor negativity, thus again pointing to the possible association of vimentin with a clinically aggressive behavior.[42,43]

Glial filament protein was initially reported as the IF protein typical of glial cells and tumors.[44-53] It was also found in the interstitial cells of the epiphysis[54-57] and in the stellate cells of the hypophysis.[58-60] Subsequent immunohistologic studies, however, showed that the distribution of GFP was broader than originally envisioned, as it has been reported in certain perisinusoidal liver cells of some rat strains but not of others,[61,62] in the lens 'epithelium' of certain rodents,[63] in subsets of Schwann cells,[24] in subsets of cells of nerve sheath neoplasms,[64-66] in choroid plexus tumors,[68] in subsets of thymic epithelial cells,[69] in some epithelial cells of salivary glands and salivary gland tumors,[24,70] in malignant mixed müllerian tumors,[71] and in respiratory tract chondrocytes and chondromatous hamartomas.[72] Unfortunately, the presence of GFP was not confirmed by independent methods in most of these studies. Several of them described remarkable species variability in the expression of GFP in certain nonastrocytic cells.[24] To date, no report has mentioned the presence of GFP-immunoreactive cells in normal or abnormal breasts. We have now shown with several GFP MAbs that reactivity is readily detectable in subsets of myoepithelial-appearing cells in normal breast ducts and acini, and that these cells are rather frequent in certain proliferative variants of FCD, including adenosis, ductal hyperplasia, and florid papillomas. These findings are reminiscent of those in the parotid gland, in which GFP positivity was noted in a subset of normal myoepithelial cells,

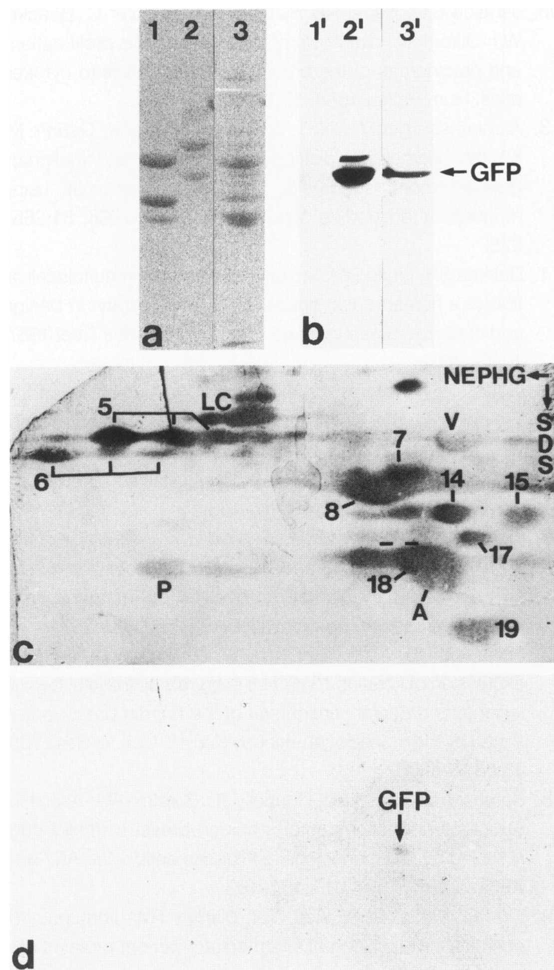

Figure 4. *Immunoblot detection of GFP in intraductal papilloma (adjacent to sample shown in Figure 1i) after (one-dimensional) SDS-PAGE* (**a, b**) *and two-dimensional gel electrophoresis* (**c, d**). **a**: *SDS-PAGE separated cytoskeletal proteins of MCF-7 cells (lane 1, bands, from top to bottom are cytokeratins 8, 18 nd 19), U333CG/343MG (lane 2, major bands are, from top to bottom, vimentin and GFP), and mammary tissue containing multiple intraductal papillomas (lane 3; microdissected to avoid "contamination" with nerves), shown after transfer to nitrocellulose membrane and Ponceau red staining.* **b**: *Corresponding immunoblot (peroxidase staining) using MAb GF 12.24. Note the presence of immunoreactive GFP not only in the cultured glioma cells (lane 2') but also in the intraductal papilloma (lane 3'); MCF-7 cells have been included as negative control (lane 1'). The minor band above the main GFP (lane 2') is a GFP modification, probably a phosphorylated form.* **c**: *Total cytoskeletal proteins of intraductal papilloma tissue shown after two-dimensional gel electrophoresis, transfer to nitrocellulose paper, and staining with India ink. NEPHG, first dimension using non-equilibrium pH gradient electrophoresis; SDS, second dimension using SDS-PAGE. The cytokeratin polypeptides present are designated with the numbers of the catalog (27); V, vimentin; A, actin; LC, nuclear lamin C; and P, 3-phosphoglycerokinase from yeast (added as marker protein).* **d**: *Immunoblot reaction of the same nitrocellulose paper using MAb GF 12.24 to GFP. Note the weak but specific reaction of one polypeptide spot (arrow) having the coordinates of GFP, the position of which is between the two bars depicted in* (**c**), *where it is not visible because of its minute amount.*

while GFP positive cells were distinctly more frequent in pleomorphic adenomas.[23,24,70,73,74]

In normal and transformed glial cells, GFP is extensively distributed in the main cytoplasmic mass and in cytoplasmic processes.[53] Notably, when GFP is expressed in many nonglial elements, eg, chondrocytes, lens epithelium, salivary gland myoepithelial cells, etc., these cells frequently exhibit a stellate shape, and GFP-containing IFs appear to aggregate in the cytoplasmic processes.[24,70,74] Our observations with regard to the presence of GFP-possessing cells in the breast seem to reflect the latter description. Furthermore our immunohistochemical findings appear to parallel and reinforce earlier electron microscopic studies that described the development of 'globoid' cell bodies and prominent cytoplasmic processes in myoepithelial cells in certain proliferative breast conditions such as adenosis.[75,76] It thus would be tempting to speculate that, in part at least, the expression of GFP in nonglial cells may be related to—or promoted by—certain structural–functional characteristics, ie, the formation of dendrite-type cell processes.

In neoplastic and non-neoplastic glial cells, GFP may be expressed alone, although it is often coexpressed and may be even coassembled with vimentin, as has been shown in detail for cultured glioma cells.[77–79] Coexpression of GFP and vimentin has also been found in developing glial cells,[80–82] ependymomas,[83] and in subsets of peripheral nerve sheath cells[24] and tumors arising therefrom.[66] The coexistence of GFP with vimentin and cytokeratin in certain nerve sheath tumors has also been described,[67] although no intracellular localization of all three IF proteins was presented in this report. In certain complex central nervous system neoplasms, eg, primitive neuroectodermal tumors, GFP may be coexpressed not only with vimentin but also with neurofilament proteins, cytokeratins, and even desmin.[20,21] Nakazato et al[22,23] reported the existence of GFP in a subpopulation of salivary gland and plemorphic adenoma cells. Achsttaeter et al[24] described the presence of GFP in certain myoepithelial cells of the parotid, and in cells of pleomorphic adenomas. By double-label fluorescence microscopy, these authors showed coexpression of GFP with cytokeratins and with vimentin in individual cells; and, in step sections, they also noted the presence of the three IF proteins in individual cells.[24] The expression of GFP in salivary gland cells and tumors has been confirmed by other observers.[70,73,74] More recently, Gustafsson et al[70] also have reported the presence of cytokeratins-vimentin-GFP in pleomorphic adenomas and in a small group of carcinomas of the salivary gland, and described occasional cells with the additional coexpression of desmin.[70] Moreover, these authors stressed that, in the normal salivary glands as well as in tumors, only subsets of myoepithelial-type cells express GFP.[70] Similarly Kasper et al[84] recently identified GFP in coexistence with cytokeratins

and vimentin in a subpopulation of folliculo-stellate cells of the human hypophysis, including cells of Rathke's cysts.

Our immunohistochemical and biochemical studies indicate that myoepithelial-type cells capable of expressing GFP, and of coexpressing it with cytokeratins and vimentin, exist also in the normal, hyperplastic, and neoplastic breast. Interestingly, the greatest number of GFP-expressing myoepithelial cells were noted in certain nonneoplastic but strongly proliferative forms of FCD, in benign neoplasms, and in 'borderline' and *in situ* carcinomas. Perhaps myoepithelial cells tend to 'switch' to GFP production as a reactive response in certain proliferative situations. Alternatively myoepithelial cells with 'constitutive' GFP expression may proliferate preferentially, resulting in dysplasias and tumors with a relatively high proportion of GFP-containing cells.

The finding of GFP in normal and diseased breast epithelium also represents an interesting demonstration of GFP-containing IFs in desmosome-bearing cells. The attachment of IF proteins to desmosomal plaques is most characteristic of cytokeratin IFs as formed in epithelial cells and tumors.[85] Yet the attachment of IFs containing desmin to desmosomal plaques in myocardial cells[86] and of vimentin IFs to desmosomal plaques in arachnoidal cells and meningiomas,[87] and in granulosa cell tumors,[85] have also been noted. More recently, the attachment of vimentin to desmosomal plaques has been seen in Ewing's sarcomas,[88] and in a variety of other epithelioid and nonepithelial cells.[89] Thus, the possibility of a GFP–desmosome interaction exists in the breast and in the salivary glands, although it has not been demonstrated.[24] Furthermore the identification of GFP-positive cells in normal and transformed breast epithelium, and the coexpression of GFP with cytokeratin and/or vimentin in certain cells of the breast and of the salivary glands, would add credence to the notion that this peculiar cytoskeletal profile does not merely constitute a stochastic 'oddity' of some transformed cells but may indeed define a special subset of myoepithelial cells[24] with special biologic and pathobiologic properties. Whether or to what extent GFP-expressing myoepithelial cells may differ functionally from their GFP-negative counterparts, and whether the subpopulations of cells producing GFP represent clonal lineages or reflect changes in the microenvironment of certain groups of cells[90] remains to be clarified by future studies.

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