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Expression of smooth muscle-specific proteins in myoepithelium and stromal myofibroblasts of normal and malignant human breast tissue

(smooth muscle differentiation/breast carcinoma)

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ABSTRACT **The expression of several differentiation markers in normal human mammary gland myoepithelium and in certain stromal fibroblasts ("myofibroblasts") associated with breast carcinomas was studied by immunofluorescence microscopy of frozen sections. Several antibodies to smooth muscle-specific proteins (smooth muscle α-actin, smooth muscle myosin heavy chains, calponin, α_1-integrin, and high molecular weight caldesmon) and to epithelial-specific proteins (cytokeratins, E-cadherin, and desmoplakin) were used to show that myoepithelial cells concomitantly express epithelial and smooth muscle markers whereas adjacent luminal cells express only epithelial markers. The same antibodies were used to establish that stromal myofibroblasts exhibit smooth muscle phenotypic properties characterized by the expression of all the smooth muscle markers examined except for high molecular weight caldesmon. In addition, both myoepithelium and myofibroblasts show a significant degree of heterogeneity in smooth muscle protein expression. Thus, myoepithelial cells and stromal myofibroblasts are epithelial and mesenchymal cells, respectively, which coordinately express a set of smooth muscle markers while maintaining their specific original features. The dual nature of myoepithelial cells and the phenotypic transition of fibroblasts to myofibroblasts are examples of the plasticity of the differentiated cell phenotype.**

An emerging concept in cell biology is that the differentiated state of specialized cells requires continuous regulatory input and can therefore exhibit a remarkable plasticity (reviewed in ref. 1). Experiments with heterokaryons (2) and the conversion of nonmuscle to muscle cells by the transcription factor MyoD1 (3) demonstrated that muscle genes can be activated by muscle regulatory elements in cells which did not originally exhibit a muscle phenotype.

In vivo, several nonmuscle cell types (e.g., basal cells of glands, thecal cells of the ovary, macrophages in xanthogranuloma, submesothelial stromal cells, hair follicle dermal cells, bone marrow stromal cells, and myofibroblasts in stromal cells associated with tumors and in granulation tissue of wound repair) show a partially smooth muscle (SM)-like phenotype. Ultrastructural and immunomorphological studies have shown an increased amount of microfilament bundles and contractile proteins (actin, myosin, α-actinin, etc.) in these cells compared with other nonmuscle cells. Moreover, it was recently shown that they are positive for SM α-actin, and more rarely, for SM myosin (reviewed in refs. 4 and 5). It has not been determined, however, whether this phenomenon reflects the coordinated expression of several cytodifferentiation-related SM-specific proteins (i.e., the activation of a complex myogenic differentiation program) or whether only these two SM proteins are expressed in such cells. To distinguish between these two possibilities, we have studied the SM-α-actin-expressing myoepithelial cells (6) of human mammary glands and the myofibroblasts in the stromal reaction of breast carcinomas.

The epithelium of mammary glands consists of a luminal layer of milk-secreting cells and a basal layer of contractile, basket-like myoepithelial cells surrounded by a connective tissue stroma. Breast carcinoma is thought to arise from abnormal proliferation of luminal cells in ducts. Myofibroblasts frequently appear in the stroma of neoplastic breast tissue. These cells, whose origin is still unknown, are characterized by a fibroblastic appearance with ultrastructural features reminiscent of SM, hence their name.

In this study we analyzed the expression in normal and malignant human breast tissue of five proteins which are considered to be markers for SM cell phenotype [SM α-actin (7, 8), SM myosin heavy chains (SM-MHCs) (9), α_1-integrin (10), calponin (11), and high molecular weight caldesmon (h-caldesmon) (12)] and four epithelial cell-type markers [total cytokeratins (13), cytokeratin 17 (14), E-cadherin, and desmoplakin (15)]. We have found that in myoepithelium all five SM-specific and four epithelial cell-specific markers are simultaneously expressed and that a concerted SM phenotypic expression can be induced during myofibroblast formation in certain stromal cells.

MATERIALS AND METHODS

Tissue Samples. Twelve samples were examined: 2 normal breast samples, 1 intraductal carcinoma, and 9 infiltrating ductal carcinomas were obtained immediately after surgery from the Department of Pathology of the Institut Curie, Paris. The two normal breast samples were obtained from 40-year-old women undergoing mastectomy for invasive ductal breast carcinoma. They were taken in the quadrant opposite to the tumor and their histological aspect appeared normal upon microscopic examination, without any evidence of malignancy.

Frozen tissue sections, 5 μm thick, were applied to poly(L-lysine)-precoated glass slides, fixed in acetone at −20°C, and stored at −70°C until used.

Antibodies. Murine monoclonal antibodies specific for the following proteins were used: SM α-actin [no. A2547, Sigma (7)], pan-cytokeratins (KL1, Immunotech, Luminy, France),

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Abbreviations: SM, smooth muscle; MHC, myosin heavy chain; h-caldesmon, high molecular weight caldesmon.

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α_1-integrin [TS2/7, T Cell Sciences, Cambridge, MA (16)], desmoplakin [anti-DP I and II, kindly provided by W. Franke, German Cancer Research Center, Heidelberg, Germany (17)], cytokeratin 17 [mAb E3, kindly provided by S. Troyanovsky, Cancer Research Center, Moscow (14)], E-cadherin [HECD-1, kindly provided by S. Hirohashi, National Cancer Center Research Institute, Tokyo (18)], SM-MHCs (SMMS-1), h-caldesmon (h-CD), and calponin (CALP) (19). In addition we used affinity-purified rabbit antibodies to α_1-integrin [kindly provided by V. Belkin, Institute of Biological and Medical Chemistry, Academy of Medical Sciences, Moscow (10)], SM-MHCs [kindly provided by R. Adelstein, National Heart, Lung, and Blood Institute, National Institutes of Health (20)], and calponin (kindly provided by V. Shirinsky, Cardiology Research Center, Moscow).

Staining Procedures. Frozen tissue sections were processed for immunofluorescence microscopy by using a biotin–streptavidin staining kit (Amersham). Sections were first incubated with phosphate-buffered saline containing 5% bovine serum albumin (blocking solution) for 45 min. Immunoreactivity was revealed by incubating the sections overnight with primary antibodies diluted in blocking solution. In the case of monoclonal antibodies this was followed by incubation with biotin-conjugated anti-mouse IgG for 1 hr and subsequent incubation with fluorescein-conjugated streptavidin for 45 min. In the case of rabbit antibodies the sections were incubated with Texas Red-conjugated anti-rabbit IgG for 1 hr. For double-labeling experiments murine monoclonal antibodies and rabbit antibodies were applied together, followed by simultaneous incubation with biotin-conjugated anti-mouse IgG and Texas Red-conjugated anti-rabbit IgG. Fluorescein-conjugated streptavidin was subsequently applied. Each step in the staining procedure was followed by three 10-min washes in phosphate-buffered saline. All commercial antibodies were applied at the dilutions recommended by the supplier. Hybridoma culture supernatants were used undiluted (cytokeratin 17, SM-MHC, calponin, h-caldesmon), and monoclonal antibodies in ascites fluid (to E-cadherin and desmoplakin) were diluted in blocking solution 1:300 and 1:100, respectively. Rabbit antibodies to α_1-integrin, calponin, and SM-MHCs were diluted in blocking solution 1:10, 1:50, and 1:250, respectively.

Sections were examined under epi-illumination on a Leitz Orthoplan Microscope and photographed with Kodak T-MAX 400 film.

Table 1. Expression of several SM and epithelial markers in normal and malignant breast tissue

	Normal tissue			Malignant tissue	
Markers	SM cells*	Luminal epithelium	Myoepithelium	Myofibroblasts	Carcinoma
SM α-actin	+	−	+	+	−
SM-MHCs	+	−	+	±†	−
Calponin	+	−	+	+	−
h-Caldesmon	+	−	±‡	−	−
α_1-Integrin	+	−	+	+	−
Cytokeratins§	−	+	+	−	+
Cytokeratin 17	−	−	+	−	−
E-Cadherin	−	+	+	−	+
Desmoplakin	−	+	+	−	+

*SM cells in vessels.
†Some myofibroblasts were negative for SM-MHC immunoreactivity.
‡Immunoreactivity to h-caldesmon was observed in the myoepithelial component of galactophorous sinuses but not in ducts or lobules.
§Immunoreactivity to antibody against total cytokeratins.

RESULTS

SM and Epithelial Differentiation Markers in Myoepithelial Cells. Myoepithelial cells of several secretory glands express SM α-actin and cytokeratins (6, 7, 21). Therefore they are good candidates to establish whether some cell types can simultaneously express *in vivo*, under normal physiological conditions, both SM and epithelial differentiation markers. We have stained frozen sections of normal human mammary glands by double immunofluorescence labeling with a panel of five antibodies to proteins characteristically synthesized in adult SM cells (SM α-actin, SM-MHC, calponin, h-caldesmon, and the α_1-integrin subunit) and four antibodies to proteins considered to be epithelial markers (total cytokeratins, cytokeratin 17, E-cadherin, and desmoplakin).

SM cells of blood vessel walls were brightly stained by all five antibodies to SM markers and not at all by the antibodies to epithelial markers (Table 1). Double labeling with antibodies to SM markers in conjunction with antibodies to epithelial markers (Fig. 1 *C, D; E, F; K, L*; and *M, N*) clearly

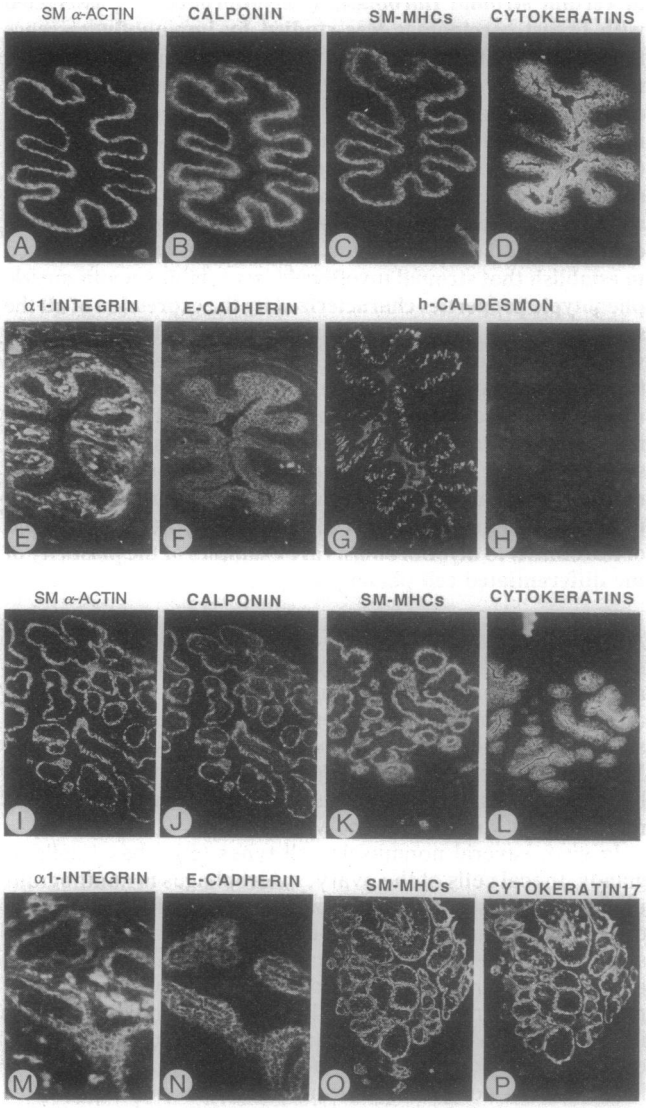

FIG. 1. Immunofluorescent labeling of frozen sections of normal human breast epithelial structures (*A–G*, galactophorous sinus; *H–P*, lobules) with antibodies to SM and epithelial markers as indicated above the photographs. (*A, B*; *C, D*; and *E, F*) Pairs of double-labeled serial sections of the same sinus. (*G* and *H*) Single labeling with antibody to h-caldesmon of a sinus and lobules, respectively. (*I, J*; *K, L*; *M, N*; and *O, P*) Pairs of double-labeled sections of lobular tissue. (×110 except for *M* and *N*, which are ×160.)

distinguished two layers of cells in the normal mammary gland. Anti-pan-cytokeratin and anti-E-cadherin labeled both layers, while anti-SM-MHCs labeled only the external layer, which consisted of myoepithelium. The myoepithelial cell layer in lobules and ducts expressed all the SM markers examined except for h-caldesmon, as well as all the epithelial markers (Fig. 1 *H* and *P*; Table 1). An identical pattern of expression was observed in the galactophorous sinuses, but there h-caldesmon was also expressed in the myoepithelial cell layer in a characteristic, discontinuous pattern (Fig. 1*G*), indicating that only a subset of myoepithelial cells expressed this marker. The internal cell layer, or luminal epithelium, did not stain for any of the SM markers but was positive for the epithelial markers pan-cytokeratin, E-cadherin, and desmoplakin (Fig. 1 *D, F, L,* and *N*; Table 1). Cytokeratin 17, a specific myoepithelial and basal cell marker in several glandular epithelia, was expressed only in the myoepithelial cells, as expected (Fig. 1*P*). Interestingly, immunoreactivity to total cytokeratins, as revealed by antibody KL1, was less pronounced in the myoepithelial layer than in the luminal layer (Fig. 1 *D* and *L*) whereas E-cadherin reactivity was equally strong in both layers (Fig. 1 *F* and *N*), indicating that total cytokeratin content of myoepithelial cells could be significantly less than in luminal cells.

SM Differentiation Markers in Myofibroblasts. Double immunofluorescence labeling of sections of breast carcinomas and normal breast tissue was performed with the same panel of antibodies in order to characterize stromal myofibroblasts. In all 10 carcinoma cases examined, numerous brightly stained elongated stromal cells were positive for SM α-actin (e.g., Fig. 2 *D* and *F*), whereas no immunoreactivity was detected in fibroblasts of two normal breast samples. Tumor cells were positive for the epithelial markers cytokeratin, E-cadherin, and desmoplakin, and negative for all the SM markers exam-

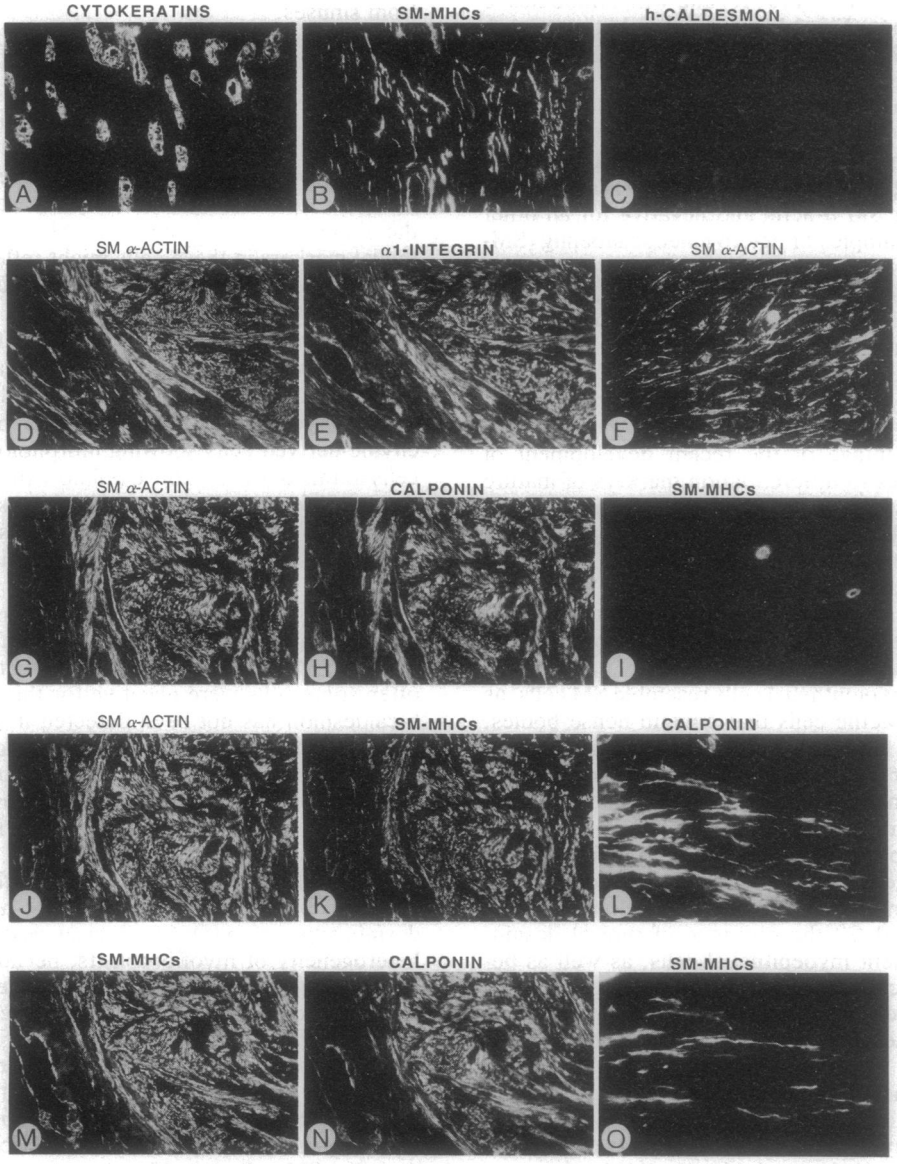

FIG. 2. Immunofluorescent labeling of stromal myofibroblasts in frozen sections of human breast carcinoma with antibodies to SM and epithelial markers as indicated. (*A* and *B*) Double labeling of a section showing the relationship between tumor cells (labeled with anti-pan-cytokeratins) and stromal myofibroblasts (labeled with anti-SM-MHCs). (*C*) Immunoreactivity of stromal myofibroblasts to anti-h-caldesmon was never observed. The field includes many myofibroblasts, which were readily labeled by anti-SM-MHCs (data not shown). (*D, E; G, H; J, K;* and *M, N*) Double labeling of serial sections with antibodies to SM markers, showing a dense stroma in which every myofibroblast is positive for the four markers. (*F, I* and *L, O*) Double labeling of sections with anti-SM α-actin and anti-SM-MHCs (*F, I*), and anti-calponin and anti-SM-MHCs (*L, O*), showing regions of heterogeneity in SM-marker expression. Note two small vessels positive for SM-MHCs in *I*. (×150 except for *L* and *O*, which are ×240.)

ined. Conversely, myofibroblasts positive for SM markers were negative for these epithelial markers (Table 1). Myofibroblasts were often seen to be aligned in the same direction, in close apposition to nests of infiltrating tumor cells (Fig. 2 *A*, *B*, and *F*). Occasionally we observed regions with a high density of myofibroblasts and few tumor cells, in which the former were not arranged in an ordered array (e.g., Fig. 2*G*).

Double labeling was performed with different combinations of antibodies to SM markers in order to determine whether myofibroblasts could express several of these markers simultaneously. Stromal myofibroblasts were positive for SM α-actin (Fig. 2*D*), SM-MHCs (Fig. 2*K*), calponin (Fig. 2*H*), and α_1-integrin (Fig. 2*E*) but not for h-caldesmon (Fig. 2*C*). Moreover, in areas of dense stromal reaction such as that shown in Fig. 2*D*, all of the SM-α-actin-positive cells were also positive for SM-MHCs, calponin, and α_1-integrin, as can be seen in the four serial sections double-labeled with antibodies to SM α-actin and to each of the three other markers (Fig. 2 *D*, *E*; *G*, *H*; and *J*, *K*) and with antibodies to SM-MHCs and calponin (Fig. 2 *M* and *N*). In addition to such groups of cells which simultaneously express the four SM markers, we also often observed zones of strong immunoreactivity to SM α-actin which were entirely or partly devoid of reactivity to SM-MHCs. In general, stromal regions positive for SM α-actin and negative for SM-MHCs were positive for calponin and α_1-integrin. Only rarely did we observe myofibroblasts positive for SM α-actin and negative for all other SM markers. Two examples of heterogeneous labeling (SM-α-actin-positive/SM-MHC-negative and calponin-positive/SM-MHC-negative) are given in Fig. 2 *F*, *I* and *L*, *O*.

Our findings on the expression of SM and epithelial markers in the parenchyma and the stroma of normal and malignant breast tissue are summarized in Table 1.

DISCUSSION

We have taken advantage of the recent development of several new antibodies to differentiation markers of mature SM or epithelial cells in order to characterize the differentiation state of glandular and stromal components of human breast tissue under normal and pathological conditions. More specifically, we have addressed two questions: (*i*) What is the nature of myoepithelial cells? (*ii*) What is the range of SM differentiation features exhibited by stromal myofibroblasts in breast carcinoma tissue?

Morphologically, myoepithelial cells resemble SM cells, as they are highly contractile cells that contain dense bodies, endocytotic vesicles, and large amounts of contractile elements (21). Moreover, myoepithelial cells have been shown to express SM α-actin (6) and SM-MHCs (9), indicating that morphological features of SM differentiation may have a biochemical counterpart in these cells. The major criteria indicative of the epithelial nature of myoepithelial cells are (*i*) that they contain intermediate-size filaments belonging to the cytokeratin family and (*ii*) the presence of typical desmosomes between adjacent myoepithelial cells, as well as between myoepithelial cells and luminal milk-secreting epithelial cells (21). However, some special types of SM cells also are able to synthesize cytokeratins 8 and 18 under certain conditions (22–24).

Our results demonstrate that myoepithelial cells have a complex phenotype, combining epithelial and SM features. We have found that myoepithelial cells express two epithelial markers, E-cadherin and desmoplakin, in addition to myoepithelial cytokeratin 17, indicating that myoepithelial cells exhibit a true epithelial phenotype. Furthermore, the extensive SM-like character of myoepithelium was demonstrated by the expression of the differentiation-related proteins calponin, h-caldesmon, and α_1-integrin together with SM α-actin and MHCs. All of these proteins were found to be expressed in myoepithelial cells of galactophorous sinuses, ducts, and lobules; only h-caldesmon was not detected in myoepithelium of ducts and lobules.

Interestingly, the SM phenotypic expression in mammary gland myoepithelial cells seems to parallel that of vascular SM cells from adult human aorta. Similar to myoepithelium of ducts and lobules, subendothelial intimal cells in the aorta have a very low level of h-caldesmon expression and, accordingly, are thought to represent a less mature or partially dedifferentiated SM phenotype (25). Myoepithelial cells of sinuses seem to be very similar to mature medial SM cells of large vessels, which express significant amounts of h-caldesmon. It is important to mention that in contrast to medial SM cells, two populations of myoepithelial cells were observed in galactophorous sinuses, one expressing h-caldesmon and the other not expressing it. Myoepithelial cells of ducts and lobules possibly originate from h-caldesmon-negative cells; alternatively, ductal and lobular myoepithelium could be partially dedifferentiated derivatives of myoepithelial cells from sinuses.

Our observations lead us to conclude that myoepithelial cells of the human mammary gland express several SM- and epithelial-related differentiation markers, strongly suggesting a dual nature of the myoepithelial phenotype. In contrast to muscle cells, myoepithelial cells of the mammary gland are derived from ectoderm (21) and are permanently separated from the surrounding stroma by a basal lamina. Therefore, the simultaneous existence of SM (mesenchymal) and epithelial markers in these cells might reflect the occurrence of an epithelial–mesenchymal transition during mammary gland development.

The availability of new probes specific for SM differentiation also provided an opportunity to examine the phenotypic properties of mesenchymal (stromal) cells in malignant breast tissue. Myofibroblasts are the major population of mesenchyme-derived cells showing morphological features of SM cells in this tissue (5, 26). Until now the only reliable marker for SM differentiation in myofibroblasts was SM α-actin (27, 28). Two other SM proteins, SM myosin and desmin, have also been reported to occur in certain subpopulations of fibroblasts (5, 9).

We have found that three SM proteins, SM α-actin, calponin, and α_1-integrin, were present in all myofibroblasts. In addition, SM-MHCs were detected in a majority of cases, but often some cells were negative for this marker. In contrast, h-caldesmon has not been detected in myofibroblasts. This indicates that only a partial SM phenotype is activated in myofibroblasts, involving the coordinated expression of several SM-specific proteins. However, this phenotype may be modulated, as we have found some heterogeneity in the myofibroblast population.

Previously, four main types of myofibroblasts have been described based on the analysis of SM-α-actin, vimentin, and desmin expression (5). Our data suggest a possible functional heterogeneity of myofibroblasts, because some of the proteins which we used as markers (h-caldesmon, calponin, and myosin) are directly involved in the regulation of SM contractility. Since all myofibroblasts were h-caldesmon-negative and calponin-positive but some calponin-positive cells were also SM-MHC-negative, these two subsets of myofibroblasts display different patterns of contractile regulatory proteins, as previously described in human subendothelial SM cells (19, 25).

The histological origin of myofibroblasts is uncertain. Most studies suggest that they originate from mesenchymal derivatives such as fibroblasts or SM cells. Because they are associated with the stromal reaction in neoplastic tissue, it is also possible that they originate from the carcinoma cells themselves through an epithelial–mesenchymal transition mechanism. However, this is not likely to be the case, since

we have not observed the expression of epithelial markers in myofibroblasts.

In conclusion, our study has extended the phenotypic characterization of myoepithelial cells and of myofibroblasts and, it is hoped, will provide new insights regarding their origin.

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