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Snail and Slug play distinct Roles during Breast Carcinoma Progression

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Abstract

Purpose: Carcinoma progression is linked to a partially dedifferentiated epithelial cell phenotype. As previously suggested, this regulation could involve transcription factors Snail and Slug, known to promote epithelial-mesenchymal transitions during development. Here, we investigate the role of Snail and Slug in human breast cancer progression.

Experimental design: We analyzed Snail, Slug and E-cadherin RNA expression levels and protein localization in large numbers of transformed cell lines and breast carcinomas, examined correlation with tumor histological features and described at the cellular level Snail and Slug localization in carcinomas, using combined in situ hybridization and immunolocalization.

Results: In contrast with transformed cell lines, Slug was found to colocalize with E-cadherin at the cellular level in normal mammary epithelial cells and all tested carcinomas. Snail also colocalized at cellular level with E-cadherin in tumors expressing high levels of Snail RNA. In addition, Snail was significantly expressed in tumor stroma, varying with tumors. Slug and Snail genes were significantly overexpressed in tumors associated with lymph node metastasis. Finally, presence of semi-differentiated tubules within ductal carcinomas was linked to Slug expression levels similar or above normal breast.

Conclusions: These results suggest that Snail or Slug expression in carcinoma cells does not generally preclude significant E-cadherin expression. They emphasize a link between Snail, Slug and lymph node metastasis in a large sampling of mammary carcinomas and suggest a role for Slug in the maintenance of semi-differentiated structures. Snail and Slug proteins appear to support distinct tumor invasion modes and could provide new therapeutic targets.

Introduction

Breast cancer is a major cause of female mortality in the Western world. Breast cancer is characterized by the considerable histological heterogeneity of tumors, generating a complex classification based on cell phenotype, proliferation and invasiveness criteria. Identification of distinct classes is crucial for therapy decisions. Typically, carcinoma cells show reorganization of cell-cell adhesion structures and cytokeratin mesh reorganization. This partial phenotype modulation evokes more complete epithelial-mesenchymal transitions (EMT) found during developmental stages involving full dissociation between individualized cells (1, 2). Phenotype modulation in breast carcinomas depends on tumor type. In infiltrating lobular carcinomas (ILC), cells express an individualized phenotype associated with the loss of E-cadherin (E-cad), the main epithelial cell-cell adhesion molecule. This downregulation is linked to distinct mechanisms including mutations, deletion events (3), and transcriptional control (4). In breast invasive ductal carcinomas (IDC), the dominant type of breast cancer, most carcinoma cells still express E-cad, actually used as a tumor type marker (5, 6). E-cad is mostly membrane-linked but may also show intracytoplasmic localization. This maintenance of some level of cell-cell adhesion is not inconsistent with cell migration and invasiveness observed in IDC. It is typically described during other migratory events throughout developmental stages and wound healing (2). Members of the Snail family of zinc-finger proteins have been found to be directly involved in developmental EMT, implicating cadherin regulation (reviewed in 7). Snail and Slug have also been described in several transformed cell lines to correlate negatively with E-cad expression levels. Both Snail and Slug genes have been found to repress E-cad promoter at the level of E2 boxes (8, 9). In addition, members of the Snail family have been found to be involved in apparently unrelated functions linked to cell survival (10, 11). In recent years, the roles of Snail and Slug in cancer progression have been postulated by several groups, based on distinct lines of evidence involving RNA studies, cell models and clinical correlations (12-14). Snail gene was found to be regulated in human breast cancer by an estrogen-dependent corepressor, MTA3 (15). Slug was also linked to clinical progression of lung carcinoma (16). Finally, three studies in distinct mouse models have recently shown a direct involvement of Snail (17, 18) and Slug (17) in tumor progression. However, the lack of reliable antibodies has hampered a precise cellular localization of Snail or Slug in human tumors, making it difficult to assume a precise role. Therefore, no studies have been published yet comparing Snail, Slug and E-cad cellular localization within human tumors. Recently, the description of a new rat monoclonal antibody targeting human Snail has finally allowed this approach (19).

Considering the converging data suggesting a role for Snail genes during breast carcinoma progression, we initiated an exhaustive screening of normal and transformed cells to detect links between Slug, Snail, E-cad and tumor progression status. The screening was extended to more than a hundred breast cancer clinical samples, examined by RT-PCR, in situ hybridization and immunolocalization, using the new monoclonal antibody directed against Snail. A new and intriguing expression pattern of Snail and Slug emerged from these studies and suggests distinct functions for the two transcription factors.

Materials and Methods

Tumor samples and clinical material

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The breast tissue samples included benign lesions (five fibroadenomas, two gynecomastias). Malignant tumors were composed of 56% invasive ductal and 44% invasive lobular carcinomas. Among these carcinomas, 45% had positive lymph nodes (N+) and 55% had negative lymph nodes (N-). The histologic grade was evaluated by a certified clinical pathologist (F. Bibeau) according to the Nottingham combined histologic grade: grade 1-11%, grade 2-53%, grade 3-36%. Steroid receptor status was determined using ligand-binding assay: 74% ER+ (>10 fmoles/mg protein), 26% ER-, 77% PR+ (>10 fmoles/mg protein) and 23% PR-. Histological types were determined according to the last WHO classification. Normal breast tissue was obtained, upon written consent, from 3 donors undergoing reductive mammoplasty and from two commercial sources (Invitrogen, Stratagene).

RNA extractions and Reverse Transcription

RNA extraction from tumor samples and cell lines was done using a Qiagen RNA extraction kit. Prior to extraction, tumor samples were pulverized with a pestle under liquid nitrogen. After extraction, RNA quality was checked by spectrometer analysis and gel migration. One μ g of total RNA was then reverse transcribed using hexanucleotides, in combination with Superscript II Reverse Transcriptase (Invitrogen).

Real Time-Quantitative PCR

RT-PCR was performed using an ABI SDS7000 (Applied Biosystems), in combination with SYBR Green I. Specific primers for each gene of interest were chosen using the software Primer-express (Applied Biosystems). Primer sequences are reported in supplementary data (Table 2S). All the amplifications were done using the SyberGreen master mix (Applied Biosystems) in a final volume of 25 μ l using standard PCR conditions (40 cycles with an annealing/elongation step at 60°C). Results are derived from the average of at least two independent experiments or RT-QPCR estimations. Gene expression was reported relative to housekeeping gene 36B4.

Statistical analysis

Statistical analysis was performed using Student's t test and Pearson correlation rank. Primary data generated by quantitative PCR were expressed as the difference in the number of cycles between the studied gene and a control housekeeping gene, 36B4, during the linear amplification phase. In some cases, values were related to a specific sample (normal breast or HMEC). These values were used for graphs, then normalized by logarithmic transformation when necessary in order to apply Student's T test and Pearson correlation test.

Cell Culture

BRCA MZ01, BRCA MZ02, VP 267, SUM 149-1, SUM 159-1, and SUM 185-1 cell lines were cultured as described previously (20). They were cultured in DMEM supplemented by 10% fetal calf serum. HMEC were graciously provided by Dr. Stampfer and cultured according to her instructions. Briefly, finite life span 184 and extended life span culture 184 A1 HMEC were cultured in serum-free MCDB 170 medium (MEGM, Clonetics Corporation, San Diego, CA) as described (21). Cells were routinely subcultured at split ratios of 1:8. All other cell lines were obtained from American Type Culture Collection (ATCC) and cultured according to ATCC recommendations.

Mouse

The Slug-LacZ mouse line was initially generated by in-frame insertion of the β -galactosidase

gene into the zinc finger coding region of the Slug gene (22) and graciously provided by Dr. Thomas Gridley (Jackson Laboratory). Animals were treated in accordance with institutional guidelines (INSERM).

Immunolocalization

Cells were grown on glass coverslips for 48h before processing. Cells were fixed with methanol at -20°C for 6'. For E-cad and Plakoglobin cells were fixed and permeabilized with formaldehyde (4%) + triton (0.05%). For immunohistochemistry, formalin-fixed material was embedded in paraffin and processed for sectioning. After rehydration (for paraffin sections) and washing in PBS, cells or tissue sections were incubated for 1 hour at room temperature with primary antibodies, as detailed on Table 3 (Supplementary data). For Snail antibody, antigen retrieval was performed using citrate buffer (90°C, 10') followed by slow cooling to room temperature. Goat secondary antibody was added to cells for a 30' incubation at room temperature. Alternatively, immunoreactivity was detected using the appropriate ABC kit (Vector). Diaminobenzidine was used as a chromagen, and slides were counterstained with hematoxylin before dehydration and mounting. Non-specific staining was blocked by adding 10% goat serum to both primary and secondary antibodies. Some tumor sections were stained with hematoxylin-eosin-safran (HES). Phase contrast microscopy and immunofluorescence of cell cultures was performed using a Leica DM IRB inverted microscope (Leica Microsystems) and images were acquired with a CoolSNAP HQ camera (Roper).

Histochemistry

To demonstrate β -galactosidase activity, Slug-lacZ heterozygous mice were sacrificed humanely by CO₂ inhalation. Tissue samples were embedded in OCT, frozen in liquid nitrogen, and sectioned at 10 μ m. Sections were air-dried for 30 min, then stained for 24 hr at 37°C in a humidified chamber using the Beta-Gal staining kit (PanVera). Slides were counterstained with 0.1% nuclear fast red in 5% (NH₄)₂ SO₄, dehydrated, and mounted.

In situ hybridization

Paraffin-embedded tissue was gathered and sectioned at 3–6 μ m onto coated slides (Fisher) and kept at 4°C until used. All slides were used within 3 months of sectioning. In situ hybridization was performed in accordance with a published protocol for paraffin sections (23). Probes for Slug were designed to avoid any risks of cross-detection of other members of the Snail family. They have been published previously (2). The signal was detected with BM Purple AP (Roche).

Results

Slug and Snail are negatively correlated with E-cad expression in breast cancer cell lines, but not in human mammary epithelial cells.

We used RT Q-PCR to screen thirty different human breast cancer cell lines in addition to finite lifespan primary human mammary epithelial cells (HMEC) 184, and an immortalized variant cell line called 184 A1 (24) for Slug, Snail, and E-cad mRNA expression levels (Fig 1). Globally, Slug, Snail and E-cad expression levels in HMEC cells were in the same range as normal breast (Fig 1, arrow). Overall, average Slug expression was found to be significantly higher than Snail expression (about ten times), as found by a paired t test ($p < 0,05$). The dispersion range of expression among distinct cell lines was very wide, spanning four levels of magnitude. As expected from published work (8, 9, 25), E-cad displayed a statistically significant negative

correlation ($p < 0,05$) overall with Slug and Snail when compared one to one. Pearson correlation index was $r = 0.50$ and $r = 0.36$, respectively.

Slug and Snail gene expression is strongly correlated with cell phenotype

In order to establish a putative link between cell phenotypes and Slug, Snail and E-cad expression, we screened all cell lines for cell-cell adhesion status, a reliable marker for epithelial phenotype. Studies included immunodetection and immunolocalization for desmoplakin, plakoglobin, cytokeratin and E-cad. E-cad expression levels were also confirmed semi-quantitatively by Western blotting. We defined functional markers for cell adhesion structures. Desmosomes were identified and located by the expression of desmoplakin at the membrane level and colocalization with anchoring cytokeratin filaments. Adherens junctions were identified by plakoglobin location at membrane level, in addition to, in most cases, E-cad. Based on these criteria, we classified 32 primary or transformed cell lines into four groups, categorized in Table 1 (Supplementary data) and shown in Fig. 1, panel B. A majority of cells (29 out of 32) expressed at least some cell-cell adhesion structures. We sorted the cell lines by increasing expression levels of E-cad mRNA (Fig. 1, panel C) to observe a putative link between Slug, Snail, E-cad expression levels and phenotype groups. As expected, group 1 included cells expressing the most E-cad. No group 1 cells expressed significantly less E-cad mRNA than normal breast. We then looked at Slug expression levels in normal and transformed cell lines. When looking specifically at the transformed cell lines, we also found Slug to be a very good indicator of cell-cell adhesion status (columns without arrows on Fig. 1, panel C). Group 1 “epithelial” transformed cells expressed very little Slug - ten to one hundred times less than normal breast and primary HMEC levels. All group 4 “mesenchymal” cell lines expressed Slug at levels similar or above normal breast. Conversely, the most intriguing finding was the high level of expression of Slug among primary and immortalized HMEC. In fact, these two categories were found to express significantly more Slug than any transformed cell lines displaying desmosomes (groups 1 and 2). Cell confluency was also found to modulate E-cad, Slug and Snail RNA expression levels in HMEC cells. Similar results were observed with Snail, but to a lesser extent, potentially reflecting the very low amount of Snail expression versus Slug expression in the studied cell populations.

Slug or Snail and E-cad expression levels are correlated in invasive ductal carcinomas

In order to study involvement of Slug, Snail and E-cad genes during breast cancer progression, we performed quantitative PCR on 128 breast tumor samples belonging to the four most representative clinical types, covering 90% of breast cancer cases: invasive ductal carcinomas (IDC), with or without in situ component (DCIS) and infiltrating lobular carcinomas (ILC) with or without in situ component (LCIS). To this group, we added 7 samples of benign breast tumors (gynecomastias and fibroadenoma) and 5 different normal breast samples, obtained from healthy subjects. A global comparison of Slug, and Snail expression patterns (Fig. 2, panel A) and E-cad expression pattern (Supplementary Fig. 1) indicated tumor expression levels were distributed evenly around normal breast level. Distribution was relatively narrow for normal breast samples (Fig. 2, panel A), justifying the use of the normal breast mean value as a reference level for cell lines and tumors in this study. Similar to the cell lines, the average expression level of Snail was found by a paired t test to be significantly lower than Slug ($p < 0.01$) or E-cad ($p < 0.05$). Normal breast samples expressed a very significant amount of Slug. As determined by paired t test, Slug expression level was not significantly down- or upregulated in any of the tumor types when compared to normal breast levels. In contrast, we observed that non-invasive tumors

express significantly less Snail than IDC or ILC ($p < 0.01$). As expected from previous findings (5, 6), levels of E-cad were significantly decreased ($p < 0.0001$) in ILC when compared to IDC ($p < 0.001$). We found the expression levels of E-cad to be the highest in DCIS, significantly above normal breast levels ($p < 0.05$) (Supplementary Fig. 1).

Finally, for each tumor type, mRNA levels for E-cad were plotted versus the mRNA levels for Slug and Snail (Fig. 2, panel B). Analysis of 45 IDC showed a clear positive correlation between Slug and E-cad expression ($r = 0.35$; $p < 0.05$) and between Snail and E-cad mRNA expression ($r = 0.57$; $p < 0.0001$). DCIS exhibited the same positive correlation for Slug ($r = 0.60$; $p < 0.001$) and Snail ($r = 0.62$; $p < 0.001$). Conversely, no significant correlation was observed in ILC, with or without in situ component (data not shown).

Slug and Snail are overexpressed in IDC associated with lymph node metastasis.

We studied a potential link between Slug and Snail expression and lymph node invasion among breast carcinomas. When compared to IDC that were not associated with lymph node metastasis (encompassing about 50% of our clinical samples), average levels of Slug or Snail expression were significantly higher in IDC associated with lymph node metastasis, as determined using unpaired t test ($p < 0.05$). The link was also observed within the DCIS samples. The same observation was made for Snail ($p < 0.05$). No significant differences were found in other tumor types. We report the expression of Slug and Snail as a function of the number of invaded lymph nodes (Fig. 2, panel C). These observations reinforce a role for Slug and Snail in overall tumor invasiveness (7, 17, 26).

Slug is mostly expressed by E-cadherin-positive carcinoma cells

In the absence of reliable antibodies for Slug, and in order to locate Slug-expressing cells in tumors, we performed in situ hybridization on four tumor samples. Serial sections from two IDC samples were analyzed simultaneously by immunolocalization to compare Slug, E-cad and cytokeratin expression patterns (Fig 3, panel A). Carcinoma cells were distinguished from stromal cells by cytokeratin labeling and were found to express E-cad at cytoplasmic or membrane level as reported earlier (5, 27). We found all carcinoma cells to express significant amounts of Slug. In samples including apparently normal mammary structures or benign breast tumors (Fig. 3, panel B, a), mammary epithelial cells were also found to express high level of Slug. Some expression was also found in certain stromal cells in normal and tumor samples, but only in a small percentage of cells. Control section showed no significant hybridization (Supplementary Fig. 2).

To confirm at protein level Slug expression by mammary epithelial cells, we analyzed a Slug-LacZ mouse (22). It was shown that in this mouse model, tissues that express full length Slug mRNA coexpress the fusion protein, as detected by β -galactosidase activity (22), suggesting that β -galactosidase activity faithfully reflects the presence of Slug protein. We analyzed mammary gland sections from heterozygous mice to find a strong Slug expression in mammary epithelial cells from tubules (Fig. 3, panel B, b-d).

Snail is mostly expressed by carcinoma cells but also by tumor stromal cells co-expressing vimentin.

We used a recently characterized Snail antibody (19) to locate Snail expression at the cellular level. Since we found an intriguing E-cad and Snail correlation at the RNA level in IDC, we focused on IDC. Tumor cells were identified in all cases by cytokeratin expression. We analyzed six samples and found two distinct expression patterns (Fig. 4), reflecting Snail RNA expression

levels. In the first group, immunoreactivity was restricted to stromal cells (Fig. 4, A-C). Tumors in this group expressed the lowest levels of Snail RNA (lower quartile of the entire IDC group). In the second group, carcinoma cells were also found in addition to stromal cells to express Snail in nuclei at various expression levels (Fig. 4, D-N). Tumors in this group expressed significantly higher levels of Snail RNA than group 1 (above median value of IDC group). In these carcinoma cells, Snail was co-expressed with E-cad, usually located at the membrane level (Fig. 4, D-I). More generally, Snail was found to be expressed in stroma in all IDC, in fibroblast-like cells expressing vimentin in most cases, but also in leukocytes expressing CD45 (Fig. 4, K-L). Not all vimentin- or CD45-positive cells expressed Snail and no correlation was observed with overall Snail RNA tumor expression levels or with tumor grade. We also looked for Snail expression by endothelial cells labeled with an anti-CD31 antibody. As expected, blood and lymphatic vessels were found to express CD31, but did not show significant Snail immunoreactivity (Supplementary Fig. 3). Overall, Snail immunostaining patterns suggest that carcinoma cells are responsible for variations in Snail RNA expression levels observed in tumor samples. We did not detect any carcinoma cells, identified by cytokeratin expression, which were simultaneously negative for E-cad and positive for Snail.

Morphological analysis suggests a link between Slug and the maintenance of semi-differentiated tubes in IDC

In order to investigate a putative role for Slug or Snail in tumor cells during IDC progression, we analyzed the samples for morphological features. Using standard criteria, a certified clinical pathologist (F. Bibeau) performed a blind screen of all the available IDC samples for the expression of morphologically discernable tubes within the tumor. As seen on Fig.5, only tumors expressing levels of Slug similar or above normal breast levels were found to include semi-differentiated tubes. This restriction was not observed for Snail (data not shown). We also systematically checked the IDC stromal component for changes in structure, inflammatory cell infiltration and global extent versus tumor component. We found no correlation between Slug or Snail expression and stroma variations. We conclude that Slug could be involved in the maintenance of tubule-like structures in IDC.

Discussion

This report combines for the first time extensive in vitro and in vivo analysis of breast carcinoma expression patterns for Slug, Snail and E-cad in a panel of 35 cell lines and 144 breast clinical samples, including evaluation of RNA and protein levels. In addition to in situ hybridization, we used a quantitative PCR technique to increase sensitivity and to avoid the risks of cross-detection associated with the significant similarities between members of the Snail family. Protein expression was estimated using a newly described antibody for Snail and a mouse reporter gene model for Slug. Overall, protein expression pattern was found to validate RNA expression analysis for Slug, mostly expressed by normal and transformed epithelial cells. For Snail, elevated Snail RNA expression level was found to correlate with substantial carcinoma cell immunoreactivity. However, we also found a significant subpopulation of stromal cells co-expressing vimentin and Snail in most tumor cases, with an unsteady pattern. In addition, we found that both genes were overexpressed in IDC that metastasized to lymph nodes and that Slug appears to be required for progression or maintenance of semi-differentiated tubules within IDC.

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In accordance with previous work on a smaller sampling (8, 9, 28), we found Slug and Snail to correlate negatively with E-cad in transformed cell lines. However, we observed the opposite in normal HMEC cells and in a large panel of breast tumor samples, with normal or transformed epithelial cells coexpressing E-cad and Slug or Snail. Slug and Snail factors were found in several transformed cell models to target E-boxes in E-cad promoter to repress E-cad transcription (8, 9). Similarly, we observed that induced overexpression of Slug or Snail in HMEC cells repress an E-cad promoter/reporter gene construct (Come, C. et al. , unpublished observations). This observation indicates that Snail or Slug, when overexpressed in HMEC, can repress discrete E-cad promoter elements, even though the actual gene is not significantly downregulated. This persistence of E-cad underscores the role of other regulatory elements in physiological E-cad transcription regulation, as determined by several authors (29, 30). Taken as a whole, our observations are not compatible with a general downregulation of E-cad gene in breast carcinomas by transcription factors Snail or Slug. However, these observations do not preclude local and transient Slug or Snail overexpression at particular sites, potentially linked to E-cad and cytokeratin downregulation during epithelial-mesenchymal transition phases that could occur during the initiation of invasive events (1, 31).

Slug or Snail expression levels in breast carcinomas were found to be distributed along a wide range around normal breast level, encompassing two orders of magnitude. We found no correlation with any carcinoma histological subtype, suggesting Slug and Snail role during tumor progression could relate to general mechanisms involved in all subtypes. Regarding Slug, our findings unveil a new morphogenetic role during tumor progression. Slug appears to be necessary to the formation or maintenance of tubular structures in ductal carcinomas. It is tantalizing to suggest Slug could participate in the semi-cohesive migration mode displayed by invasive structures within IDC. Accordingly, we found a link between low confluency state in HMEC, associated with a less differentiated and more motile phenotype, and higher expression levels of Slug and Snail, in accordance with previous reports for Slug (32). In complement, we also found that HMEC grown on Matrigel display a more differentiated phenotype, as described previously (33), and express significantly less Slug (Come, C., unpublished data). Finally, we found recently that keratinocytes, exemplifying a distinct population of epithelial cells involved in a cohesive migration, express large amounts of Slug and E-cad in vivo during wound healing re-epithelialization (2). In this case, Slug appeared to be required for cell migration to occur. During breast carcinoma progression, a related migration mode could involve semi-differentiated structures such as tubule-like structures, cordons, or less organized groups of cells and would translate into more invasive tumors, as suggested by the link between Slug expression levels and an increase in the number of invaded lymph nodes. The presence of tubules is a morphological criteria used to estimate tumor differentiation, one of the three criteria for Scarff Bloom and Richardson (SBR) grading in complement to nuclear pleiomorphism and mitotic count. However, only a combination of these histopathological markers provides a reliable prognostic tool. Probably linked to tumor heterogeneity, presence of tubules does not itself represent a prognostic factor. Consequently, we did not find any significant correlation between Slug and Snail expression levels and tumor histological SBR grade (data not shown).

We found an intriguing downregulation of Snail expression in our non invasive breast tumors when compared to invasive carcinomas, but also to normal breast samples. When added to the association we found with the number of invaded lymph nodes, this observation suggests a specific role for Snail in tumor progression, potentially linked to stroma involvement. This

could involve new functions of Snail as a survival and motility inducer during cancer progression, as suggested recently (34).

In conclusion, we established at cellular level the links between transcription factors Snail, Slug and E-cad in breast carcinomas, as compared to transformed or normal mammary epithelial cells. Modulation of Snail and Slug expression appears to reflect distinct tumor invasion modes and could provide functional classification tools to better categorize ductal carcinomas and ultimately design appropriate therapeutical approaches.

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Figure legends

Fig. 1. Slug and Snail are linked to epithelial phenotype in mammary epithelial cells. A) Expression levels of Slug, Snail, and E-cadherin (E-cad) in breast cancer cells. Messenger RNA from 35 HMEC and human breast cancer cell lines was quantified by real time quantitative PCR analysis. Arrows point to normal breast tissue expression level, added as a reference. Open arrowheads point to HMEC grown at low confluency. Filled arrowheads point to HMEC grown at high confluency. Horizontal bars indicate column mean. Axis is in logarithmic scale. RNA expression level relative to 36B4 level is estimated using the value $2^{-\Delta CT}$ where ΔCT is the number of cycles between the gene of interest (Slug, Snail or E-cad) and the reference gene 36B4. B) Phenotype profiling using cell-cell adhesion structure expression. Cell-cell adhesion status was established by phase contrast (g, h) and immunofluorescence using antibodies against desmoplakin (a, b), cytokeratins (e, f) used in double-labeling with anti-desmoplakin antibodies on panels a and e, and Plakoglobin (c, d). Cell lines shown here belong to phenotype group 1 (VP-267: a, e), 2 (CAMA: b, f), 3 (SUM 159: c, g) and 4 (MDA-MB-436: d, h). Scale bar = 10 μ m. C) Phenotype profiling using E-cad, Slug and Snail expression levels. Cell lines were ranked by increasing levels of E-cad expression, as indicated, and related to normal breast levels. Columns were filled using phenotype-specific theme corresponding to the four groups defined in Table 1. Open arrowhead points to the reference normal breast (expression level = 1). Arrows

point to primary HMEC 184 (open arrow) grown at high (HC) or low (LC) confluency or immortalized HMEC 184 A1 (filled arrow) grown at high (HC) or low (LC) confluency.

Fig. 2. Slug and Snail are correlated with E-cad and lymph node invasion in IDC. A) Expression level ranges of Slug and Snail in 140 human samples. Tumors samples were quantified by real time quantitative PCR analysis and divided into the four main histological types, Invasive ductal carcinomas (IDC), Invasive ductal carcinomas with in situ component (IDC+DCIS), Infiltrating lobular carcinomas (ILC) and Infiltrating lobular carcinomas with in situ component (ILC+LCIS). Non-invasive breast tumors (Non Inv) were reported and normal breast samples (NB) were indicated for reference. RNA expression level relative to 36B4 level is estimated using the value $2^{-\Delta CT}$, where ΔCT is the number of cycles between the gene of interest (Slug, Snail) and the reference gene 36B4. Horizontal bars indicate column mean. Snail expression in non-invasive tumors (*) was found by a paired T-test to be significantly lower than in IDC or ILC. B) Comparative analysis of mRNA expression for Slug, Snail and E-cad on human invasive ductal carcinomas. Tumor samples were divided into two categories IDC and IDC+DCIS, E-cad mRNA quantification was normalized to normal breast tissues. Black lines represent linear regression: E-cad was found to be significantly positively correlated to Slug and Snail ($p < 0.05$). Axes are in logarithmic scale. C) Slug and Snail expression levels correlate with the number of invaded axillary lymph nodes in IDC. Invasion of axillary lymph nodes was monitored and the number of invaded nodes plotted versus the relative expression levels of Slug and Snail on semi-logarithmic axes. Average Slug or Snail expression levels in IDC associated with lymph node invasion was found to be significantly higher ($p < 0.05$) than the levels found in IDC not associated with lymph node invasion ($n = 0$).

Fig. 3. Normal and carcinoma mammary epithelial cells express Slug and E-Cad. A) Two IDC samples (a-d and e-j) were analyzed by in situ hybridization (a, b, e, h) for Slug expression. Subsequent serial sections were double-immunolabeled for E-cad (c, f, i) and cytokeratins (d, g, j). Scale bar = 10 μm (h-j), 25 μm (b-g) or 50 μm (a). B) Non invasive tissues such as a human fibroadenoma (a) and mouse mammary gland (b-d) were also found to express Slug within the epithelial compartment as found by in situ hybridization (asterisk, a: epithelial compartment), and β Gal enzymatic detection in five-weeks old Slug-LacZ heterozygous mouse (b-d). Asterisk, b: tubule lumen. Arrow: tubule wall. Scale bar = 25 μm (b-d) or 50 μm (a).

Fig. 4. Snail is expressed by epithelial and stromal cells within breast ductal carcinomas. Four IDC (A-C, D-I, J, K-N) expressing respectively low (A-C) or high (D-N) levels of Snail were analyzed for E-cad, Snail and cytokeratin (CK) expression by immunofluorescence, as indicated. Snail (green in color panels) was found to be expressed by stromal cells (B, E, H, J -N) and carcinoma cells (E, H, J, K, L, N), usually colocalizing in carcinoma cells with E-cad (D, red in F) and cytokeratin (G). Some Snail-positive stroma cells are characterized by vimentin expression (red in K) or CD45 expression (red in N). Snail nuclear localization is clear at higher magnification (F, J-L). HES staining was provided to appreciate tumor structure (C, I, M). Scale bar = 10 μm (F, J-L, N), 20 μm (A-E, G-I, M).

Fig. 5. Semi-differentiated tubules are only found in IDC expressing levels of Slug similar or above normal breast level. Two IDC expressing respectively low (A) and high (B) amounts of Slug were sectioned and analyzed for E-cad expression. The high amount of Slug appears to be linked to the expression of numerous tubules (arrowhead). C. Our sampling of 45 IDC was

ranked by increasing level of Slug mRNA, related to the level expressed in normal breast (NB). Tumor sections were examined blindly for occurrence of differentiated tubes. Tumors in which tubes were identified are indicated by a filled column, suggesting a clear link to Slug mRNA expression levels. In both tumors, E-cad was found to concentrate mostly at cell membrane level (arrow). Scale bar = 20 μ m.

Supplementary Fig. 1. Expression range of E-cad in IDC. Tumors samples were quantified by real time quantitative PCR analysis and divided into the four main histological types, Invasive ductal carcinomas (a), Invasive ductal carcinomas with in situ component (b), Infiltrating lobular carcinomas (c) and Infiltrating lobular carcinomas with in situ component (d). Non-invasive breast tumors were reported on line e and normal breast samples were indicated for reference on line f. RNA expression level relative to 36B4 level is estimated using the value $2^{-\Delta CT}$, where ΔCT is the number of cycles between E-cad and the reference gene 36B4. Horizontal bars indicate column mean.

Supplementary Fig. 2. Negative controls for Slug in situ hybridization and β -galactosidase activity in Slug-LacZ mouse included (A) IDC frozen section incubated with sense slug probe and (B) frozen section from wild-type mice. No specific activity was detected in either case.

Supplementary Fig. 3. Blood vessels in invasive ductal carcinoma were found to express CD31 (arrow), but not Snail. Snail was expressed by other stromal cells.

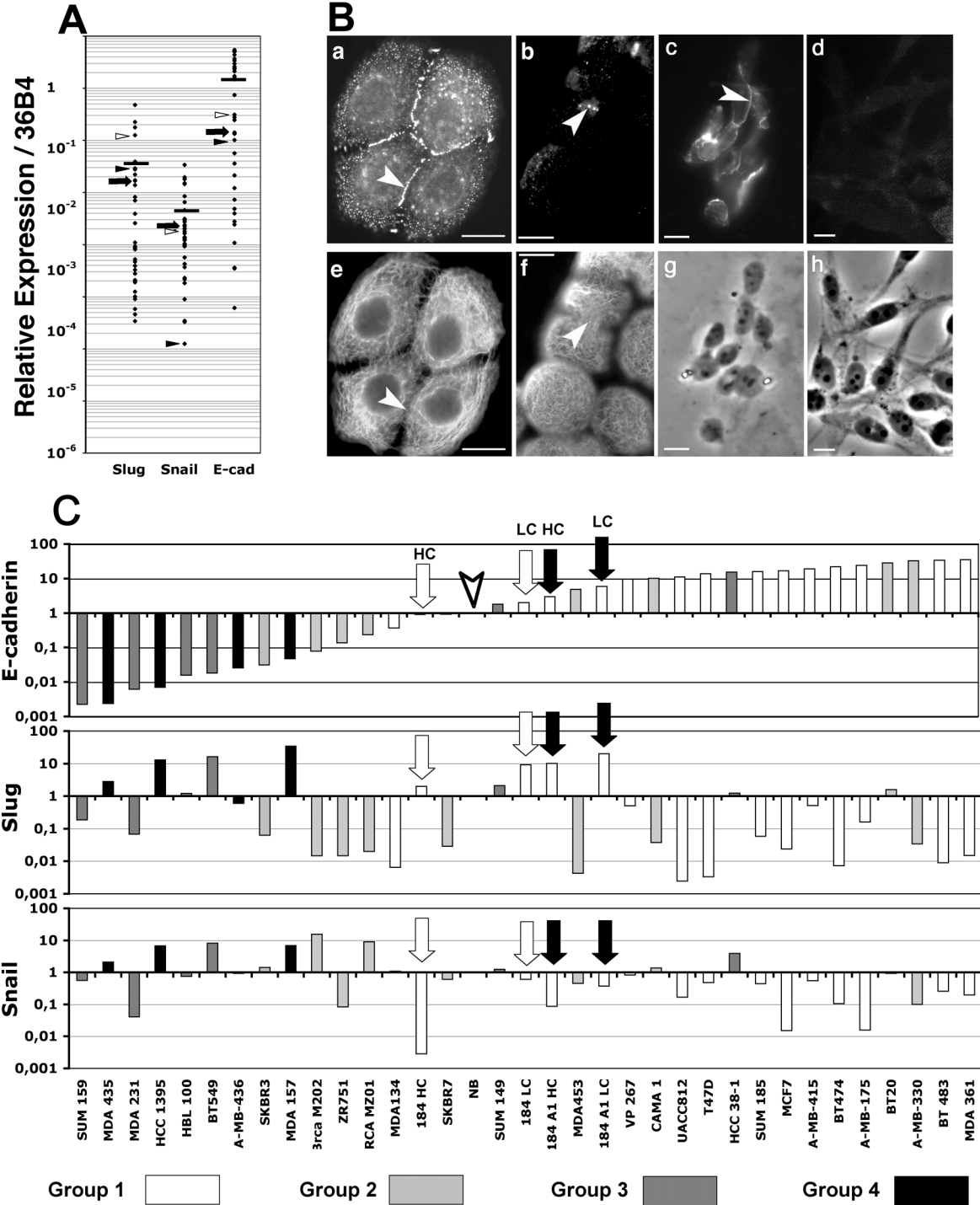


Fig. 1

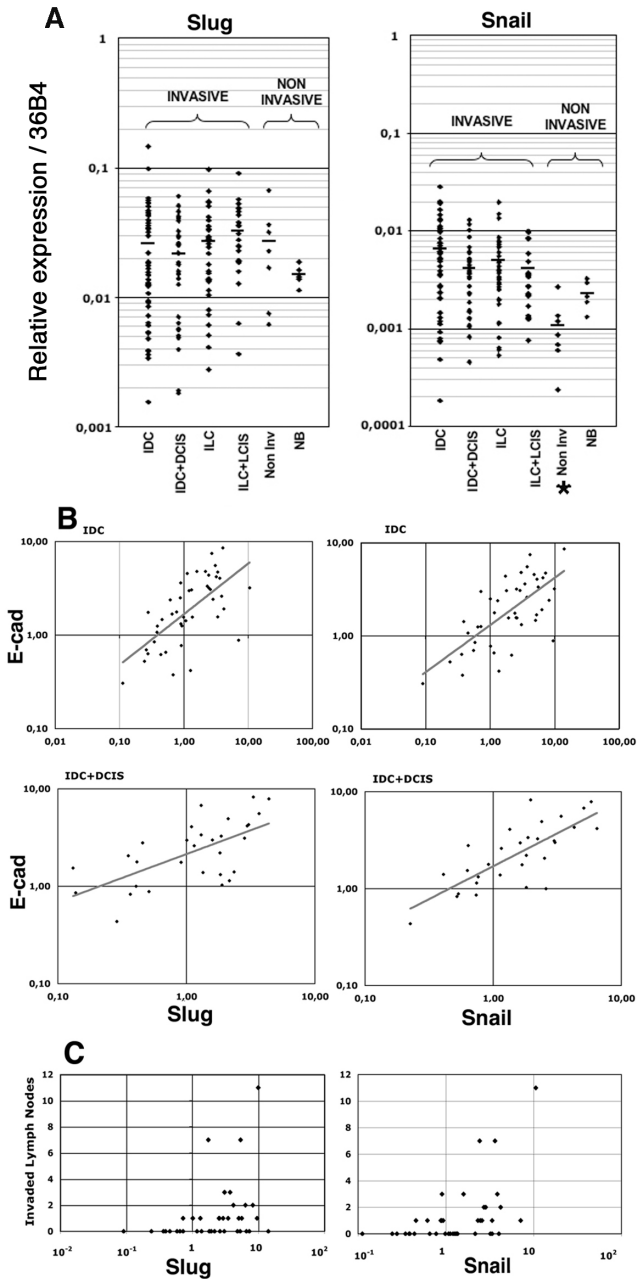


Fig. 2

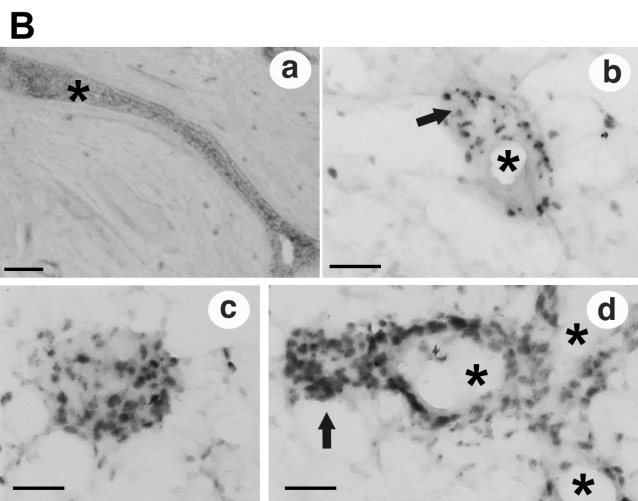
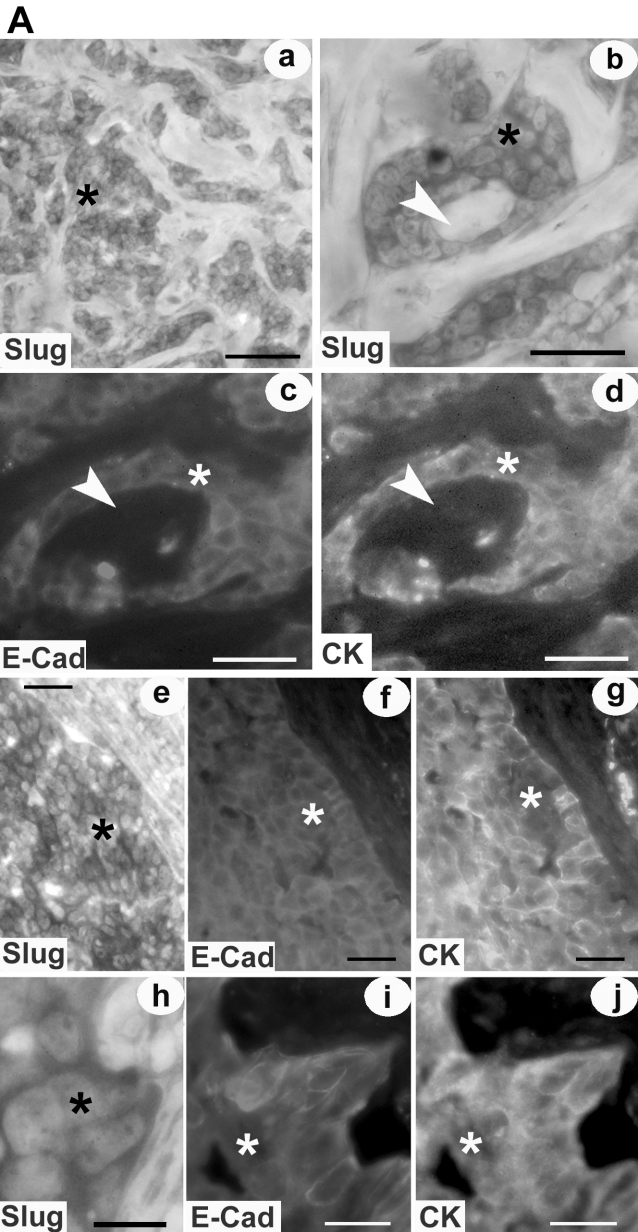


Fig. 3

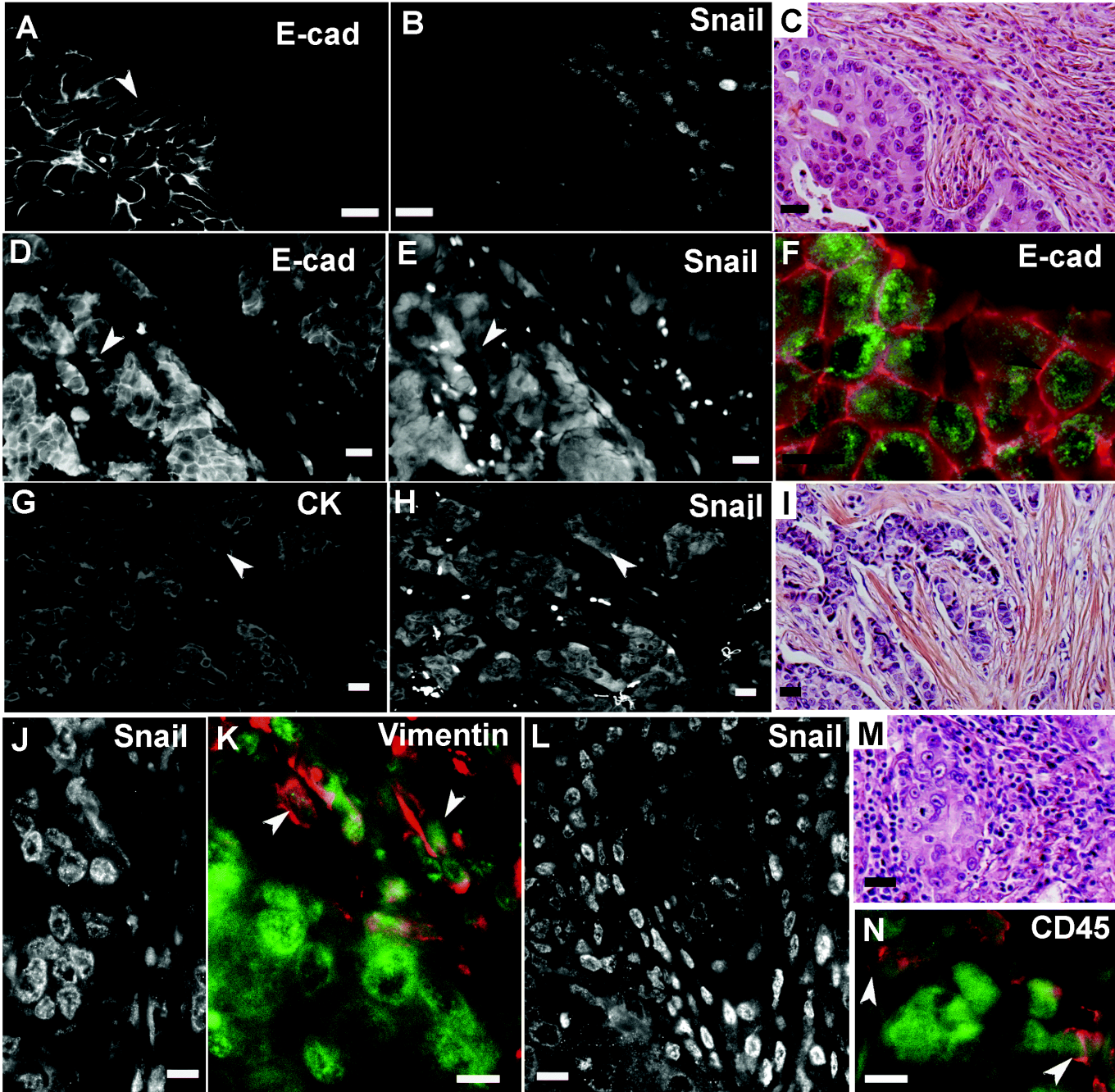


Fig. 4

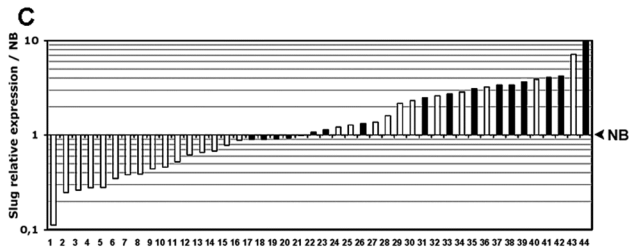
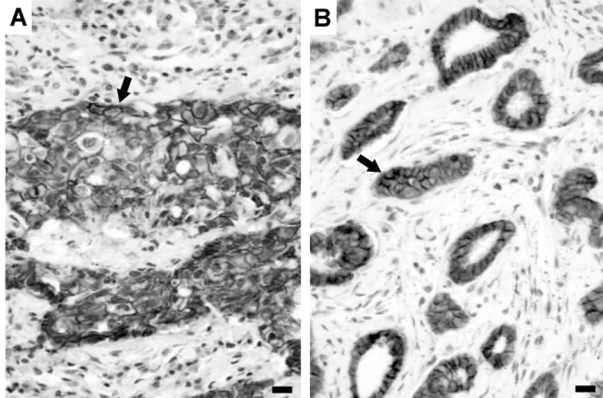


Fig. 5

	CK IF	DP IF	E-Cad W	E-Cad IF	PG IF	Desmosome	AJ	Phenotype
BRCA MZ01	+	+	0	+	++	+	+	2
BRCA MZ02	+	+	0	+	++	+	+	2
BT 20	++	++	++	++	+++	+	+++	2
BT 474	+++	+++	+++	+++	+++	+++	+++	1
BT 483	nd	nd	nd	nd	nd	+++*	nd	1
BT 549	0	0	0	0	+	0	+	3
CAMA 1	+++	+	++	+++	+++	+	+++	2
HBL 100	0	0	0	0	+	0	+	3
HCC 38-1	+	0	+	+	++	0	+	3
HCC 1395	0	0	0	0	0	0	0	4
HMEC 184	+++	+++	+++	+++	+++	+++	+++	1
HMEC 184 A1	+++	+++	nd	+++	+++	+++	+++	1
MCF7	+++	+++	+++	+++	+++	+++	+++	1
MDA-MB-134	+	+	0	nd	+	+	+	2
MDA-MB-157	0	0	0	0	0	0	0	4
MDA-MB-175	+++	+++	+++	+++	+++	+++	+++	1
MDA-MB-231	+	0	0	0	+	0	+	3
MDA-MB-330	++	++	++	++	+	+	+	2
MDA-MB-361	+++	+++	+++	+++	+++	+++	+++	1
MDA-MB-415	+++	+++	+++	+++	++	+++	+++	1
MDA-MB-435	0	0	0	0	0	0	0	4
MDA-MB-436	0	0	0	0	0	0	0	4
MDA-MB-453	+	+	0	0	+	+	+	2
SKBR3	+++	+	0	0	++	+	+	2
SKBR7	+	+	+	+	+	+	+	2
SUM 149	0	0	+	+	++	0	+	3
SUM 159	0	0	0	+	+	0	+	3
SUM 185	+++	+++	+++	++	++	+++	+++	1
T47D	+++	+++	+++	+++	+++	++	+++	1
UACC812	+++	+++	++	+++	+++	+++	+++	1
VP 267	+++	+++	+++	+++	+++	+++	+++	1
ZR751	++	+	+++	++	++	+	+++	2

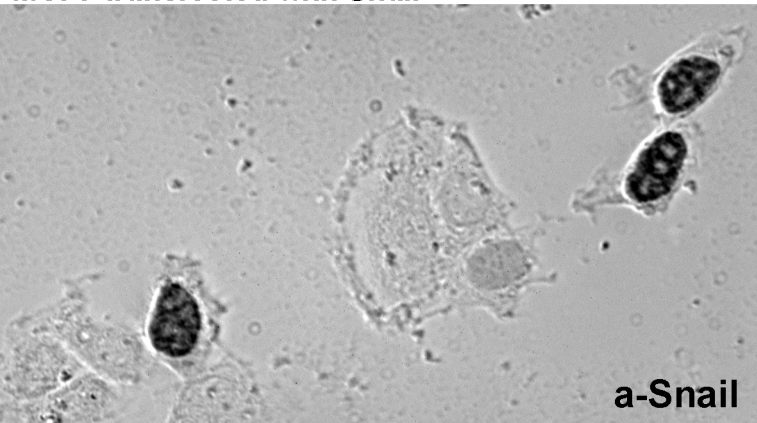
Table 1. Phenotyping of 34 breast primary or transformed cell lines. We characterized cell-cell adhesion structures in 34 human breast primary or transformed cell lines in order to classify them into four groups, based on the decreasing expression of desmosomes and adherens junctions (AJ). Group 1 included cells with numerous desmosomes including more than 50% of the cell-cell contacts areas; group 2 included cells that did express some desmosomes, covering less than 10% of the cell-cell contact areas; group 3 included cells expressing no detectable desmosomes, but showing indications of at least some adherens junction structures; group 4 cells did not express any detectable cell-cell adhesion structure and expressed a spindle-shaped phenotype. Desmosomes were defined by the combined membrane-linked expression of cytokeratins and desmoplakins by immunofluorescence (CK IF and DP IF). Adherens junctions were defined by combined membrane expression of E-Cadherin and plakoglobin by immunofluorescence (E-Cad IF and PG IF) and western analysis (E-Cad W). Expression levels were ranked semi-quantitatively into three levels: + weak, ++ significant, +++ strong.

* based on phase microscopy

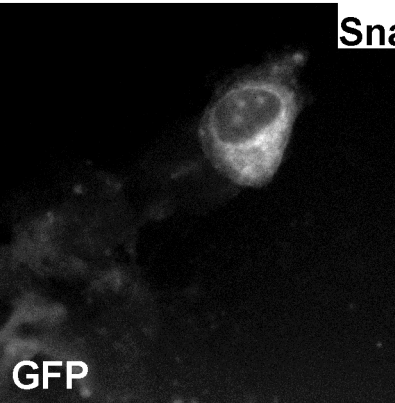
For reviewer's eyes only:

MCF7 human cells were transfected with EGFP and a validated Snail or Slug full length clone . After 48h Cells were fixed and immunostained with anti-snail antibody. Transfected cells were identified by GFP

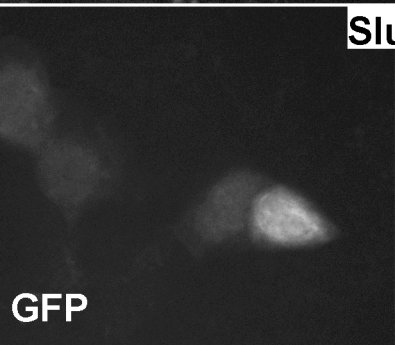
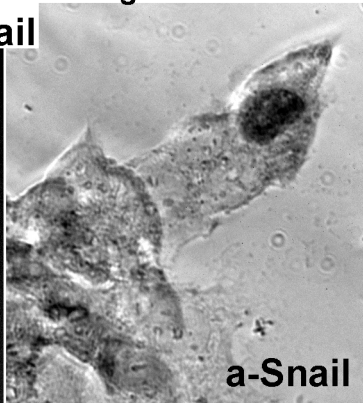
MCF7 transfected with Snail



MCF7 transfected with Snail or Slug and GFP



Snail



Slug



Fig. 2 Hybridoma supernatant Sn9H2 detects a functional Snail protein. E-cadherin-positive gastric cancer cells GC2957 (a, b) and MCF7 mammary cells (c, d) were transfected with vector as control or with Snail-His. **a** Western blot demonstrating the overexpression of Snail and the reduction of E-cadherin in the Snail-transfected cells. Alpha-tubulin was used as protein loading control. **b** Snail overexpression resulted in cell scattering. **c** Western blot showing the reduction of E-cadherin after Snail overexpression. No change is seen for alpha-tubulin. **d** Quantitative real-time RT-PCR demonstrates the reduction of E-cadherin expression by 95% after 3.7-fold Snail overexpression at the mRNA level. Results are representative of two independent transfection experiments done in triplicates and measured 48 h after transfection. The mRNA levels of vector-transfected cells are set to 100%; the mean \pm standard deviation is shown

