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Laminin-5 Is a Marker of Invading Cancer Cells in Some Human Carcinomas and Is Coexpressed with the Receptor for Urokinase Plasminogen Activator in Budding Cancer Cells in Colon Adenocarcinomas[1]

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ABSTRACT

Recombinant human γ2 chain of laminin-5 was expressed in *Escherichia coli,* and used to generate specific polyclonal antibodies which were used to study the distribution of the protein in human cancers. A total of 72 biopsies of human cancers were stained, including 23 cases of colon adenocarcinomas, 16 ductal breast carcinomas, 9 malignant melanomas, 14 squamous cell carcinomas of the skin and cervix, and 10 sarcomas. As a control for the specificity of the antibodies, we performed *in situ* hybridization on adjacent sections of a number of the cases, and in all of these cases the localization of the γ2 chain protein and mRNA was identical. We found γ2 chain immunoreactivity in cancer cells in all cases of colon adenocarcinomas and squamous cell carcinomas but not in any of the sarcomas, supporting the view that the laminin-5 protein is specific for cells of epithelial origin. Notably, in all of the cases of colon adenocarcinomas, the positive staining was invariably associated with budding cancer cells located at the tip of invading malignant epithelium, whereas the cancer cells deeper in the tumors were most often negative. The staining was cytoplasmic in all cases and only in one case did we see additional extracellular immunoreactivity, indicating that this laminin isoform in cancer tissue is not laid down in the extracellular matrix but probably exerts its function at the cell surface or in its immediate vicinity. Using *in situ* hybridization to analyze the coexpression of laminin-5 and components of the plasminogen activation system, we found that the histological distribution of laminin-5-positive budding cancer cells at the invasion front in colon adenocarcinomas was identical to that of the receptor for urokinase-type plasminogen activator. These findings suggest that laminin-5 is a marker of invading cancer cells in at least some human malignancies, and that it therefore might represent a valuable marker for the invasive potential of these cancers. The colocalization of laminin-5 and urokinase-type plasminogen activator receptor in a subset of cancer cells in colon cancer also suggests that a controlled up-regulation of a number of gene products is a characteristic of budding colon cancer cells, and that these gene products serve functions crucial for the invasive phenotype of these cancer cells.

INTRODUCTION

Laminins are a family of structurally related basement membrane proteins composed of a heterotrimer of polypeptide chains: one heavy α chain and two light chains designated β and γ (1, 2). At present nine genetically distinct laminin chains are known to exist (1–3),[3] but it is likely that the repertoire *in vivo* is larger. These chains can form a variety of trimeric isoforms, but relatively little is still known about their biological roles.

Laminin-5, also referred to as kalinin, nicein, and epiligrin, is composed of the α3, β3, and γ2 chains, and the genes encoding these three chains have all been cloned in recent years (4–6). Immunoultrahistological studies have localized laminin-5 to epithelial basement membranes. Thus, Rousselle *et al.* (7) showed laminin-5 to be a component of anchoring filaments, and Carter *et al.* (8) localized the protein to the basement membranes of human skin, lung, and intestine using a mAb. Laminin γ2 chain gene expression has also been shown to be a characteristic of some epithelial cells in the human and mouse embryo as determined by *in situ* hybridization (4). This laminin isoform may also be present in different variants, as there is evidence for alternative splicing of the primary transcripts for the γ2 and α3 chains (4, 6).[4]

It is evident that both the β3 and the γ2 chains, and thus presumably laminin-5, are essential for the adhesion of basal keratinocytes to the basal lamina, because defects in these genes have been associated with junctional forms of epidermolysis bullosa, a severe skin blistering disease in which the epidermis de-adheres from its supporting basement membrane (9–12).

In pathological conditions, laminin-5 gene expression and immunoreacticity has been found in migrating keratinocytes in healing skin wounds (6, 13). By *in situ* hybridization of human cancer tissues, we recently found that mRNA for the γ2 chain of laminin-5 was expressed only in cancer cells in a number of different carcinomas, whereas cancer cells of mesenchymally derived tumors were devoid of signal (13). In contrast, the laminin chains α1, β1, and γ1 constituting laminin-1 (also known as "classical" laminin) were almost exclusively expressed by stromal cells (13). Interestingly, the gene expression of the γ2 chain in colon adenocarcinomas appeared to be a characteristic of cancer cells showing a budding cell phenotype (13).

In the present study, we report the localization of laminin γ2 chain protein in human cancer biopsies and provide evidence that the β3 and γ2 chains are expressed concomitantly in the same cancer cells, indicating that functional heterotrimeric laminin-5 is secreted by these cells.

MATERIALS AND METHODS

Expression of Recombinant Laminin γ2 Chain. For the production of polyclonal antibody, a part of domain III of the γ2 chain was expressed as a GST[5] fusion protein. A DNA fragment encompassing nucleotides 1289–1818 (4) was generated by PCR and subcloned into the *Eco*RI site of the pGEX-1λT expression vector (Pharmacia, Uppsala, Sweden). The sequence of the PCR product was confirmed by DNA sequencing. The fusion protein was produced in JM101 cells (14) upon induction with isopropyl-β-D-thiogalactoside and purified on a glutathione-Sepharose 4B column as recommended by the manufacturer (Pharmacia).

Preparation and Characterization of Polyclonal Antibodies. Two rabbits were immunized s.c. four times using 100 μg of the γ2-GST fusion protein per immunization and rabbit. The first immunization was carried out in

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[2] To whom requests for reprints should be addressed.

[3] A. Iivanainen, K. Sainio, H. Sariola, and K. Tryggvason. Primary structure and expression of a novel human laminin α4 chain, manuscript submitted for publication.

[4] T. Airenne, H. Haakana, T. Kallunki, P. Kallunki, and K. Tryggvason. Structure of the human laminin γ2 chain gene (LAMC2): evidence for alternative splicing, manuscript submitted for publication.

[5] The abbreviations used are: GST, glutathione *S*-transferase; TBS-Triton, 10 mM Tris-HCL (pH 7.4) with 150 mM NaCl containing 0.1% Triton X-100; uPAR, urokinase-type plasminogen activator receptor.

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Freund's complete adjuvant, and the next three in Freund's incomplete adjuvant. The specificity of the antiserum was tested by Western blotting against full-length recombinant γ1 (15) and γ2 chains.[6] Briefly, 0.1 μg of the recombinant chains was electrophoresed on a 6% SDS-PAGE gel under reducing conditions, and the proteins were then transferred to Immobilon membranes (Millipore, Bedford, MA) and reacted with the antisera at a dilution of 1:200 and visualized with a horseradish peroxidase-conjugated second antibody. Protein A-Sepharose purification of IgG from the rabbit showing the highest immune response was performed according to the manufacturer's recommendations (Pharmacia).

Tissues. Seventy-two routinely processed, formalin-fixed, and paraffin wax-embedded specimens from cancer surgery performed from 1992 to 1994 were drawn from pathology department files at Herlev Hospital. The specimens were evaluated according to standard criteria, and included 23 cases of colon adenocarcinomas, 16 cases of ductal mammary carcinomas, 14 squamous cell carcinomas (6 skin, 7 cervix, and 1 vulva), 9 malignant melanomas, and 10 sarcomas (4 leiomyosarcomas, 3 malignant fibrous histiocytomas, 1 neurofibrosarcoma, 1 myxoid liposarcoma, and 1 pleomorphic sarcoma, Table 1).

Immunohistochemistry. For immunohistochemistry, 5-μm paraffin sections were subjected to a horseradish peroxidase-avidin-biotin complex method. In brief, slides were deparaffinized in xylene, then heated to 90°C in a microwave oven in 10 mM citrate buffer (pH 6.0) for 10 min, and were then allowed to cool in the buffer at room temperature for 20 min. After a brief rinse in TBS-Triton, the sections were digested with 0.03% trypsin (catalogue no. L2133; Biochrom KG, Berlin, Germany) for 10 min at 37°C, blocked with 5% newborn rabbit serum in TBS-BSA (TBS with 0.25% BSA), and then incubated for 1 h at 37°C with the polyclonal antibodies diluted in TBS-BSA to 2–5 μg/ml. After that, a biotinylated swine antirabbit antibody was applied (code no. E431, 1:300; DAKO A/S), followed by horseradish peroxidase-avidin-biotin complex diluted as recommended by the manufacturer (code no. K355; DAKO A/S) before the slides were developed in diaminobenzamidine for 15 min. Slides were washed between each step in three shifts of TBS-Triton for a total of 15 min. All incubations were conducted for 30 min at room temperature, except where otherwise stated above. The slides were lightly counterstained with hematoxylin, dehydrated, and mounted. Negative controls consisted of a nonimmune rabbit IgG applied to adjacent sections at the same concentrations.

Preparation of ^{35}S-labeled RNA Probes. Plasmid constructs phb2t-03 and phb2t-05 for the γ2 chain of human laminin-5, and pHUR04 and pHUR06 for human urokinase-type plasminogen activator receptor were used as previously described for preparation of RNA probes (13, 16). RNA probes specific for the long and the short transcripts of the γ2 chain were generated from two transcription vectors containing bp 3493–4026 of sequence Z15008 (4) and bp 3457–3860 of sequence Z15009 (4). These were a kind gift from Dr. T. Airenne. The human β3 laminin-5 chain cDNA was made using reverse transcriptase-PCR as follows: a pool of cDNAs from human cultured cells was made on RNA purified from cells and primed with a poly(dT) primer with reverse transcriptase (17). This pool was used for PCR amplification of a β3 cDNA fragment with two 24-mer oligonucleotide primers designed to amplify the coding sequence 2897–3506 of laminin β3 chain, numbers referring to sequence L25541.EMPRI (5). The PCR product was cut out of an agarose gel and blunt-end ligated into the *Eco*RV site of pBluescriptIIKS(+) (Stratagene, La Jolla, CA) using standard DNA methodology. The sequence of the plasmid insert was confirmed by dideoxy sequencing of one strand. Sense and antisense RNA transcripts were made by *in vitro* transcription using T3 and T7 polymerase and ^{35}S-labeled UTP (13, 16).

***In Situ* Hybridization.** *In situ* hybridization was performed with ^{35}S-labeled RNA probes as described previously (13, 16). Negative control experiments consisted of sense RNA probes at the same specific radioactivity applied to adjacent sections of all samples. Positive controls for the γ2 chain of laminin-5 and for uPAR were performed on five of the colon cancer samples, and consisted of the application to adjacent sections of two antisense RNA probes derived from two nonoverlapping cDNA clones (13, 16).

[6] S. Salo *et al.*, unpublished data.

RESULTS

Characterization of Recombinant Laminin γ2 Chain and Polyclonal Antibody

The recombinant γ2-GST fusion protein purified on the glutathione-Sepharose 4B column had the expected M_r 47,000 (data not shown). This purified protein was used to immunize rabbits. Following three immunizations, the antiserum was tested for immunoreactivity against purified human laminin γ chains using Western blotting. Purified full-length recombinant human γ1 and γ2 chains were electrophoresed by SDS-PAGE, and staining of the electrophoresed proteins with Coomassie brilliant blue confirmed an expected size of 210,000 for the γ1 chain and 140,000 for the γ2 chain (Fig. 1*A*). These protein preparations were transferred to nylon membranes and immunoreacted with the antiserum generated against the recombinant γ2-GST fusion protein. As can be seen in Fig. 1*B*, the antiserum reacted with the full-length γ2 chain, while no reactivity was shown with the closely related γ1 chain.

Immunostaining for the γ2 Chain of Laminin-5

Table 1 summarizes our findings of the γ2 chain immunoreactivity in 72 paraffin-embedded biopsies of human cancers. The relative number of positive cells was scored on a visual basis for each case (Table 1). As a negative control we applied a nonimmune rabbit IgG at the same concentration, which did not give any staining. In addition to the Western blotting experiment described above, the specificity of the antilaminin γ2 chain antibody was ensured by the exact colocalization of γ2 chain protein and γ2 chain mRNA, when immunohistochemistry and *in situ* hybridization for the γ2 chain was performed on adjacent sections (see below).

Colon Adenocarcinomas. We stained a total of 23 specimens of colon adenocarcinomas with the polyclonal antibody to the γ2 chain of laminin-5. All specimens were positive, and the staining was almost exclusively confined to the cancer cells. The staining intensity was moderate to strong, and the reactivity was most prominent at the invasion front of tumors (Fig. 2*A*). In one specimen, we observed extracellular staining in a basement membrane-like pattern surrounding positive cancer cells, but apart from this

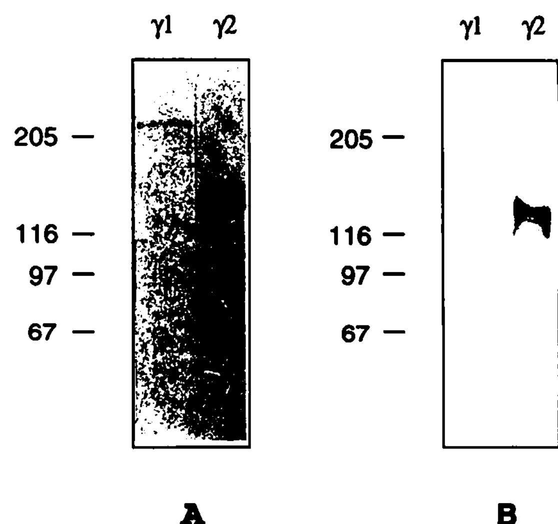

Fig. 1. Characterization of the polyclonal antilaminin γ2 chain antibody. Purified full-length recombinant laminin γ1 chain and partially purified full-length recombinant laminin γ2 chain were run on a 6% SDS-PAGE gel under reduced conditions and stained with Coomassie brilliant blue (*A*), or electroblotted onto nylon membrane and immunostained with a polyclonal antibody raised against the recombinant human γ2 chain (*B*). The antiserum only recognizes the γ2 chain.

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Table 1 *Immunohistochemical staining for the γ2 chain of laminin-5*

Case	Sex/age	Cancer type	Histology[a]	Laminin γ2 chain[g]
1[b,c]	F/80	Colon adenocarcinoma	MD, Duke's, B	+++
2	M/62	Colon adenocarcinoma	MD, Duke's, B	+
3	F/82	Colon adenocarcinoma	LD, Duke's, C	+
4[b,c]	F/77	Colon adenocarcinoma	MD, Duke's, B	+
5[b,c]	M/68	Colon adenocarcinoma	MD, Duke's, B	++
6	F/61	Colon adenocarcinoma	MD, Duke's, C	+
7[b,c]	M/66	Colon adenocarcinoma	MD, Duke's, A	++
8	F/82	Colon adenocarcinoma	LD, Duke's, B	+
9	M/52	Colon adenocarcinoma	MD, Duke's, B	+
10[b,c]	F/65	Colon adenocarcinoma	MD, Duke's, C	++
11[b,c]	M/84	Colon adenocarcinoma	LD, Duke's, B	+
12	F/70	Colon adenocarcinoma	MD, Duke's, B	+
13[d]	F/75	Colon adenocarcinoma	MD, Duke's, A	+
14[d,e]	M/58	Colon adenocarcinoma	MD, Duke's, C	+
15	M/74	Colon adenocarcinoma	MD, Duke's, B	+
16[e]	M/69	Colon adenocarcinoma	HD, Duke's, A	+
17[d,e]	M/73	Colon adenocarcinoma	MD, Duke's, B	+++
18[d,e]	M/78	Colon adenocarcinoma	MD, Duke's, B	++
19	M/75	Colon adenocarcinoma	LD, Duke's, C	+++
20[d]	M/39	Colon adenocarcinoma	LD, Duke's, B	+
21[e]	M/82	Colon adenocarcinoma	HD, Duke's, B	+
22	F/75	Colon adenocarcinoma	LD, Duke's, B	+
23	F/65	Colon adenocarcinoma	LD, Duke's, B	+
24	F/49	Malignant melanoma, SSM	Level 5 (6.9 mm)	–
25	F/49	Malignant melanoma, SSM	Level 2, (.34 mm)	++[f]
26[b]	M/73	Malignant melanoma, SSM	Level 4, (4.4 mm)	++[f]
27[b]	M/39	Malignant melanoma, SSM	Level 4, (3.5 mm)	++[f]
28[b]	F/47	Malignant melanoma, SSM	Level 4, (1 mm)	+++[f]
29	M/75	Malignant melanoma, SSM	Level 2, (.75 mm)	+++[f]
30[b]	M/71	Malignant melanoma, SSM	Level 4, (12 mm)	–
31	F/45	Malignant melanoma, SSM	Level 4, (1.3 mm)	+++[f]
32	M/39	Malignant melanoma, SSM	Level 4, (1.1 mm)	++
33	F/55	Mammary ductal carcinoma	Grade II	–
34	F/42	Mammary ductal carcinoma	Grade II	–
35	F/65	Mammary ductal carcinoma	Grade II	+
36	F/76	Mammary ductal carcinoma	Grade III	+++
37	F/61	Mammary ductal carcinoma	Grade III	–
38	F/61	Mammary ductal carcinoma	Grade III	++
39	F/62	Mammary ductal carcinoma	Grade I	+++
40[b]	F/69	Mammary ductal carcinoma	Grade III	++
41[b]	F/50	Mammary ductal carcinoma	Grade III	++
42	F/60	Mammary ductal carcinoma	Grade III	+
43	F/44	Mammary ductal carcinoma	Grade III	–
44[b]	F/21	Mammary ductal carcinoma	Grade II	+
45[b]	F/78	Mammary ductal carcinoma	Grade II	–
46[b]	F/75	Mammary ductal carcinoma	Grade II	+++
47[b]	F/80	Mammary ductal carcinoma	Grade III	++[f]
48[b]	F/63	Mammary ductal carcinoma	Grade III	+
49	F/31	Squamous cell carcinoma, cervix	MD	++
50[b]	F/46	Squamous cell carcinoma, cervix	MD	++[f]
51	F/63	Squamous cell carcinoma, cervix	MD	+
52	F/28	Squamous cell carcinoma, cervix	MD	++
53[b]	F/40	Squamous cell carcinoma, cervix	MD	+++
54[b]	F/39	Squamous cell carcinoma, cervix	MD	+++
55	F/37	Squamous cell carcinoma, cervix	MD	++
56[b]	F/77	Squamous cell carcinoma, vulva	MD	+++
57	M/73	Squamous cell carcinoma, skin	HD	+++
58[b]	F/47	Squamous cell carcinoma, skin	HD	++
59	M/79	Squamous cell carcinoma, skin	MD	+++
60[b]	M/70	Squamous cell carcinoma, skin	HD	+++
61	M/79	Squamous cell carcinoma, skin	HD	+++
62[b]	F/88	Squamous cell carcinoma, skin	HD	+++
63	F/69	Leiomyosarcoma, skin	Grade II	–
64[b]	M/82	Leiomyosarcoma, ileum	Grade I	–
65[b]	F/25	Leiomyosarcoma, duodenum	Grade II	–
66[b]	F/84	Leiomyosarcoma, skin	Grade III	++[f]
67[b]	M/46	Neurofibrosarcoma, retroperitoneum	Grade I	–
68[b]	F/42	Myxoid Liposarcoma, cervix	Grade I	–
69[b]	F/75	Pleomorphic Sarcoma, cervix	Grade I	–
70[b]	M/56	Malignant fibrous histiocytoma	Grade III	–
71	M/97	Malignant fibrous histiocytoma, femur	Grade III	–
72	M/78	Malignant fibrous histiocytoma, skin	Grade III	–

[a] MD, moderately differentiated; SSM, superficial spreading type melanoma; LD and HD, low and highly differentiated.
[b] Immunohistochemistry and *in situ* hybridization for the laminin γ2 chain performed on adjacent sections.
[c] Laminin γ2 chain and uPAR *in situ* hybridization performed on adjacent sections.
[d] Laminin β3 chain *in situ* hybridization was performed.
[e] *In situ* hybridization was performed for both the long and the short laminin γ2 chain mRNA variants.
[f] Only nonmalignant epidermal cells involved in a wound healing response were positive for the γ2 chain.
[g] +, ++, and +++, relative number of laminin γ2 chain immunopositive cells; –, no immunoreactivity detected.

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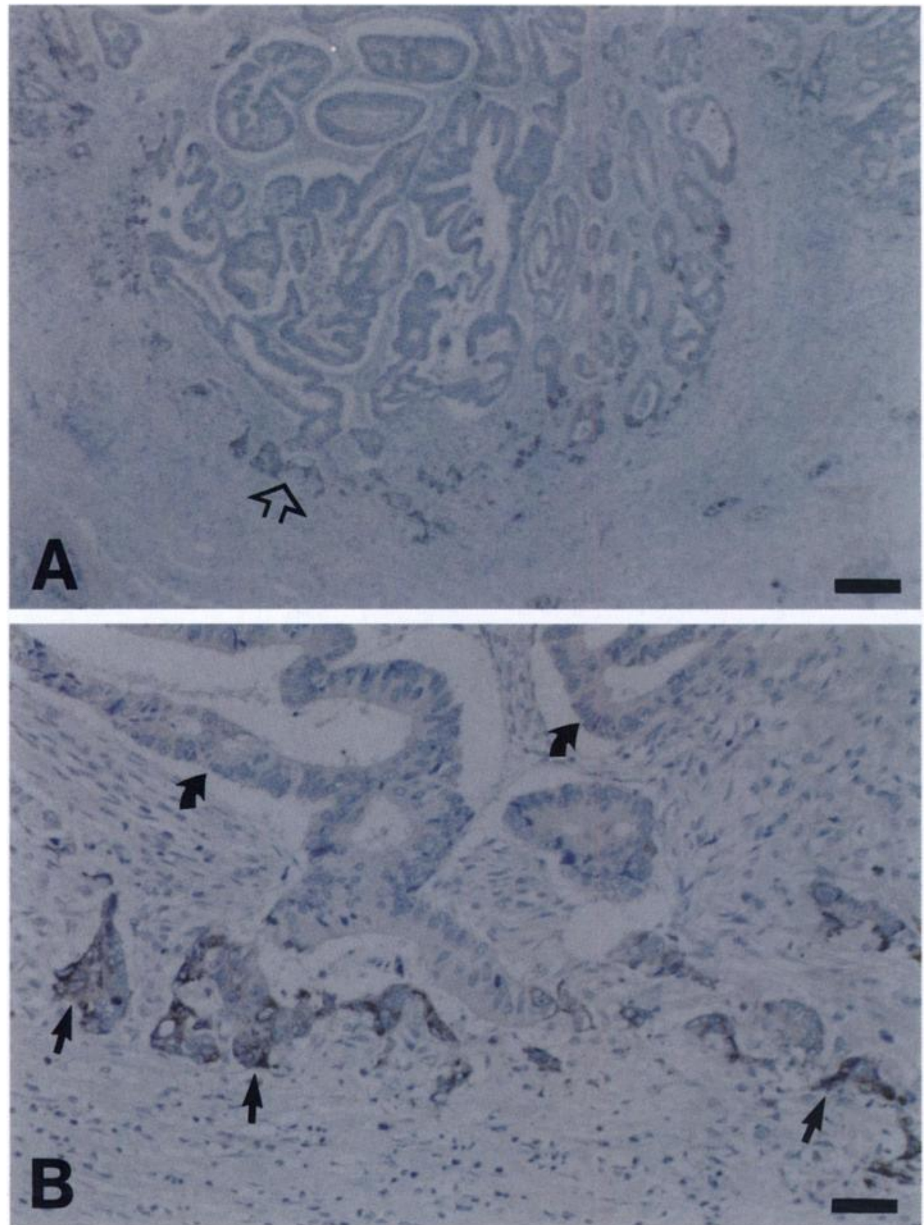

Fig. 2. Immunohistochemistry for the γ2 chain of laminin-5 in a case of human colon adenocarcinoma. *A*, immunohistochemical staining of cancer cells located along the tumor/stroma interface. *Area marked by the open arrow*, shown at higher magnification in *B*. Here, strong cytoplasmic staining for laminin-5 is evident in a number of cancer cells (*straight arrows*) apparently budding from adjacent malignant epithelium showing a higher differentiation and negative for laminin-5 (*curved arrows*). *Bars*, 170 μm (*A*) and 25 μm (*B*).

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specimen the staining was always cytoplasmic (Fig. 2*B*). On a morphological basis, the positive cells often constituted a characteristic subpopulation of cancer cells that appeared to be engaged in budding activities (Fig. 2*B*), as was previously described for γ2 chain mRNA in this cancer type (13). In adjacent normal-appearing colon mucosa, γ2 chain staining was seen in the epithelial cells in a few crypts that showed signs of degeneration and leukocyte infiltration in five of the specimens, and occasionally weak reactivity was seen in luminal epithelial cells (data not shown).

Ductal Mammary Carcinomas. Of 16 cases of mammary cancer, 11 were positive for the γ2 chain when examined immunohistochemically. Of these, 10 contained positive cells immediately surrounding nests of cancer cells. This staining was weak to moderate, and was present only around a very small proportion of the malignant cells. Based on morphological examination it was evident that most, if not all, of these cells were myoepithelial cells (data not shown). The cancer cells themselves were negative in all cases. One case showed only reactivity in keratinocytes in a part of the epidermis located above the malignant lesion and apparently engaged in a wound healing response (data not shown).

Malignant Melanomas. Nine specimens of malignant melanomas were stained for the γ2 chain. Six of them showed moderate to strong staining in apparently nonmalignant keratinocytes. These cells were in all cases judged to be engaged in wound healing responses secondary to the growth of the tumor. It was difficult to determine definitely whether melanocytes were also positive for the γ2 chain protein due to their heavy content of melanin, but when *in situ* hybridization for the γ2 chain was performed on adjacent sections, these cells were found not to contain γ2 chain mRNA (data not shown).

Squamous Cell Carcinomas. Fourteen cases of squamous cell carcinomas originating from the cervix (seven specimens), vulva (one specimen), and skin (six specimens) were stained for the γ2 chain and showed immunopositivity in all cases. In all cases, staining was seen only in cancer cells, except one case of cervix cancer where nonmalignant epidermal cells overlying the lesion and apparently participating in a wound healing process were also positive. In most of the cases, cancer cells located immediately adjacent to the surrounding stromal tissue showed the most intense labeling (Figs. 3 and 4), and

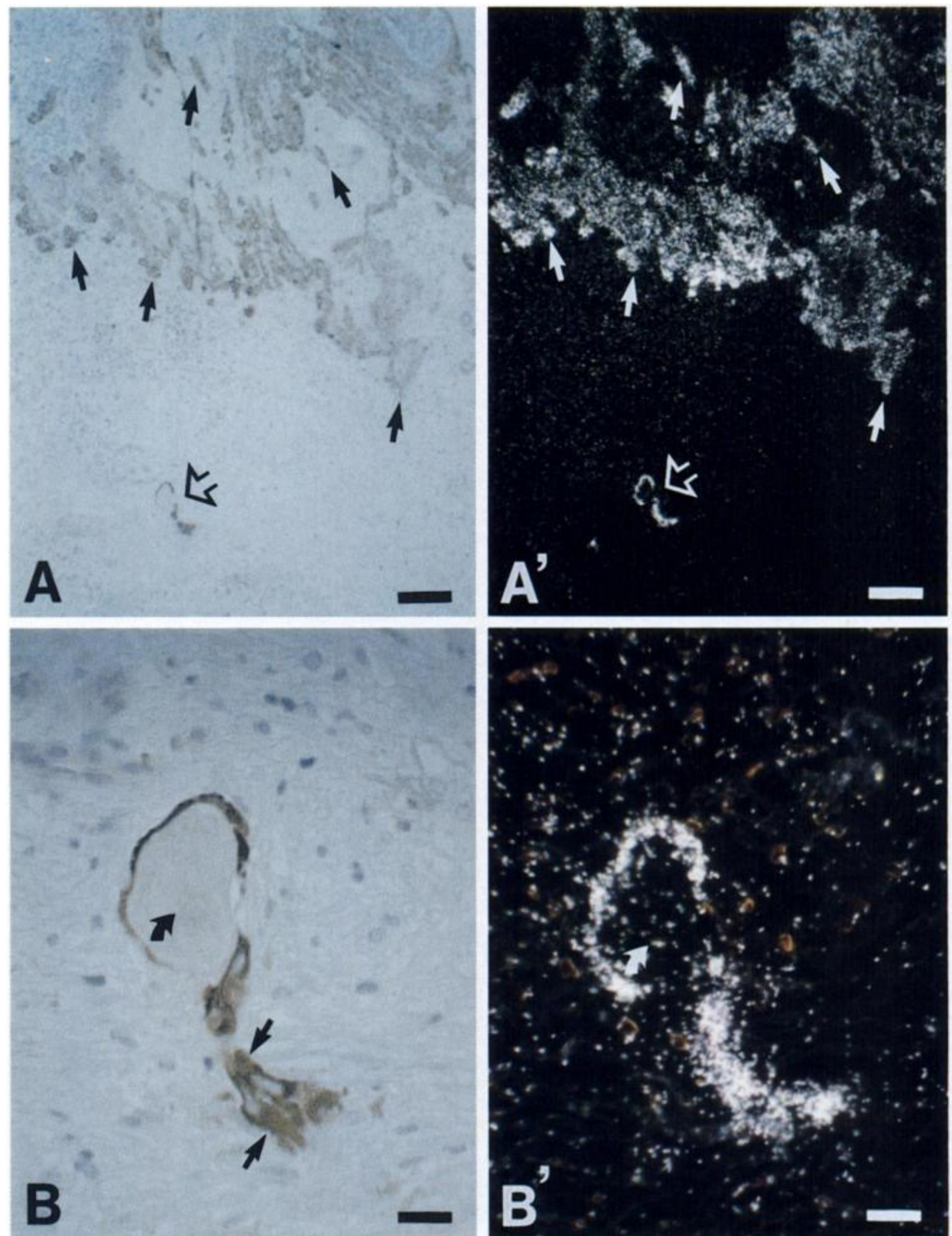

Fig. 3. Immunohistochemistry (*A* and *B*) and *in situ* hybridization (*A'* and *B'*) for the γ2 chain of laminin-5 on adjacent sections of a sample of squamous cell carcinoma of the skin. *A'* and *B'* are dark-field images to highlight the hybridization signal. In *A* and *A'*, numerous cancer cells are strongly positive for the γ2 chain protein and mRNA, respectively. Note that the signals obtained with the two methods are identical (compare positive cells marked by *straight arrows*). *B* and *B'* are higher magnifications of the *area marked by open arrows* in *A* and *A'*. A small nest of deeply invading cancer cells show strong γ2 chain protein (*B*) and mRNA (*B'*) labeling. Note that the staining is cytoplasmic (*straight arrows*). A keratin pearl (*curved arrow*) is associated with the malignant cells. *Bars*, 170 μm (*A* and *A'*) and 17 μm (*B* and *B'*).

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often small nests of isolated, deeply invading γ2 chain-positive cancer cells were observed (Fig. 3).

Sarcomas. We stained 10 sarcomas (Table 1). Of these, none showed immunostaining, except for one case, in which keratinocytes of skin overlying the lesion were positive (Table 1).

Colocalization of the Laminin γ2 Chain Protein and mRNA

To ensure that the antibody used in the present study indeed labeled genuine γ2 chain protein, we performed concomitantly immunohistochemistry and *in situ* hybridization on adjacent sections of 31 of the samples used (Table 1). In all of the positive cases, we found an identical staining pattern for γ2 chain mRNA and protein (Figs. 3 and 4), and all of the cases that were devoid of γ2 chain immunostaining showed no signal for γ2 chain mRNA. In these experiments and in all subsequent *in situ* hybridizations, a sense RNA probe from the same plasmid was applied to adjacent sections. In none of these cases did we find any signal.

In Situ Hybridization for the β3 Chain of Laminin-5

Using reverse transcriptase-PCR (see "Materials and Methods"), we prepared a probe for the β3 chain believed to constitute intact laminin-5 along with the γ2 and α3 chains. When applied to adjacent sections of five of the cases of colon adenocarcinoma (Table 1), we observed a complete colocalization of signals for both chains, indicating that the positive cancer cells possess the potential to produce functional laminin-5.

In Situ Hybridization for an Alternatively Spliced Form of the Laminin γ2 Chain

Two different cDNA clones for the laminin γ2 chain have been isolated, probably arising by alternative splicing and predicted to give rise to two γ2 chain proteins differing in their carboxyl-terminal ends (4). To determine whether one or both of these forms were present in tumors *in vivo*, we prepared RNA antisense probes specific for each

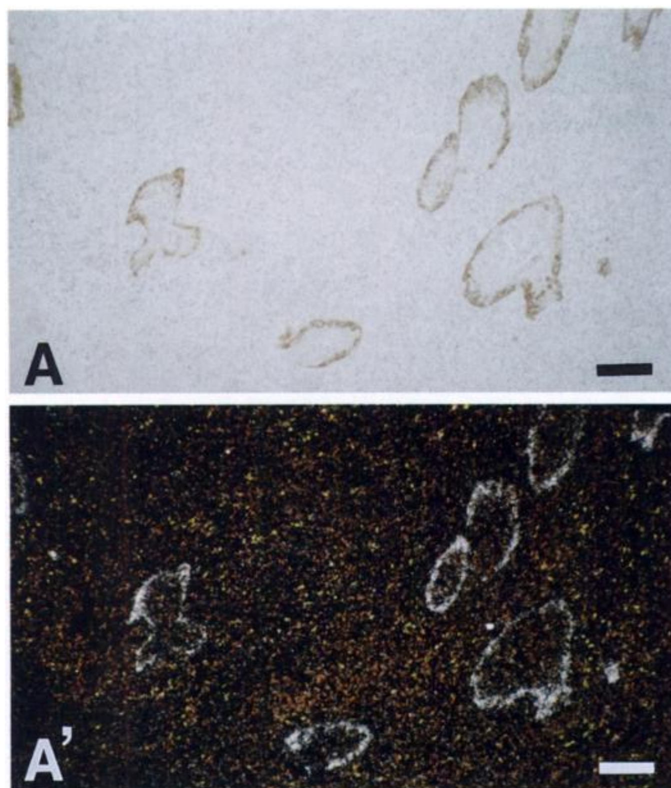

Fig. 4. Immunohistochemistry (*A*) and *in situ* hybridization (*A'*) for the γ2 chain of laminin-5 on adjacent sections of a sample of squamous cell carcinoma of the cervix. *A'* is a dark-field image to highlight the hybridization signal. The distribution of the γ2 chain protein (*A*) and mRNA (*A'*) is identical and restricted to cancer cells bordering the surrounding stroma. *Bars*, 170 μm.

form. When applied to sections of five of the samples (Table 1), we found signal only for the longer transcript, corresponding to the sequence z15008 reported by Kallunki *et al.* (4).

Laminin-5 and Urokinase Receptor Are Colocalized in Budding Colon Cancer Cells

The distribution of laminin-5 protein and mRNA in colon adenocarcinomas appeared to be very similar to that previously reported for uPAR mRNA in regard to its localization in the malignant cells (16). To clarify this matter further, we performed *in situ* hybridization for both the γ2 chain of laminin-5 and uPAR on adjacent sections of six of the samples used (Table 1). This allowed us to establish that the cancer cell population that contains γ2 chain mRNA and protein also contains uPAR mRNA (Fig. 5). It should be noted that uPAR mRNA in addition to this population of cancer cells at the invasion front also could be seen in a population of tissue macrophages in the same areas as was also reported earlier (16), whereas γ2 chain mRNA and protein were never found in this cell type. As positive controls we applied, for both γ2 chain and uPAR, antisense RNA probes from two nonoverlapping cDNA clones to adjacent sections yielding identical hybridization signals.

DISCUSSION

Using a polyclonal antibody against recombinant γ2 chain of laminin-5, we have determined the localization of this protein in 72 biopsy specimens of human cancers, of which 62 specimens derived from carcinomas and 10 specimens from sarcomas. In colon adenocarcinomas and in all cancers showing squamous cell differentiation, laminin γ2 chain immunoreactivity was located exclusively in a subpopulation of cancer cells, whereas the sarcomas were negative. To our knowledge this is the first report of laminin-5 protein localization in human malignancies. The findings agree with and extend our previous study of laminin-5 mRNA localization in the same cancer types by *in situ* hybridization (13). In that study, extended use of controls assured the specificity of the hybridization signal, and we now report that the localization of γ2 immunostaining exactly matches that of γ2 mRNA.

In contrast to a previous report on laminin-5 immunoreactivity in basement membranes of normal human skin and intestine (8), we observed no laminin γ2 chain immunoreactivity in normal skin and colon tissue adjacent to carcinomatous areas. This might be due to a lack of reactivity of the polyclonal antibody against an extracellular matrix-linked form of laminin-5. Speaking against this possibility, however, we found extracellular staining in a basement membrane-like pattern in one case of colon cancer. As an alternative explanation of this apparent discrepancy, it is possible that the sensitivity of immunodetection in our study might be too low to detect laminin-5 associated with the basal lamina of normal skin and intestinal epithelium. Supportive of this explanation, we found no signal for γ2 chain mRNA in normal skin and colon tissue, except for a very weak signal in luminal colonic epithelium in some cases, nor did we see signal for the laminin β3 chain by *in situ* hybridization (13; the present study). Additional immunohistochemical studies using a panel of mono- and polyclonal antibodies against the three chains of laminin-5 are warranted to settle this issue.

A consistent finding in our study was the prominent γ2 chain immunoreactivity in cancer cells at the invasion front of all cases of colon adenocarcinomas. In these cancers, the strongest signal was found in the cancer cells that appeared to be budding from more well-differentiated malignant epithelium, a cell phenotype that has been correlated to a more aggressive behavior (18). The high expression of laminin-5 in only a minor population of cancer cells and the preferential localization of the positive cells at the invasion front in colon adenocarcinomas make it attractive to investigate further the use of this molecule as a marker for disease progression and malignancy. The finding in the present study that the γ2 chain protein is present almost exclusively in the cytoplasmic compartment, *i.e.*, probably in an extractable form, and the availability of a specific polyclonal antibody to the γ2 chain make possible future studies using immunochemical methods of the extract levels of this interesting molecule. It is noteworthy that such extract measurements of uPAR antigen levels have recently shown this molecule to be of prognostic value in colon as well as in lung cancer tissue (19, 20).

In mammary ductal carcinomas and in malignant melanomas, we previously reported the presence of γ2 chain mRNA in a subset of cancer cells as determined by *in situ* hybridization (13). There are, however, some limitations to the identification of positive cells using *in situ* hybridization alone. First, the tissue morphology is often poor following the denaturing conditions of the procedure itself, and, second, the positive signal being silver grains in an emulsion overlying the tissue section, it is often difficult to obtain a resolution fine enough to allow a definite cell identification. Consequently, we could not in the former study determine whether the positive cells in the mammary cancers investigated might be of myoepithelial origin (13). Using immunohistochemistry, we were in the present study able to identify accurately the positive cells in mammary cancers as myoepithelial cells. Similarly, with the improved resolution of immunostaining compared to *in situ* hybridization, we have verified that the γ2 chain containing cells in malignant melanomas appear in fact to be nonmalignant keratinocytic cells engaged in wound healing responses. This phenomenon was seen in several of the samples from different cancer types, and agrees well with the previous findings by us and others of strong γ2 chain expression by the basal keratinocytes in skin wound healing (6, 13).

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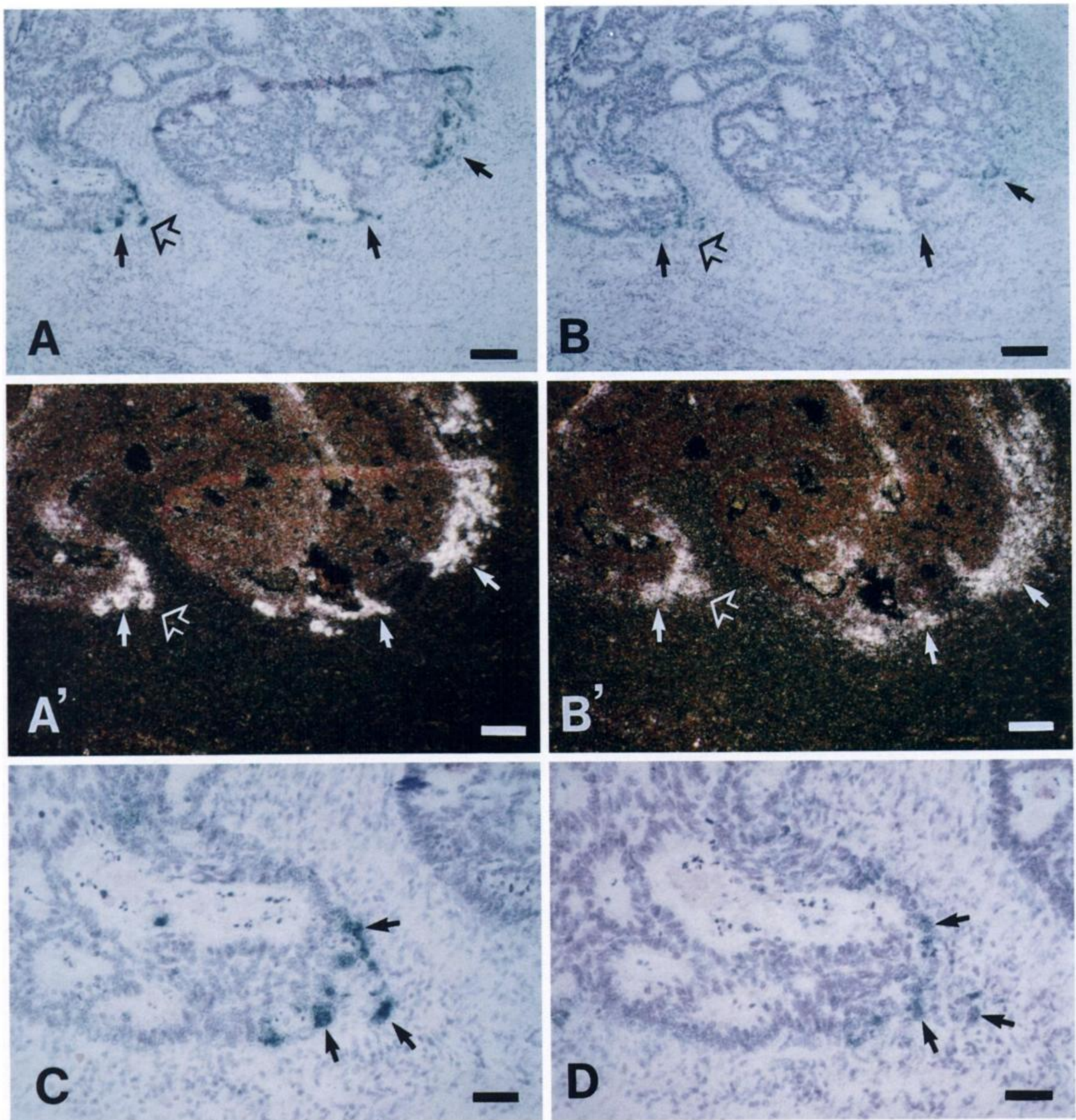

Fig. 5. *In situ* hybridization performed on adjacent sections of a sample of colon adenocarcinoma for the γ2 chain of laminin-5 (*A* and *C*) and uPAR (*B* and *D*). *A'* and *B'* are dark-field images of *A* and *B*, respectively, to highlight the hybridization signal. *C* and *D*, higher magnification of *area marked by open arrow* in *A* and *B*, respectively. Both γ2 chain mRNA (*A*) and uPAR mRNA (*B*) are located predominantly in cancer cells at the invasion front (*straight arrows*, *A* and *B*). At higher magnification individual cancer cells showing strong signal for the γ2 chain mRNA (three different cells marked by *arrows*, *C*) can be seen to express also uPAR mRNA (the same three cells marked by *arrows* in *D*). *Bars*, 170 μm (*A*, *A'*, *B* and *B'*) and 25 μm (*C* and *D*).

Using *in situ* hybridization for the uPAR, we have provided histological evidence for a coexpression of the γ2 chain of laminin-5 and uPAR in the budding cancer cells at the invasion front in colon cancer. We have previously proposed a function of uPAR in these cells in the invasion process via their binding of the ligand for uPAR, urokinase plasminogen activator secreted by the surrounding stromal cells (16, 21). Subsequent activation of plasminogen is proposed to lead to extracellular matrix degradation and to facilitate cancer cell invasion (16, 22). The fact that this interesting subpopulation of cancer cells express at the same time laminin-5 and uPAR has several important implications. First, the correlation between a budding cell morphology and the concomitant expression of laminin-5 and uPAR suggest that both proteins are needed for the acquisition of this potentially invasive cell phenotype. Second, in the budding cancer cells there must be a tight regulation of these two gene products, *i.e.*, the γ2 chain of laminin-5 and uPAR, since adjacent cancer cells with a nonbudding

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morpholgy do not show expression of any of the two proteins. This may reflect that the two genes belong to a set of genes that function in the same cell process, namely, cancer cell migration and invasion. This assumption is supported by an analogous coexpression of the γ2 chain of laminin-5 and uPAR in migrating keratinocytes in superficial skin wounds (13, 23). It is conceivable that mutational defects in yet unknown genes that control complex epithelial cell behavior like that seen in migrating keratinocytes during reepithelialization in wound healing could lead to an acquisition of a migrating and invading epithelial cell phenotype that is a prerequisite for malignancy.

The concept of control genes regulating complex epithelial cell behavior can be extended to account also for the prominent stromal cell activation so frequently found in human cancers. We and others have previously proposed that certain stromal cell populations lying close to the malignant cells are induced to assist in the invasion process by signals sent out by the cancer cells, stimulating in stromal cells the synthesis of gene products that facilitate cancer cell invasion and migration (for review, see Ref. 22). As an example, in colon adenocarcinomas, there is a high expression in stromal tumor-infiltrating fibroblasts of proteins involved in extracellular proteolysis, *e.g.*, urokinase plasminogen activator (16, 21, 24, 25) and several matrix metalloproteinases (26–28). It is possible that signal molecules secreted by the cancer cells are produced in response to epithelial cell control genes that in the normal organism function to coordinate processes like wound healing, organogenesis, and involution, where epithelial cells and intimately associated stromal cells cooperate to accomplish tissue remodeling. A recent report identifies such a control gene and describes its dramatic physiological impact in organogenesis (29). We would like to propose that in carcinomas, epithelial cell control genes have been activated, causing uncontrolled expansion of both cancer cells and their associated stromal cells. More histological evidence of analogous gene expression patterns in normal and malignant processes are needed to further substantiate this hypothesis.

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