Epithelial-Mesenchymal Transition in Breast Cancer Relates to the Basal-like Phenotype

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Abstract

Epithelial-mesenchymal transition (EMT) is defined by the loss of epithelial characteristics and the acquisition of a mesenchymal phenotype. In carcinoma cells, EMT can be associated with increased aggressiveness, and invasive and metastatic potential. To assess the occurrence of EMT in human breast tumors, we conducted a tissue microarray–based immunohistochemical study in 479 invasive breast carcinomas and 12 carcinosarcomas using 28 different markers. Unsupervised hierarchical clustering of the tumors and statistical analysis showed that up-regulation of EMT markers (vimentin, smooth-muscle-actin, N-cadherin, and cadherin-11) and overexpression of proteins involved in extracellular matrix remodeling and invasion (SPARC, laminin, and fascin), together with reduction of characteristic epithelial markers (E-cadherin and cytokeratins), preferentially occur in breast tumors with the "basal-like phenotype." Moreover, most breast carcinosarcomas also had a basal-like phenotype and showed expression of mesenchymal markers in their sarcomatous and epithelial components. To assess whether basal-like cells have intrinsic phenotypic plasticity for mesenchymal transition, we performed *in vitro* studies with the MCF10A cell line. In response to low cell density, MCF10A cells suffer spontaneous morphologic and phenotypic EMT-like changes, including cytoskeleton reorganization, vimentin and *Slug* up-regulation, cadherin switching, and diffuse cytosolic relocalization of the catenins. Moreover, these phenotypic changes are associated with modifications in the global genetic differentiation program characteristic of the EMT process. In summary, our data indicate that in breast tumors, EMT likely occurs within a specific genetic context, the basal phenotype, and suggests that this proclivity to mesenchymal transition may be related to the high aggressiveness and the characteristic metastatic spread of these tumors. [Cancer Res 2008;68(4):989–97]

Introduction

Breast cancer is a heterogeneous disease, which includes a wide range of histologic subtypes and a diversity of clinical behaviors and patient's outcome (1). Recent studies by gene expression profiling enabled the identification of different subgroups of breast tumors with distinct molecular signatures (2–4). According to this molecular classification, breast carcinomas can be divided into at least four biologically different phenotypes: normal-like phenotype (expression profile similar to noncancerous breast tissue); luminal phenotype [generally estrogen receptor (ER)-positive tumors, with expression of epithelial markers, such as E-cadherin, and cytokeratins CK8, 18, and 19]; ER-negative tumors, comprising the subgroups of HER2 (overexpressing *ERBB2* oncogene); and basal-like phenotypes [tumors expressing markers that are characteristic of the myoepithelium of the normal mammary gland, such as epidermal growth factor receptor (EGFR), p63, and basal cytokeratins CK14, CK5/6, and CK17; refs. 2–4]. This classification has also been reproduced to a certain extent in immunohistochemical studies (5, 6).

Importantly, this molecular taxonomy has important clinical value because some of the molecular phenotypes (especially HER2 and basal-like) show unfavorable prognosis and/or resistance to chemotherapy (3, 4). Additionally, it has been shown that basal-like tumors show a special proclivity for distant metastasis to characteristic tissues (lung and brain; ref. 7). The different biological behaviors and metastatic patterns observed among the distinct breast cancer phenotypes may suggest different mechanisms of invasion and metastasis for breast tumors. Carcinomas can invade as multicellular aggregates in a process known as collective cell migration in which the carcinoma cells may retain their epithelial characteristics (including adherens junctions and apical-basal polarity; refs. 8, 9). Nonetheless, epithelial-mesenchymal transition (EMT) can also play a relevant role in tumor invasion and metastasis (10–13). It has been proposed that EMT-like processes might occur during tumor progression in carcinomas, particularly at specific stages (i.e., invasion and intravasation) where tumor cells disassemble and migrate to tissue/organ sites distant from the primary tumors (10–13). EMT is an essential developmental process by which cells of epithelial origin lose epithelial characteristics and polarity, and acquire a mesenchymal phenotype with increased migratory behavior (10–14). Thus, EMT is characterized by loss of intercellular adhesion (E-cadherin and occludins); down-regulation of epithelial makers (cytokeratins); up-regulation of mesenchymal markers [vimentin and smooth muscle actin (SMA)]; acquisition of fibroblast-like (spindle) morphology with cytoskeleton reorganization; and increase in motility, invasiveness, and metastasic capabilities (10–14). In addition, the process known as "cadherin switching" (down-regulation of E-cadherin and up-regulation of mesenchymal cadherins such as N-cadherin or cadherin-11; refs. 15, 16) and the accumulation of β-catenin have also been associated with EMT (12, 14).

The complex genetic changes necessary to accomplish the phenotypic changes associated with EMT are, at least in part, mediated

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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by a number of specific transcription factors, here called "EMT inducers." These transcription factors include Snail (also known as Snail1; ref. 17), Slug (also known as Snail2; ref. 18), SIP-1 (ZEB-2; ref. 19), δEF1 (ZEB-1; ref. 20), E12/E47 (21), and Twist (22). When expressed in a variety of cell types, these factors act as transcriptional repressors of *E-cadherin* (23, 24) and modulate directly or indirectly the expression of a wide number of genes involved in cancer invasion and metastasis (such as matrix metalloproteinase 9 or SPARC), and consequently promote complete EMT *in vitro* (25, 26).

Additionally, the expression of some of these EMT inductors has been detected in a variety of human cancer biopsies, including breast carcinomas, and their overexpression is usually related to increased tumor aggressiveness or recurrence, unfavorable clinicopathologic variables, and poor prognosis (reviewed in ref. 23).

However, much of the evidence for the association of tumor invasion with EMT comes from studies in cancer cell lines and in animal models (12, 13), as pathologists cannot easily or often identify EMT in human tumors because the events defining a full EMT process *in vitro* are rarely observed together *in vivo* (27). Therefore, its actual occurrence and relevance in human cancer is a matter of intense debate (8, 12, 13, 27). Some authors have proposed that EMT may be transient and reversible, and may only occur in reduced groups of cells or even in isolated cells of the tumor invasive areas (12, 23). Furthermore, although full EMT might not be easy to achieve *in vivo* (8, 27) it has been suggested that carcinosarcomas (also known as metaplasic carcinomas or spindle cell carcinomas) may represent true examples of complete EMT (12, 13). Carcinosarcomas are uncommon but aggressive neoplasias with biphasic histology of carcinomatous and sarcomatous elements (1). Recent molecular studies have shown the monoclonal origin of these neoplasms, as the carcinomatous and sarcomatous elements share common genetic alterations (such as p53 mutations; ref. 28). Moreover, their sarcomatous component may express epithelial markers, such as cytokeratins (29), suggesting an epithelial origin. Nonetheless, carcinosarcomas might not completely reflect the occurrence of EMT in human tumors and, in other types of neoplasms, the expression of mesenchymal markers, loss of epithelial markers (E-cadherin), and/or the cadherin switching might be independently considered as signs of partial EMT (12).

To study the phenotypic and biological context within breast tumors where EMT is thought to occur and to analyze its biological significance, we conducted a tissue microarray (TMA)-based immunohistochemical study in 479 carcinomas and 12 carcinosarcomas of the breast. In addition, *in vitro* studies on immortalized breast MCF10A cells were performed to assess the phenotypic and genetic changes associated with mesenchymal transition of basal-like breast cells.

Materials and Methods

Human tumors. A series of 491 formalin-fixed paraffin-embedded human breast tumors were acquired from the archives of the Pathology Department of La Paz Hospital, Madrid, Spain. Patients had undergone surgery between 1993 and 2001. The mean patient age at surgery was 53 years (range, 27–87 years). Fifty-three percent of the tumors showed axillary lymph node metastasis at the time of diagnosis. The series included 455 infiltrating ductal carcinomas, 24 invasive lobular carcinomas, and 12 breast carcinosarcomas. Fourteen percent of the tumors were grade I, 39% were grade II, and 46% were grade III. For breast carcinosarcomas, careful examination of the H&E-stained sections by three pathologists (JP, SMRP, and DH) confirmed the diagnosis on the basis of histologic criteria (1). This study was approved by the ethic committee of the institution.

TMA construction and immunohistochemistry. Representative areas of breast tumors were carefully selected on H&E-stained sections, and two 1-mm diameter tissue cores were obtained from each specimen. The cores were precisely arrayed into new paraffin blocks using a TMA workstation (Beecher Instruments). For breast carcinosarcomas, only the carcinomatous component was included in the TMAs. All studied TMAs also included normal breast tissue as an internal control. Immunohistochemistry was carried out on sequential TMA sections, using the Envision method (Dako) or the LSAB method (DAKO). Detailed information of the immunohistochemical procedures and the antibodies used is listed in the Supplementary Table S1. The primary antibodies were omitted in negative controls.

Immunohistochemistry scoring and statistical analysis. Expression data for each immunohistochemical marker were transformed to a binary categorical variable (0, negative; 1, positive expression; according to the threshold of positive staining for each marker, as indicated in Supplementary Table S1).

To analyze the immunohistochemical data from breast carcinomas, hierarchical unsupervised clustering was performed using the UPGMA method, and assuming Euclidian distances among markers. The statistical test and the clustering were implemented using the GEPAS package.[5] The χ^2 contingency test with a Yates correction when appropriate or the Fisher's exact test was used to determine the association between variables. The statistical package "SPSS 13.0 for Windows" (SPSS, Inc.) was used for this analysis.

Cell culture, immunofluorescence, and Western blot. MCF-10A cells were obtained from American Tissue Culture Collection (ATCC)[6] and grown according to ATCC recommendations. Cells were grown at the indicated cell densities, 10% to 30% confluence (sparse cultures) or 80% to 90% confluence (confluent) in a humidified 5% CO_2 atmosphere at 37°C. For immunofluorescence analysis, cells were plated onto sterile 12-mm glass coverslips and grown to the desired confluence. They were then fixed in either methanol (−20°C, 5 min) or 3.7% formaldehyde (for 30 min at room temperature) and then incubated with the primary and secondary antibodies as described elsewhere (26). For immunofluorescence of cadherins and catenins, we used the antibodies listed in the Supplementary Table S1, except for p120, where the polyclonal rabbit anti-p120 was used (Santa Cruz Biotechnology). The Alexa-594–coupled phalloidin (Molecular Probes) was used to stain actin cytoskeleton, and antipaxillin antibody (Abcam) was used to detect focal adhesions. Cell nuclei were stained using 4,6-diaminidino-2-phenylindole (Molecular Probes). Fluorescence was examined using a confocal ultraspectral microscope (TCS-SP-2-AOBS-UV; Leica). For Western analysis, cells were grown to the desired confluence, and total cell extracts were obtained in radioimmunoprecipitation assay buffer and analyzed as described elsewhere (26).

Quantitative real-time reverse transcription-PCR. Quantitative real-time reverse transcription-PCR (qRT-PCR) was performed with gene-specific fluorescent TaqMan probes (Assays on demand; Applied Biosystems) using an ABI PRISM 7700 Sequence Detection System Instrument and the associated software (Applied Biosystems), following the manufacturer's instructions. Each reaction was performed in triplicate from two cDNA dilutions. The standard human β2-microglobulin gene (*B2M*; Applied Biosystems) was used to normalize variations in the quantities of input cDNA. The amount of target and endogenous reference was determined using the standard curve method. The standard curve was constructed by 5-fold serial dilutions of cDNA generated from Universal Human Reference RNA (Stratagene).

cDNA microarrays. MCF10A cells were grown to 10% to 20% (sparse) or 80% to 100% (confluent) confluence. Total RNAs from sparse and confluent cultures were extracted using RNAeasy Extraction kit (QIAGen). The experiment was repeated, giving two RNA samples for sparse and confluent conditions, respectively. RNAs were amplified by *in vitro* transcription as described before (28) and then fluorescently labeled with Cy5-dUTP (sparse

[5] http://gepas.bioinfo.cipf.es/cgi-bin/tools
[6] http://www.atcc.org

cells) or Cy3-dUTP (confluent cells; Amersham). Samples were directly hybridized onto the "Centro Nacional de Investigaciones Oncológicas (CNIO) Oncochip" cDNA microarray v 2.0, as described previously (26). For each experiment, three hybridizations were performed, making a total of six hybridizations. Slides were washed, dried, and then scanned in a Scanarray 5000 XL scanner (GSI Lumonics). Data from the fluorescence intensity measurements were quantified using GenePix Pro 6.0 program (Axon Instruments, Inc.). For data analysis, we selected the genes whose median expression was up- or down-regulated by a factor of at least 2-fold in sparse cells with respect to confluent cells. All of the microarray raw data tables have been deposited in the Gene Expression Omnibus[7] under the accession number of GSE8430.

Results

Expression of EMT markers in breast tumors. To investigate whether the events associated with *in vitro* EMT processes occurred in specific biological contexts or breast tumor subtypes *in vivo*, we studied a series of 479 invasive breast carcinomas together with 12 carcinosarcomas (only evaluating the epithelial component) for the immunohistochemical expression of 28 markers. The series of markers included those associated with EMT (vimentin, SMA, and SPARC), several cadherins (E-, N-, P-cadherin, and cadherin-11), cell cycle and proliferation proteins (cyclins, p27, p21, survivin, and Ki67), together with markers currently used for the identification of specific subgroups of breast tumors with biological relevance (such as hormonal receptors, basal and luminal keratins, HER2, EGFR, p63, etc.), among others (for complete list see Supplementary Table S1).

Unsupervised hierarchical clustering of the expression data subdivided the tumors into two main clusters (Fig. 1*A*):

"Cluster A", encloses two subgroups: "A1", containing the majority of hormonal receptor–positive tumors, and "cluster A2" of mostly ER-negative tumors. The majority of the tumors in "cluster A" expressed typical epithelial markers, such as CK8 and CK19 (96.4% and 86.7%, respectively), which identify the luminal phenotype (refs. 3, 6; Supplementary Table S2).

"Cluster B", consisted a group of 73 tumors, mostly grade III, hormonal receptor–negative, and expressed basal/myoepithelial markers (CK5/6, CK14, EGFR, CD10, p63, P-cadherin, and caveolin), characteristic of basal-like tumors (2–4). Importantly, we observed that EMT markers (vimentin, SMA, and SPARC), as well as cadherin switching (reduced expression of E-cadherin and up-regulation of N-, P-, and cadherin-11) were significantly more frequent in the tumors within cluster B than in cluster A (Fig. 1; Supplementary Table S2). Therefore, to further test if EMT markers occurred preferentially in basal-like tumors, we decided to use two different established immunohistochemical criteria to identify basal-like tumors. Nielsen et al. (6) proposed that ER and HER2 negativity, and positivity for EGFR and/or CK5 precisely identify basal-like tumors, whereas Rakha et al. (30) stated that irrespective of HER2 and ER status, basal-like tumors can be identified solely by the positive expression of cytokeratins 5 and 14. Applying these criteria to our tumor series, we first observed that the majority of the tumors in cluster B exhibited a basal-like phenotype (65% and 70%, according to Nielsen et al. and Rakha et al., respectively). Second, we showed that EMT markers, cadherin switching, and expression of myoepithelial markers (CD10, p63, and CK14) statistically correlated with the basal-like phenotype (Table 1). Additionally, basal-like tumors showed positive staining of proteins functionally related to extracellular matrix remodeling (laminin) or invasion (fascin), as well as diffuse β-catenin cytoplasmic delocalization, more frequently than nonbasal tumors (Table 1). No nuclear β-catenin staining was evident in any carcinoma analyzed.

[7] http://www.ncbi.nlm.nih.gov/geo/

Because basal-like tumors characteristically are high grade (Table 1) we analyzed whether the positive association of EMT markers with these tumors was a general consequence of tumor dedifferentiation rather than a direct association with the basal-like phenotype. For this, we selected only grade III tumors and observed that most of EMT markers still associated with the basal-like phenotype (Supplementary Table S3).

Regarding histology, basal-like tumors were mostly invasive ductal carcinomas. By contrast, lobular carcinomas, previously suggested as likely examples of EMT (22) due to their lack E-cadherin and diffuse invasion pattern, do not frequently express EMT markers (Supplementary Fig. S1), with only one of 24 cases (4%) positive for vimentin, SPARC, or cadherin-11.

Breast carcinosarcomas show basal-like phenotype. As shown in Fig. 1, the carcinomatous component of most breast carcinosarcomas (8 of 12, 67%) were found in cluster B, whereas 5 (42%) and 9 (75%) from these 12 carcinosarcomas complied with the criteria for basal-like tumors proposed by Nielsen et al. (6) and Rakha et al. (30), respectively. Comparative immunohistochemical analysis between the epithelial and mesenchymal component of breast carcinosarcomas showed that most of them expressed the EMT markers SPARC, vimentin, and cadherin-11 in the sarcomatous component (Fig. 2; Table 2). As expected, the epithelial component of the tumors expressed E- and P-cadherin, and, importantly, a proportion of them also show focal expression of SPARC, vimentin, or cadherin-11. Moreover, whereas p120 and β-catenin were membrane restricted in the epithelial component, they were frequently found in the cytoplasm or nucleus in the sarcomatous cells, although they were absent in some cells (Fig. 2; Table 2).

Collectively, the immunohistochemical studies in breast carcinomas and carcinosarcomas indicate that expression of EMT markers, together with the cadherin switching, predominantly are found in the context of a basal-like phenotype, and suggest that neoplastic cells with basal-like features may be especially prone to mesenchymal transition.

The basal-like cell line MCF10A suffers spontaneous EMT-like phenotypic changes. To study the intrinsic plasticity of basal-like cells to undergo EMT, we used the human breast cell line MCF10A. This cell line exhibits a basal-like phenotype but shares many features of mesenchymal cancer cell lines (31–34). Although nontumorigenic in nude mice, these cells are highly motile *in vitro* (35) and exhibit higher invasive activity relative to primary breast epithelial cells (32). We first confirmed that MCF10A suffer spontaneous morphologic changes depending on cell confluence, showing a "fibroblast like" spindle morphology in sparse culture conditions and an epithelial-like compact morphology in dense cultures (ref. 16; Fig. 3*A*). Moreover, in sparse cultures (spindle morphology), these cells displayed an important increase in actin stress fibers and focal adhesions, and in the organization of the vimentin cytoskeleton (Fig. 3*A*), suggesting they might be

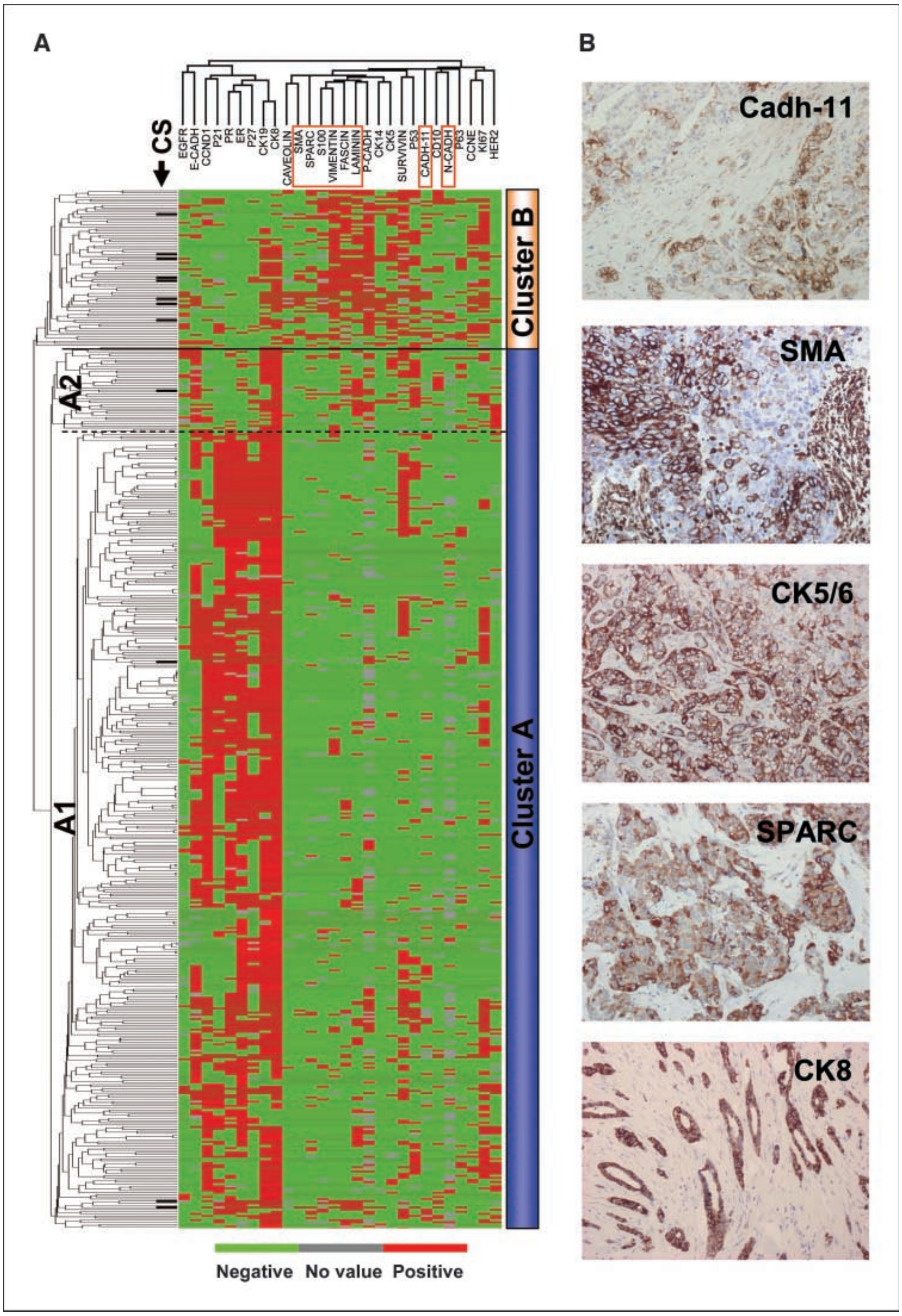

Figure 1. EMT markers are expressed in a specific subgroup of breast tumors. *A,* unsupervised hierarchical clustering of the expression of 28 immunohistochemical markers in 479 breast adenocarcinomas and 12 carcinosarcomas (*CS*, marked by "I") identifies phenotypically distinct subgroups of tumors. Each column represents an immunohistochemical variable (indicated on the right), and each row represents a tumor sample. Intensity of color is a function of the immunohistochemical expression level as depicted in the scale bar below. Tumors were divided into two main clusters (*A* and *B*, separated by a black line). Cluster A was further subdivided (*dotted line*) into groups "A1" (mostly hormonal receptors–positive) and "A2" (mostly hormonal receptors–negative). Positive expression of many EMT-related markers (enclosed within an orange rectangle on the right) occurred more frequently in cluster B than cluster A of tumors. *B,* representative examples of immunohistochemical staining of selected markers.

susceptible to EMT. Additionally, we showed that cadherin switching also occurred during the morphologic changes (Fig. 3*B–D*). Immunofluorescence, Western blot, and/or qRT-PCR detected a decrease in E- and P-cadherin, and the up-regulation of mesenchymal cadherins (N-cadherin and cadherin-11) in subconfluent cultures (Fig. 3*B–D*). Regarding the catenins, only γ-catenin showed an increase in total protein levels as the cells became confluent (Fig. 3*C*). However, similar to the mesenchymal component of carcinosarcomas, p120 and, to a lesser extent, β-catenin exhibited to a nucleocytoplasmic localization (Fig. 3*B*) in sparse spindle-shaped cells.

We next investigated whether the expression of some of the transcription factors identified as EMT regulators may correlate with these phenotypic changes. By qRT-PCR, we observed a significant increase in *Slug* (*SNAI2* gene) expression in sparse confluent cells, whereas the *TCF3* gene, encoding E12/E47transcription factors, was reduced (Fig. 3*D*). Expression of *Snail* (*SNAI1*) although very low in both culture conditions, was also increased in sparse cells.

To further study the molecular events associated with the plasticity of MCF10A cells, we characterized the genetic programs modulated during the phenotypic changes by cDNA microarrays analysis. After microarray data processing, a total of 356 transcripts (including 304 named genes with known function) were identified, whose expression was modulated at least 2-fold in response to cell confluence (the complete list of genes arranged by function is provided in the Supplementary Table S4). Focusing on the genes potentially associated with EMT, fibroblastic cells (sparse cultures) had increased the expression of some genes involved in cytoskeleton organization (*ANLN, KIF, PLEK2*, and *ARPC*), chemotaxis and cell motility (*FGF2, SDF2L1*, and *TGFB2*), and extracellular matrix remodeling and invasion (*MMP14, PLAUR, SERPINB2, SERPINE2, GSPG2*, and *HMMR*). In contrast, confluent cells increase the expression of genes related to cell-cell adhesion (*CDH1, JUP, DSC2/3*, and *DSP*), epithelial markers, and a number of cytokeratins (*KRT13, 14, 16*, and *19*; Supplementary Table S4). Microarray data corroborated the increase of E-cadherin (*CDH1*), γ-catenin (*JUP*), and E47 (*TCF3*) levels in confluent cells, as previously shown in Fig. 3*B* to *D*. Expression of *Slug* and *N-cadherin* showed a median change of <2-fold in the microarrays data (data not shown) and, thus, were omitted in the data preprocessing. However, they showed a statistically significant change using the highly sensitive method of qRT-PCR (Fig. 3*D*). The microarray data were further validated by qRT-PCR analysis. Specifically, levels of transcripts of the proinvasive factors *TGFB2* and *VEGFC*, as well as those of the tumor-promoting gene *STMN1* (Stathmin/oncoprotein 18), were significantly higher in fibroblastic (sparse) relative to epithelial (confluent) cells, whereas the opposite occurred regarding expression of the epithelial differentiation gene *FXYD3* (Fig. 4*A*).

Recent gene expression profiling of breast carcinoma cell lines has allowed the identification of a number of genes characterizing each of the breast tumor phenotypes (luminal, basal, and mesenchymal; ref. 31). Therefore, we next analyzed if any of those genes were modulated in MCF10A cells during the phenotypic switch. Relative to confluent cells, sparse-growing cells decreased expression of number of genes characteristic of luminal cells but increased expression of mesenchymal genes (Fig. 4*B*; Supplementary Table S4). Importantly, sparse-growing cells down-regulated the expression of some genes characteristic of the basal-like phenotype, including the typical myoepithelial markers CD10 (*MME* gene), CK14 (*KRT14*), and p63 (*TP73L*).

Overall, these data suggest that MCF10A cells have intrinsic phenotypic plasticity that makes them especially prone to undergoing spontaneous changes suggestive of EMT initiation, including morphologic modifications, cytoskeleton reorganization, vimentin and *Slug* up-regulation, cadherin swiching, and catenins delocalization.

Discussion

Recent gene expression profiling studies on breast cancer cell lines have shown that the most undifferentiated, fibroblastic, invasive, and metastasic cell lines [designated as "mesenchymal" (31) or "BasalB" (34)] and basal-like cells [also named "Basal" (31) or "BasalA" (34)] were closely related in terms of overall gene expression profile but were clearly different to the generally less invasive luminal-phenotype cells. Therefore, it was proposed that

Table 1. Association between selected immunohistochemical markers and the basal-like phenotype [defined according to Nielsen et al. (6) and Rakha et al. (30) criteria]

Variable	Nielsen et al. criteria			Rakha et al. criteria		
	Basal group	Nonbasal group	χ^2 test	Basal group	Nonbasal group	χ^2 test
E-Cadh	23/64 (35.9%)	198/399 (49.6%)	$P = 0.042$	29/83 (34.9%)	192/380 (50.5%)	$P = 0.010$
P-Cadh	47/62 (75.8%)	94/327 (28.7%)	$P < 0.001$	56/81 (69.1%)	85/308 (27.6%)	$P < 0.001$
N-Cadh	10/58 (17.2%)	28/325 (8.6%)	$P = 0.043$	12/73 (16.4%)	26/310 (8.4%)	$P = 0.038$
Cadh-11	17/64 (26.6%)	47/390 (12.1%)	$P = 0.002$	19/83 (23.0%)	45/371 (12.1%)	$P = 0.011$
CK 8	48/67 (71.6%)	397/425 (93.4%)	$P < 0.001$	69/88 (78.4%)	376/404 (93.1%)	$P < 0.001$
CK 19	41/67 (61.2%)	353/421 (83.8%)	$P < 0.001$	57/88 (64.8%)	337/400 (84.3%)	$P < 0.001$
CK 5/6*	53/67 (79.1%)	24/418 (5.7%)	$P < 0.001$	77/88 (87.5%)	0/397 (0%)	$P < 0.001$
CK 14 *	20/67 (29.9%)	13/423 (3.1%)	$P < 0.001$	33/88 (37.5%)	0/402 (0%)	$P < 0.001$
CD10	15/67 (22.4%)	33/417 (7.9%)	$P < 0.001$	16/87 (18.4%)	32/397 (8.1%)	$P = 0.004$
P63	14/65 (21.5%)	41/420 (9.8%)	$P = 0.005$	22/87 (25.3%)	33/398 (8.3%)	$P < 0.001$
Laminin	35/65 (53.8%)	86/417 (20.6%)	$P < 0.001$	39/87 (44.8%)	82/395 (20.8%)	$P < 0.001$
Fascin	29/65 (44.6%)	71/418 (17.0%)	$P < 0.001$	39/85 (45.9%)	61/398 (15.3%)	$P < 0.001$
Vimentin	44/67 (65.7%)	61/418 (14.6%)	$P < 0.001$	48/88 (54.5%)	56/399 (14.0%)	$P < 0.001$
SMA	14/67 (20.9%)	14/420 (3.3%)	$P < 0.001$	19/88 (21.6%)	9/399 (2.3%)	$P < 0.001$
SPARC	20/66 (30.3%)	31/411 (7.5%)	$P < 0.001$	25/87 (28.7%)	26/390 (6.7%)	$P < 0.001$
β-catenin †,‡	10/59 (16.9%)	12/392 (3.1%)	$P < 0.001$	10/75 (13.3%)	12/376 (3.2%)	$P < 0.001$
P120 †,‡	8/58 (13.8%)	22/377 (5.8%)	$P = 0.026$	5/72 (6.9%)	25/363 (6.9%)	NS
Grade I †	4/53 (7.5%)	55/365 (15.1%)		5/68 (7.4%)	54/350 (15.2%)	
Grade II	7/53 (13.2%)	157/365 (43.0%)		14/68 (20.6%)	150/350 (43.0%)	
Grade III	42/53 (79.2%)	153/365 (41.9%)	$P < 0.001$	49/68 (72.1%)	146/350 (41.8%)	$P < 0.001$

Abbreviations: NS, not statistically significant ($P > 0.05$). Cadh, Cadherin.
*Variables included in Nielsen et al. or Rakha et al. criteria to define the basal phenotype.
†Variables not included in the clustering analysis.
‡Only evaluating cytoplasmic staining.

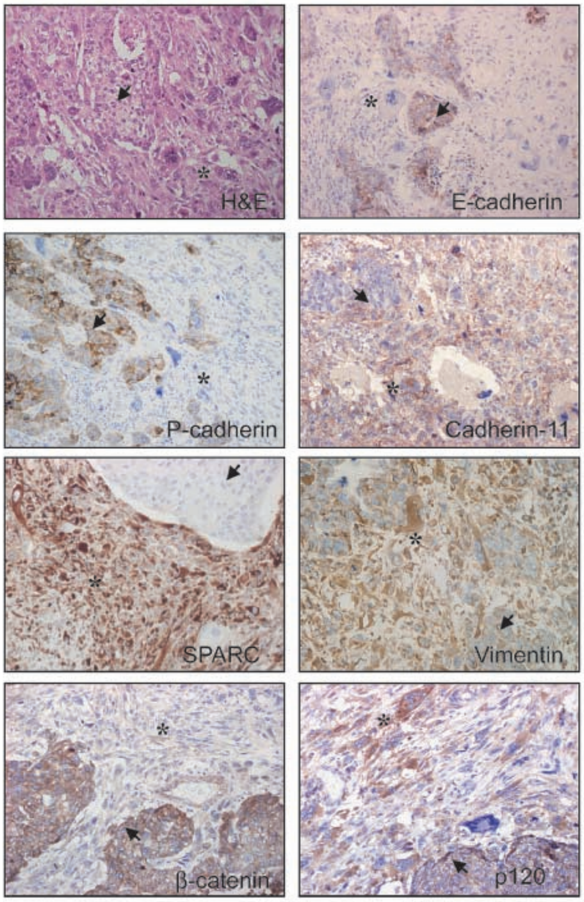

Figure 2. Immunohistochemical expression of cadherins, catenins, and mesenchymal markers in breast carcinosarcomas. *H&E,* H&E staining of a representative carcinosarcoma. *Arrows,* carcinomatous component; *, sarcomatous areas of the tumors.

EMT may preferentially occur within a specific genetic context, the basal-like phenotype (31, 32, 34, 36). To date, the possible association between EMT and basal-like phenotype in human breast tumors *in vivo* has not been assessed. Our study on 491 human breast tumors proves (for the first time) that the coordinated expression of EMT markers (vimentin, SMA, N-cadherin, cadherin-11, and SPARC) and of proteins involved in motility and extracellular matrix remodeling (fascin and laminin), together with a reduction in epithelial markers (E-cadherin and luminal cytokeratins), are more likely to happen in breast tumors with a basal-like phenotype. The association between the expression of EMT markers and basal-like tumors is observed regardless of the predefined criteria used by others (6, 30) to identify this phenotype and is also maintained when evaluating only grade III tumors. These data suggest that EMT may not be a sign of overall tumor dedifferentiation, but rather, the manifestation of a specific phenotype (basal-like) within aggressive breast tumors. Consistent to this hypothesis, the presence of spindle cell tumor areas is significantly more frequent in basal-like tumors than in other breast tumor types (37). Moreover, the observations presented here and previous data (29) clearly show that breast carcinosarcomas, considered as examples of complete EMT (12, 13), indeed show a basal-like phenotype. Thus, EMT may act mainly within specific tumor subtypes, such as poorly differentiated ductal tumors and carcinosarcomas, but probably not in others. For instance, although lobular breast carcinomas have a single-cell invasion pattern and lack E-cadherin (1), they do not show any other evidence of EMT (27, 38) or of the basal phenotype.

More importantly, the focal expression of mesenchymal markers (indicative of EMT) in basal-like tumors might be related with their poor prognosis and distinct metastatic spreading (2–4, 6, 7), as occurs with *in vitro* models for EMT. Thus, vimentin, N-cadherin, cadherin-11, fascin, or SPARC promote cancer migration and/or invasion for *in vitro* and *in vivo* models (15, 39–41), and their expression is associated with a poor prognosis and/or a tendency to develop visceral metastasis in breast cancer (7, 42, 43).

Nonetheless, other authors do not consider the expression of mesenchymal markers in breast cancer as a sign of EMT. For instance, Korsching et al. (44) reported that vimentin expression was also observed in some ductal carcinomas *in situ* (DCIS) and suggested a stem cell origin for vimentin-positive tumors. However, vimentin positivity in DCIS could also be interpreted as a sign of EMT proclivity, thus, the vimentin-positive cells being prone to the subsequent acquisition of mesenchymal markers and enhanced invasive potential. Accordingly, during the breast EMT cytoskeleton changes occur before the cadherin switching and invasion take place (16). Supporting this hypothesis, the frequency of expression of vimentin in breast tumors is markedly higher than that observed for other EMT markers such as SPARC, N-cadherin, or Cadherin-11 (Table 1).

Therefore, we suggest that basal-like cells (vimentin positive) may have a particular phenotypic plasticity that makes them especially prone to undergoing EMT, as exemplified by our studies with the MCF10A cell line. In response to a low cell density, these cells suffer spontaneous EMT-like phenotypic changes, including dramatic cytoskeleton reorganization, cadherin switching, and a cytosolic diffuse relocalization of catenins. Interestingly, the acquisition of this mesenchymal-like phenotype is required for these cells to migrate because the specific silencing of vimentin (39) or N-cadherin expression (16) effectively reduces the MCF10A motility and invasiveness. Although these cell density–dependent

Table 2. Differential expression of immunohistochemical markers between the epithelial and mesenchymal components of breast carcinosarcomas (n = 12)

Immunohistochemical marker	Epithelial (carcinoma)	Mesenchymal (sarcoma)
SPARC-positive	5 (42%)	10 (83%)
Vimentin-positive	10 (83%)	12 (100%)
E-cadherin–positive	12 (100%)	0 (0%)
P-cadherin–positive	11 (92%)	0 (0%)
Cadherin-11–positive	2 (17%)	10 (83%)
β-catenin:		
Membrane-conserved	10 (83%)	2 (17%)
Membrane-reduced	2 (17%)	7 (58%)
Cytoplasmic/nuclear	0 (0%)	3 (25%)
p120:		
Membrane-conserved	10 (83%)	2 (17%)
Membrane-reduced	2 (17%)	2 (17%)
Cytoplasmic/nuclear	0 (0%)	8 (67%)

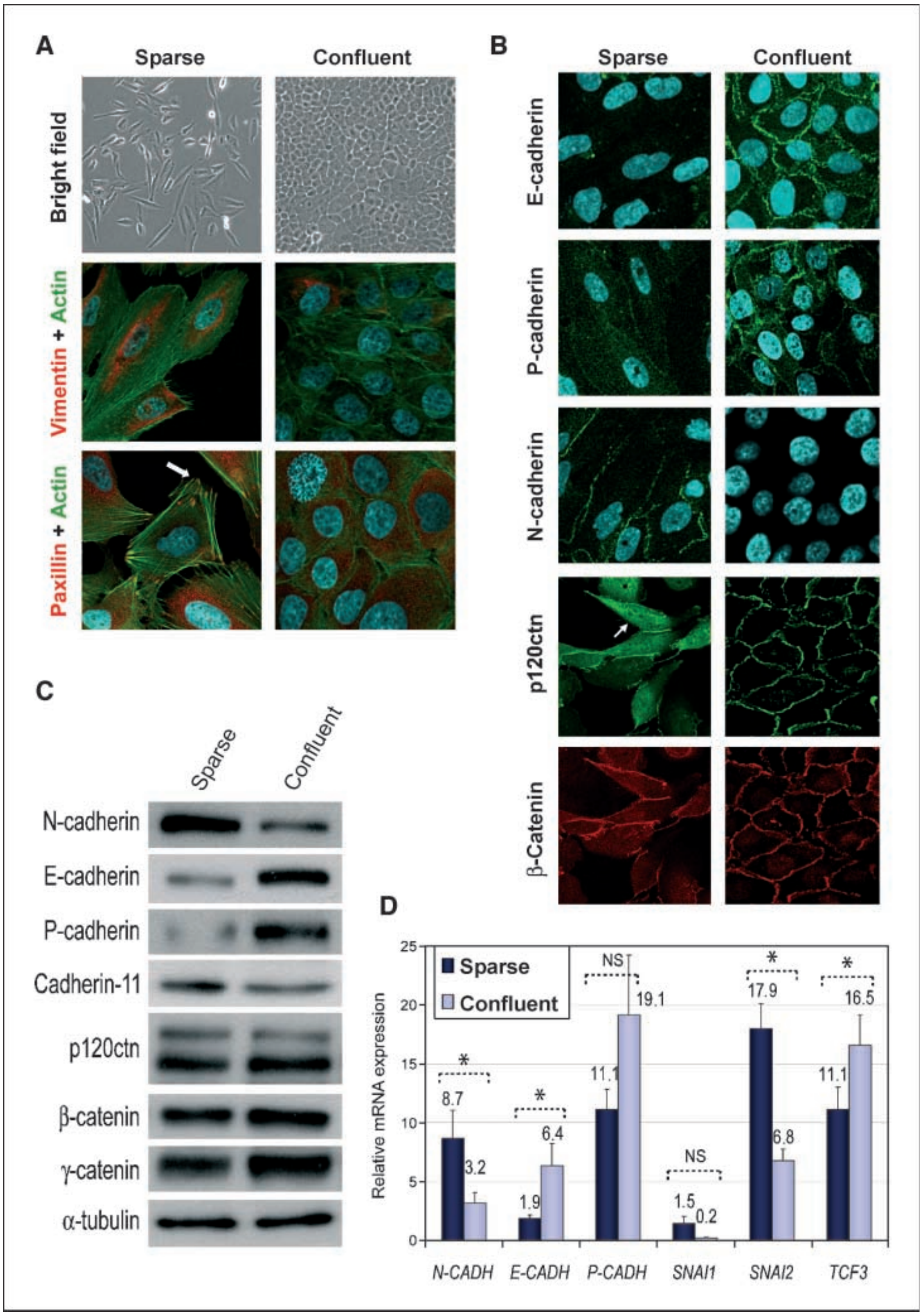

Figure 3. MCF10A cells suffer spontaneous morphologic and phenotypic EMT-like changes in response to different cell confluence. *A,* phenotypic characterization of MCF10A cells grown at low (*sparse*) and high (*confluent*) cell density. Phase contrast images (*top*) and analysis of the cytoskeleton organization and focal adhesion (*middle* and *bottom*). Sparse cells show an increase in actin stress fibers (*green*), vimentin expression (*red, middle*) and in focal adhesions (stained by paxillin; *red, arrow, bottom*). *B,* cadherin switching and catenin delocalization (*arrow*) in MCF10A sparse cells assessed by immunofluorescence staining. *C,* differential expression of cadherins and catenins by Western blot. *D,* measurement of mRNA expression of the cadherins and the EMT inducers Snail (*SNAI1*), Slug (*SNAI2*), and E47 (*TCF3*) by quantitative RT-PCR. *Columns,* mean gene expression; *bars,* SE (mRNA levels relative to control *B2M* transcript) from four different experiments under sparse or confluent growth conditions. Mean differences were compared by Student's *t* test; *, differences statistically significant at a *P* value of <0.05; *NS,* not significant.

phenotypic changes are transient, they involve the modulation of a number of EMT genes (Supplementary Table S4) and the attenuation of some typical luminal and myoepithelial characteristics in sparse cultured cells (spindle cells). Similarly, although a mesenchymal expression signature is in the main part shared by "Basal/basalA" cells and "Mesenchymal/basalB" cell lines, the latter group shows a reduction of some typical myoepithelial markers such as basal cytokeratins (31, 34, 36).

The signals and mechanisms responsible for triggering EMT processes in basal-like tumors are unknown. In some tumor types, nuclear β-catenin and the subsequent regulation of its gene targets are associated with a focal induction of EMT (12–14). However, although cytoplasmic β-catenin staining tends to be more frequent in basal-like tumors than in nonbasal ones (Table 1), no evident nuclear β-catenin was observed, except in three carcinosarcomas. Thus, no obvious relationship between β-catenin signaling and the EMT induction was observed in basal-like tumors. The activation of the transforming growth factor-β (TGFβ) signaling pathway and the subsequent up-regulation of the EMT inducers Snail, Slug, Twist, and ZEB, lead to a complete EMT in several cancer models (12, 14, 23). Moreover, "basal-like/BasalA" and "mesenchymal/BasalB" breast cancer cell lines show higher endogenous levels of *TGFB1, TGFB2, Slug, ZEB1*, and *Twist* with respect to luminal cell lines (31, 33, 36). In our MCF10A cellular model, the expression of *TGFB2* and *Slug* is significantly increased in the sparse-cultured cells relative to confluent cells, but, to achieve a more mesenchymal and motile phenotype, these cells require a long-term treatment with TGFβ

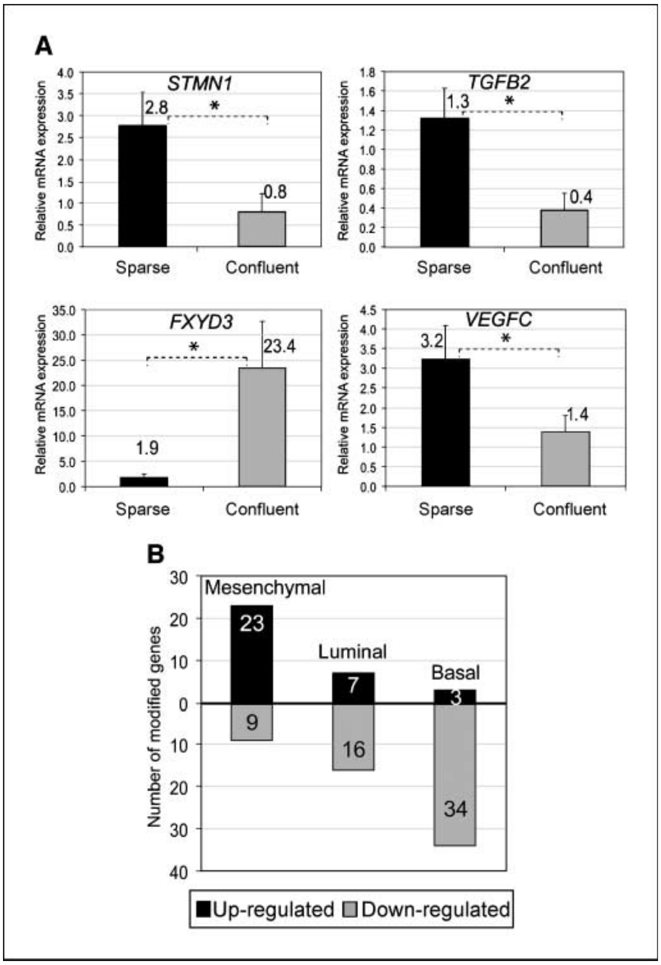

Figure 4. Gene expression modifications associated with EMT-like phenotypic changes in MCF10A cells. *A,* validation of microarray data by qRT-PCR of the indicated genes. *Columns,* mean gene expression; *bars,* SE from four independent experiments. Mean differences were compared by Student's *t* test; *, differences statistically significant at a *P* value of <0.05. *B,* number of genes previously classified as characteristic of luminal, basal, or mesenchymal phenotypes [according to Charafe-Jauffret et al. (31)], which were overexpressed (*black bars*) or down-regulated (*gray bars*) in sparse cells relative to confluent MCF10A cells.

(16). Furthermore, a recent report has shown that Slug mRNA is up-regulated in a subset of human basal-like carcinomas and points to Slug as the most likely candidate for modulating the phenotypic and invasive properties of these tumors (45). Moreover, the mesenchymal transcription factor FOXC2, which promotes EMT and metastasis *in vivo*, has also been associated with basal-like cancers (46). The development of specific antibodies against these EMT inducers that function successfully in paraffin-embedded tissues will help to clarify their role in basal-like tumors *in vivo*.

Finally, increasing evidence indicate a link among basal-like tumors, the stem cell phenotype, EMT, and the acquisition of tumorigenic, invasive, and metastatic potential (reviewed in ref. 36). Stem cells from normal and tumor breast tissue have a basal-like phenotype (47) and are enriched in the expression of genes involved in EMT (e.g., *Vimentin, Slug, CTGF, MMP9, SPARC, N-cadherin*, and *SIP1*; refs. 45, 48, 49). Subpopulations of cancer cells with stem properties are especially frequent within basal-like and fibroblastic breast cell lines (50) and show increased tumorigenic and invasive potential (47, 50). In addition, stem cell–like breast cell lines (e.g., MCF10A and PMC42-LA) are able to undergo EMT (36). Overall, these data suggest that the special proclivity of basal-like cancer cells to undergo EMT may reflect the intrinsic phenotypic plasticity of cancer stem cells. Further studies are required to clarify whether any of the EMT inducers are involved in breast stem cells differentiation and/or in the acquisition of invasive properties by cancer stem cells.

In summary, the data presented here indicate that EMT-like changes occur preferentially in the basal subtype of breast carcinomas. Furthermore, they suggest that cells with a basal-like (stem cell) phenotype may be especially prone to undergoing EMT-like changes, (breast carcinosarcomas and fibroblastic cancer cell lines being extreme examples of this phenotypic plasticity). The likely proclivity of basal-like cells to a mesenchymal transition may be related to the high aggressiveness and the characteristic metastatic spreading of these tumors.

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