

Heterogeneity for Stem Cell–Related Markers According to Tumor Subtype and Histologic Stage in Breast Cancer

So Yeon Park^{1,2,3}, Hee Eun Lee², Hailun Li^{4,5,6}, Michail Shipitsin^{3,5},
Rebecca Gelman^{4,5,6}, and Kornelia Polyak^{3,5}

Abstract

Purpose: To evaluate the expression of stem cell–related markers at the cellular level in human breast tumors of different subtypes and histologic stage.

Experimental Design: We performed immunohistochemical analyses of 12 proteins [CD44, CD24, ALDH1, vimentin, osteonectin, EPCR, caveolin 1, connexin 43, cytokeratin 18 (CK18), MUC1, claudin 7, and GATA3] selected based on their differential expression in breast cancer cells with more differentiated and stem cell–like characteristics in 47 cases of invasive ductal carcinoma (IDC) only, 135 cases of IDC with ductal carcinoma *in situ* (DCIS), 35 cases of DCIS with microinvasion, and 58 cases of pure DCIS. We also analyzed 73 IDCs with adjacent DCIS to determine the differences in the expression of markers by histology within individual tumors. CD44+/CD24– and CD24–/CD24+ cells were detected using double immunohistochemistry.

Results: CD44 and EPCR expression was different among the four histologic groups and was lower in invasive compared with *in situ* tumors, especially in luminal A subtype. The expression of vimentin, osteonectin, connexin 43, ALDH1, CK18, GATA3, and MUC1 differed by tumor subtype in some histologic groups. ALDH1–positive cells were more frequent in basal-like and HER2+ than in luminal tumors. CD44+/CD24– cells were detected in 69% of all tumors with 100% of the basal-like and 52% of HER2+ tumors having some of these cells.

Conclusions: Our findings suggest that in breast cancer, the frequency of tumor cells positive for stem cell–like and more differentiated cell markers varies according to tumor subtype and histologic stage.

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Breast cancer is not a single disease but a heterogeneous group of diseases with different pathologies, biological characteristics, and clinical behavior (1–4). Comprehensive gene expression profiling using DNA microarrays has identified five major molecular subtypes: luminal A, luminal B, HER2+, basal-like, and normal breast-like (2–4). Tumors in different subtypes follow different clinical courses and respond differently to treatment. In general, patients with basal-like tumors have worse prognosis whereas those with luminal A tumors have more favorable outcomes (3, 4). In addition to this intertumor heterogeneity, there is also a high degree of intratumor diversity that is already present in ductal carcinoma *in situ* (DCIS;

ref. 5). Specifically, a single tumor at any given time can contain tumor cell populations with distinct molecular profiles and biological properties.

Currently prevailing models explaining intratumor heterogeneity include the clonal evolution and cancer stem cell hypotheses (6, 7). The clonal evolution model emphasizes diversity for heritable traits that in combination with continuous selection allows for the expansion of tumor cells with the most favorable characteristics (8). According to the cancer stem cell hypothesis, cancer stem cells, defined as a subset of tumor cells with stem cell–like features, have the capacity to self-renew and differentiate, giving rise to a heterogeneous tumor cell population (9–12). In breast cancer, $lin^-/CD44^+/CD24^{-/low}$ cells were identified as candidate breast cancer stem cells based on xenotransplant assays in nonobese/severe combined immunodeficient mice (13). More recently, additional markers including aldehyde dehydrogenase (ALDH) activity (14), CD133 (15, 16), and ITGA6 (17, 18) have also been proposed as putative markers of mammary epithelial and breast cancer stem cells.

In a previous study, we investigated the genome-wide gene expression profiles of $lin^-/CD44^+/CD24^{-/low}$ (CD44+) and more differentiated luminal epithelial CD44–/CD24+ (CD24+) breast cancer cells and determined that these were consistent with the presumed identity of these cells (19).

Authors' Affiliations: ¹Department of Pathology, Seoul National University College of Medicine, Seoul, Korea; ²Department of Pathology, Seoul National University Bundang Hospital, Seongnam, Gyeonggi, Korea; Departments of ³Medical Oncology and ⁴Biostatistics and Computational Biology, Dana-Farber Cancer Institute; ⁵Harvard Medical School, and ⁶Harvard School of Public Health, Boston, Massachusetts

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Corresponding Author: Kornelia Polyak, Dana-Farber Cancer Institute, 44 Binney Street D740C, Boston, MA 02115. Phone: 617-632-2106; Fax: 617-582-8490; E-mail: Kornelia_Polyak@dfci.harvard.edu.

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Translational Relevance

The presence of cancer cells with stem cell–like characteristics within primary invasive breast tumors have been shown to correlate with higher risk of distant metastasis and poor clinical outcome. However, distant metastases frequently are composed of tumor cells with more differentiated luminal epithelial features. The goal of this study was to analyze if similar divergence in cellular phenotypes also occurs between *in situ* and invasive breast carcinomas and if this is associated with a particular tumor subtype. Tumor cell phenotypic plasticity and intratumor diversity have significant effect on the efficacy of cancer preventative and therapeutic strategies.

Specifically, CD44+ cells showed a more mesenchymal stem cell–like profile enriched for genes involved in cell motility, proliferation, and angiogenesis, whereas CD24+ cells highly expressed genes implicated in carbohydrate metabolism and RNA splicing. We also found that stem cell–like gene expression patterns in lymph node–negative primary invasive breast tumors correlated with shorter distant metastasis-free survival (19, 20). However, distant metastases were largely composed of more differentiated CD24+ breast cancer cells implying a phenotypic switch during metastatic colonization and tumor growth or clonal selection for CD24+ tumor cells in distant metastases.

In breast cancer cell lines, prior studies revealed an enrichment of CD44+/CD24– and CD24–/CD24+ phenotypes in basal-like and luminal subtypes, respectively (21, 22). Similarly, ALDH1 activity was only detected in basal-like breast cancer cell lines (23). In contrast, most studies analyzing primary human breast cancer samples have not revealed a significant association between CD44 and CD24 expression and tumor subtypes (24–26). Only one recent study reported the enrichment of CD44+/CD24– cancer cells in basal-like invasive breast tumors (27). The expression of ALDH1 in invasive breast tumors was also correlated with poor clinical outcome (14), but the association of this with tumor subtypes was not reported.

In this study, we investigated the expression of 12 proteins [CD44, CD24, ALDH1, vimentin, osteonectin, EPCR, caveolin 1, connexin 43, cytokeratin 18 (CK18), MUC1, claudin 7, and GATA3], selected based on our gene expression data (19) as differentially expressed in breast cancer cells with more differentiated luminal and stem cell–like characteristics, in 182 invasive ductal carcinomas (IDC; 135 with associated DCIS) and in 93 DCIS (35 with microinvasion) with the goal of correlating the expression of these 12 proteins with clinicopathologic characteristics of the tumors including molecular subtype and histologic stage. We also analyzed *in situ* and the invasive areas of 73 additional IDCs with adjacent DCIS to define progression-related changes within

individual cases. CD44+ stem cell–like and CD24+ more differentiated luminal cancer cells were further defined using double immunohistochemistry (IHC).

Materials and Methods

Tissue specimens. Three hundred ninety-seven (397) cases of surgically resected ductal breast cancers including 289 IDCs with or without adjacent DCIS and 108 DCIS with or without microinvasion were collected in Seoul National University Bundang Hospital during 2003 to 2007. Seventy-three IDCs with adjacent DCIS were evaluated using full tissue sections. Tissue microarrays (TMA) were constructed from representative tissue column (2.0 mm in diameter for IDC; 4.0 mm in diameter for DCIS) of 216 IDCs (with or without adjacent DCIS) and 108 DCIS (with or without microinvasion) as described previously (28). The TMAs were prepared with invasive tissue only from the cases of IDC with adjacent DCIS, but the cases of DCIS with microinvasion that were used in the TMAs might have included small numbers of IDC cells in addition to the DCIS cells. All patients except two (99.5%) were female, with a median age of 49 y (range, 26–83 y). Clinicopathologic information was obtained by reviewing pathology reports and H&E-stained sections. The following histopathologic variables were determined in IDCs: tumor subtype; T stage; presence of cancer in ipsilateral axillary lymph nodes; Bloom-Richardson histologic grade; estrogen receptor (ER), progesterone receptor (PR), and HER2 status; Ki-67 and P53; and presence of adjacent DCIS. For DCIS cases with or without microinvasion, we recorded tumor size; nuclear grade; ER, PR, and HER2 status; Ki-67 and P53 staining patterns; and presence of microinvasion. All cases were independently reviewed by two breast pathologists (SYP and HEL). This study was approved by the Institutional Review Board of Seoul National University Bundang Hospital (protocol # B-0809-061-301) and Dana-Farber Cancer Institute (protocol #98-229).

Immunohistochemical analyses. Four-micrometer-thick, formalin-fixed, paraffin-embedded tissue sections were cut, dried, deparaffinized, and rehydrated following standard procedures. All sections were subjected to heat-induced antigen retrieval process in citrate (pH 6.0) or Tris-EDTA (pH 9.0) buffer or by protease treatment. Following staining optimization using positive and negative controls, 12 antibodies that provided satisfactory results together with standard prognostic biomarkers were used for the study (Supplementary Table S1). Immunohistochemical staining was done using a Vectastain Elite avidin-biotin complex detection kit (Vector Laboratories) or DAKO-Envision detection kit (DAKO), and antibodies were visualized with 3,3'-diaminobenzidine (Sigma-Aldrich). In double immunohistochemical staining to detected CD44+/CD24– and CD24–/CD24+ cells, CD44 was visualized with Vectastain Elite avidin-biotin complex detection kit and diaminobenzidine as chromogen, whereas CD24 was detected with a polymer-linked alkaline phosphatase–conjugated

Table 1. Distributions of the tumors in the four histologic groups

Subtype	Luminal A		Luminal B		HER2+		Basal-like		Total
IDC alone	35	75%	1	2%	5	11%	6	13%	47
IDC with DCIS	85	63%	16	12%	18	13%	16	12%	135
DCIS with microinvasion	11	31%	6	17%	14	40%	4	11%	35
Pure DCIS	41	71%	7	12%	9	16%	1	2%	58
Total	172	63%	30	11%	46	17%	27	10%	275

NOTE: Number of patients (and raw percentage) in each of the histologic groups who had each of these tumor subtypes.

secondary antibody (DAKO) and was visualized with fast red. Sections were counterstained with Mayer's hematoxylin.

Fluorescence in situ hybridization assays for HER2 gene amplification. To determine HER2 gene amplification, the PathVysion (Vysis) assay was carried out and evaluated as described previously (29).

Definition of breast tumor subtypes. Breast tumor subtypes were defined according to Carey et al. (30) with minor modifications. Subtype definitions were as follows: luminal A (ER+ and/or PR+, HER2-), luminal B (ER+ and/or PR+, HER2+), HER2+ (ER-, PR-, HER2+), and basal-like [ER-, PR-, HER2-, basal cytokeratin+, and epidermal growth factor receptor (EGFR)+/-]. Immunostains for ER and PR were done on full sections, and cases with 10% or more positive staining were grouped as positive. Epidermal growth factor receptor and basal cytokeratins (cytokeratin 5/6, 14, and 17) were evaluated using TMAs or full sections (for 73 IDCs with adjacent DCIS), and cases with any positive membranous staining were grouped as positive using previously established and published criteria (31). HER2 positivity was determined based on fluorescence *in situ* hybridization results as described in ref. (29).

Immunohistochemical scoring. After omitting 34 cases with uninterpretable IHC results, a total of 193 IDCs (139 with DCIS) and 97 DCIS (37 with microinvasion) were informative (interpretable staining) for all antibodies in TMA and used for the analyses. For subtype analyses, a total of 182 IDCs (135 also having DCIS) and 93 cases of DCIS (35 also having microinvasion) were used after omitting 15 cases with unknown tumor subtypes (Table 1). Staining of markers was scored in 10% increments (i.e., 0%, 10%, 20%, etc.) For statistical analysis, we coded 12 proteins analyzed into binary (two valued) covariates (Supplementary Table S2). The definition of "positive" stain for each marker was made before any comparison of groups and depended on the distribution of scores for that marker; in each case, our aim was to choose a cutoff for positivity so that at least 10% of cases were categorized as positive and at least 10% of cases were categorized as "negative" (because there would be almost no power in this study to detect differences between groups if fewer than 10% of cases were in either category). The cutoff of >10% satisfied this condition for CD24, CD44, GATA3, connexin 43, claudin7, and ALDH. Osteonectin, vimentin, caveolin1, and EPCR all had fewer than 10%

of cases with staining >10%; so for these markers, the cutoff used was >0%. On the other hand, for MUC1 and CK18, the mode of staining was at 90% or 100% (unlike all the other markers that had modes of 0% or 10%); so for these two markers, a cutoff of >80% was used and called "highly positive." Because many investigators use a uniform staining cutoff for all markers, we also redid all analyses using a cutoff of >10% for all markers; the text mentions when this uniform cutoff resulted in a difference in which comparisons were significant. Two additional binary variables created by dividing the cases as having more than the median percentage of stained cells (or not) were defined for two markers (CD44 and GATA3) that seemed to have very spread out distributions (CD44 median was 20% in the two IDC groups combined and 50% in the two DCIS groups combined; GATA3 median was 70% in the two IDC groups combined and 50% in the two DCIS groups combined).

Statistical analysis. Statistical analysis was done using SAS and StatXact for the analysis. Fisher exact tests were used when comparing the binary categories of expression of proteins between groups. For 73 IDCs with adjacent DCIS, Wilcoxon signed-rank tests were used to see if the (paired) difference between the expression levels in the invasive and DCIS components was significantly different from random. Differences of continuous variables between groups were evaluated using Wilcoxon rank-sum tests. Significance levels were adjusted for multiple comparisons using Holm's method. All *P* values reported are two sided.

Results

Histologic groups and distribution of tumor subtypes. We categorized the tumors into four histologic groups: IDC alone, IDC with DCIS, DCIS with microinvasion, and pure DCIS (Table 1). The distribution of the four tumor subtypes differed significantly ($P = 0.0001$) among the four histologic groups, with luminal A tumors being the least common in the DCIS with microinvasion, luminal B tumors being the least common in the IDC alone, HER2+ tumors being the most common in the DCIS with microinvasion, and basal-like tumors being the least common in the pure DCIS groups. The distributions of the four tumor subtypes were not significantly different in the two IDC

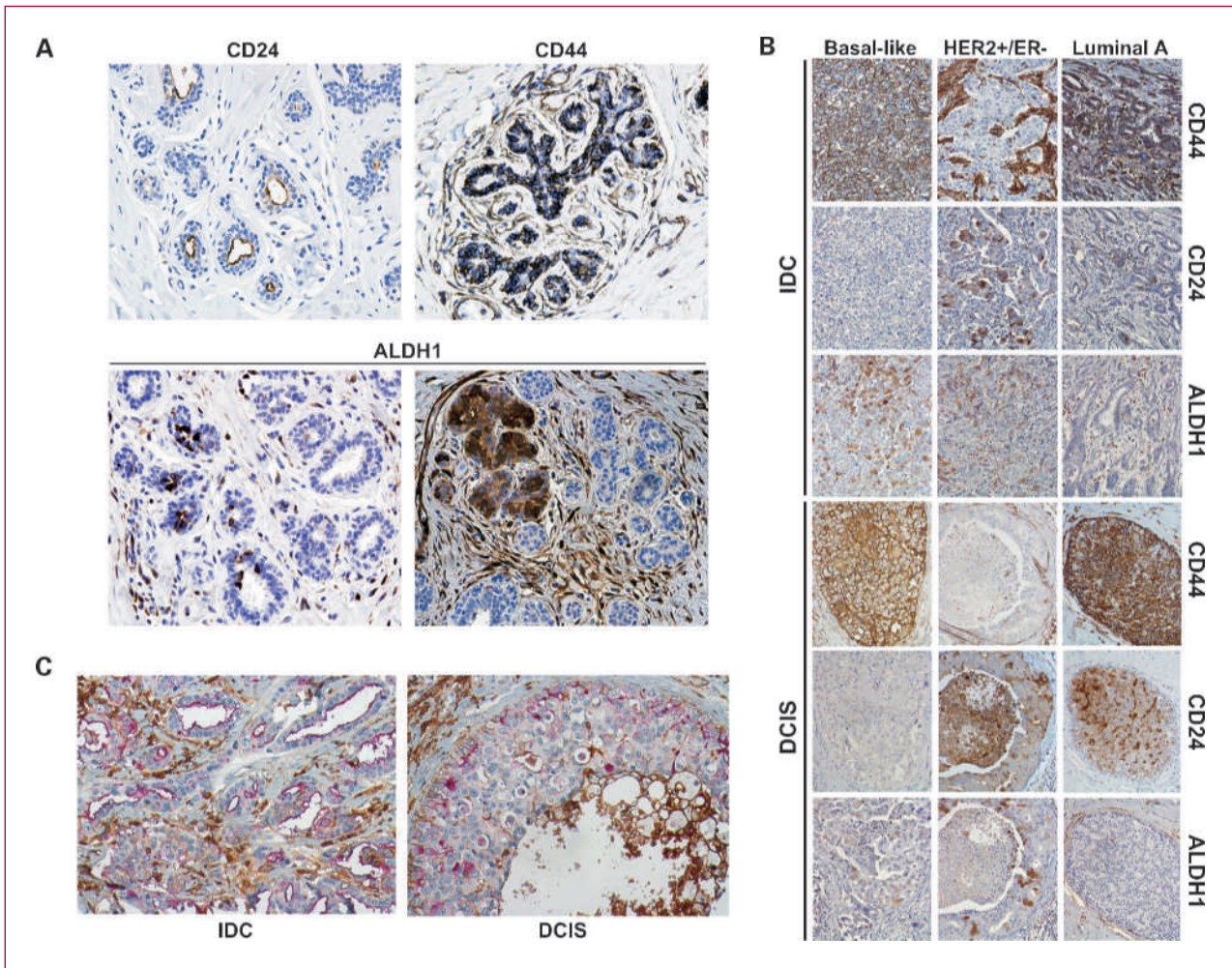


Fig. 1. Immunohistochemical analyses of CD44, CD24, and ALDH1 in breast cancer. A, immunohistochemical staining patterns in normal breast tissue. CD24 and CD44 displayed uniform luminal and basal cell-specific pattern, respectively, whereas ALDH1 expression varied depending on the area of the tissue. B, representative examples of immunohistochemical analyses of IDC and DCIS according to tumor subtype using the indicated antibodies. In luminal A subtype, tumor cells positive for CD44 and CD24 (apical membranous pattern) can be seen whereas ALDH1 was not detected. HER2+ tumor is negative for CD44, and is positive for CD24 (membrano-cytoplasmic pattern) and ALDH1. In basal-like subtype, tumor cells are positive for CD44 and ALDH1, but negative for CD24. C, representative examples of double IHC for CD44 (brown) and CD24 (red) in IDC and in DCIS.

groups (IDC alone and IDC with DCIS, $P = 0.81$), but were significantly different in the two DCIS groups (pure DCIS and DCIS with microinvasion, $P = 0.0006$). However, the DCIS with microinvasion group could be considered to have both invasion and DCIS, and might be expected to share some characteristics with the two IDC groups. Therefore, we opted to conduct the analysis using four histologic groups.

Expression of CD44, CD24, and ALDH1 in the four histologic groups. We first analyzed the expression of CD44, CD24, and ALDH1 markers previously shown to be associated with more differentiated luminal epithelial (CD24) and stem cell-like (CD44 and ALDH1) characteristics. These markers were also used for the detection of these respective cells (CD44, CD24, and ALDH1; ref. 14, 19) in 20 normal breast tissue samples to define their normal cell-

ular distribution pattern (Fig. 1A). In normal breast tissue, CD44 was localized to the cell membranes of basal/myoepithelial and a subset of luminal epithelial cells. CD24 expression was occasionally found on the apical membranes of luminal cells. ALDH1 was expressed heterogeneously in both luminal and basal cells, and in some lobules, all cells were ALDH1+ (Fig. 1A).

Next, we investigated the expression of these markers in the tumor samples (Supplementary Table S3 and S4; Fig. 1B). Strong membranous CD44 staining was detected in the majority of tumors, although a few cases displayed weak, incomplete membranous staining pattern. CD44 was expressed in 57% of IDC-only cases, in 59% of IDCs with DCIS, in 62% of DCIS with microinvasion, and in 85% of pure DCIS ($P = 0.002$). CD24 was expressed in 28% of IDC only tumors, in 38% of IDCs with DCIS,

59% of DCIS with microinvasion, and 62% of pure DCIS ($P = 0.02$). Two different CD24 staining patterns that were observed were almost mutually exclusive: apical membranous (reminiscent of expression pattern observed in normal breast tissue) and circumferential membrano-cytoplasmic. Of the total of 117 cases that were CD24 positive, 35 (30%) showed apical staining and the remaining 82 (70%) showed cytoplasmic staining, but this differed considerably by histologic group. Among the 15, 53, 22, and 27 CD24-positive cases in the four histologic groups, 47%, 26%, 9%, and 44% of the IDC alone, IDC with DCIS, DCIS with microinvasion, and pure DCIS had apical staining ($P = 0.02$). ALDH1 was expressed in few cases (9%, 6%, 3%, and 3% in each of the four histologic groups; $P = 0.56$).

To determine if the expression of these markers may be associated with some characteristics of the tumors, we compared the available clinical/pathologic features of positive and negative cases (Supplementary Table S3). After

adjusting for multiple comparisons, the following significant associations were found. In IDC alone cases, a tumor with histologic grade III was more likely to be CD44 positive than a tumor with histologic grade I or II (82% versus 41%; $P = 0.005$), and an ER-negative tumor was more likely to be ALDH1 positive than an ER-positive tumor (24% versus 0%, $P = 0.006$).

Combined expression of CD44 and CD24 according to tumor subtype and histologic stage. Next, we analyzed the combined expression of CD44 and CD24 in the same tumor according to histologic groups and tumor subtypes (Table 2). Considering only the histologic group (the column labeled "Total"), of the four possible combined expression patterns (positivity for both markers, CD44 only, CD24 only, and lack of both CD24 and CD44), the "CD44-only" pattern was the most common one in each histologic stages. However, the four histologic groups did differ significantly ($P = 0.0001$) in pattern, with pure

Table 2. CD24 and CD44 expression pattern defined based on immunohistochemical analyses of adjacent sections for the two markers according to tumor subtype and histologic groups

Histologic groups	Pattern	Tumor subtypes				Total $P = 0.0001$
		Luminal A	Luminal B	HER2+	Basal-like	
		$P = 0.001$	$P = NS$	$P = NS$	$P = NS$	
IDC alone* $P = NS$	CD44 and CD24	7 (20)	0 (0)	0 (0)	0 (0)	7 (15)
	CD44 only	9 (26)	1 (100)	3 (60)	5 (83)	18 (38)
	CD24 only	4 (11)	0 (0)	1 (20)	0 (0)	5 (11)
	Lack of both	15 (43)	0 (0)	1 (20)	1 (17)	17 (36)
	Total	35 (100)	1 (100)	5 (100)	6 (100)	47 (100)
IDC with DCIS $P = NS$	CD44 and CD24	24 (28)	3 (19)	4 (22)	2 (13)	33 (24)
	CD44 only	30 (35)	6 (37)	2 (11)	10 (63)	48 (36)
	CD24 only	10 (12)	4 (25)	5 (28)	0 (0)	19 (14)
	Lack of both	21 (25)	3 (19)	7 (39)	4 (25)	35 (26)
	Total	85 (100)	16 (100)	18 (100)	16 (100)	135 (100)
DCIS with microinvasion $P = NSAA$	CD44 and CD24	4 (36)	3 (50)	3 (21)	0 (0)	10 (29)
	CD44 only	5 (46)	2 (33)	1 (7)	4 (100)	12 (34)
	CD24 only	2 (18)	1 (17)	7 (50)	0 (0)	10 (29)
	Lack of both	0 (0)	0 (0)	3 (21)	0 (0)	3 (9)
	Total	11 (100)	6 (100)	14 (100)	4 (100)	35 (100)
Pure DCIS† $P = NS$	CD44 and CD24	15 (37)	3 (43)	3 (33)	1 (100)	22 (38)
	CD44 only	21 (51)	3 (43)	4 (44)	0 (0)	28 (48)
	CD24 only	4 (10)	1 (14)	0 (0)	0 (0)	5 (9)
	Lack of both	1 (2)	0 (0)	2 (22)	0 (0)	3 (5)
	Total	41 (100)	7 (100)	9 (100)	1 (100)	58 (100)
Total $P = 0.0006$	CD44 and CD24	50 (29)	9 (30)	10 (22)	3 (11)	72 (26)
	CD44 only	65 (38)	12 (40)	10 (22)	19 (70)	106 (39)
	CD24 only	20 (12)	6 (20)	13 (28)	0 (0)	39 (14)
	Lack of both	37 (22)	3 (10)	13 (28)	5 (19)	58 (21)
	Total	172 (100)	30 (100)	46 (100)	27 (100)	275 (100)

NOTE: Numbers in parentheses indicate column percentage. P values were calculated using Fisher's exact tests.

Abbreviations: NS, not significant (crude, unadjusted $P > 0.05$); NSAA, not significant after adjustment (using Holm's method).

*Luminal B subtype was omitted when calculating the P values, except for Total.

†Basal-like subtype was omitted when calculating the P values, except for Total.

Table 3. Presence of CD44+/CD24- and CD44-/CD24+ cells defined by double IHC according to tumor subtypes and histologic groups

	Cell staining		Tumor subtypes				Total
	CD44+/CD24- cells	CD44-/CD24+ cells	Luminal A	Luminal B	HER2+	Basal-like	<i>P</i> < 0.0001
			<i>P</i> = 0.0009	<i>P</i> = NS	<i>P</i> = NS	<i>P</i> = NS	
IDC alone* <i>P</i> = NS	Positive	Positive	1 (3)	0 (0)	0 (0)	1 (17)	2 (4)
	Positive	Negative	19 (54)	1 (100)	3 (60)	5 (83)	28 (60)
	Negative	Positive	6 (17)	0 (0)	1 (20)	0 (0)	7 (15)
	Negative	Negative	9 (26)	0 (0)	1 (20)	0 (0)	10 (21)
	Total		35 (100)	1 (100)	5 (100)	6 (100)	47 (100)
IDC with DCIS <i>P</i> < 0.0001	Positive	Positive	10 (12)	2 (13)	2 (11)	1 (6)	15 (11)
	Positive	Negative	43 (51)	8 (50)	5 (28)	15 (94)	71 (53)
	Negative	Positive	16 (19)	6 (38)	8 (44)	0 (0)	30 (22)
	Negative	Negative	16 (19)	0 (0)	3 (17)	0 (0)	19 (14)
	Total		85 (100)	16 (100)	18 (100)	16 (100)	135 (100)
DCIS with microinvasion <i>P</i> = NS	Positive	Positive	6 (55)	3 (50)	3 (21)	1 (25)	13 (37)
	Positive	Negative	4 (36)	2 (33)	4 (29)	3 (75)	13 (37)
	Negative	Positive	1 (9)	1 (17)	7 (50)	0 (0)	9 (26)
	Negative	Negative	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Total		11 (100)	6 (100)	14 (100)	4 (100)	35 (100)
Pure DCIS† <i>P</i> = NS	Positive	Positive	12 (29)	2 (29)	2 (22)	0 (0)	16 (28)
	Positive	Negative	23 (56)	3 (43)	5 (56)	1 (100)	32 (55)
	Negative	Positive	4 (10)	0 (0)	1 (11)	0 (0)	5 (9)
	Negative	Negative	2 (5)	2 (29)	1 (11)	0 (0)	5 (9)
	Total		41 (100)	7 (100)	9 (100)	1 (100)	58 (100)
Total <i>P</i> = 0.0006	Positive	Positive	29 (17)	7 (23)	7 (15)	3 (11)	46 (17)
	Positive	Negative	89 (52)	14 (47)	17 (37)	24 (89)	144 (52)
	Negative	Positive	27 (16)	7 (23)	17 (37)	0 (0)	51 (19)
	Negative	Negative	27 (16)	2 (7)	5 (11)	0 (0)	34 (12)
	Total		172 (100)	30 (100)	46 (100)	27 (100)	275 (100)

NOTE: Numbers in parentheses indicate column percentage. *P* values were calculated using Fisher's exact tests.

*Luminal B subtype was omitted when calculating the *P* values, except for Total.

†Basal-like subtype was omitted when calculating the *P* values, except for Total.

DCIS having the highest percents of CD44 only and of the combination (of CD44 and CD24), with DCIS with microinvasion having the highest percent of CD24 only, and with IDC alone having the highest percent of neither marker. Considering histologic group within each of the four tumor subtypes, the difference in pattern was significant only in the luminal A subtype (which had the largest number of cases).

Considering only tumor subtypes, the CD44-only pattern was the most common one in each subtype except for HER2+, in which CD24 only and the lack of both were more common (Table 2). The four tumor subtypes differed significantly (*P* = 0.0006) in pattern, with basal-like tumors having the lowest percent of CD24 only and of the combination, with HER2 tumors having the lowest percent of CD44 only, and with luminal B tumors having the lowest percent of tumors with neither marker. Considering tumor subtypes within each of the four histologic

groups, none were significant (although the subtypes were closest to significantly different in pattern for DCIS with microinvasion).

To investigate the combined expression of CD24 and CD44 within individual tumor cells in further detail, we performed double immunohistochemical analysis (Table 3; Fig. 1C). CD44+/CD24- stem cell-like cells were detected in 64% of IDC only, 64% IDC with DCIS, 74% of DCIS with microinvasion, and 83% of pure DCIS cases. When analyzing by tumor subtype 69% of luminal A, 70% of luminal B, 52% of HER2, and 100% of basal-like tumors contained some CD44+/CD24- cells. CD44-/CD24+ more differentiated luminal epithelial cells were detected in 19% of IDC only, in 33% of IDC with DCIS, in 63% of DCIS with microinvasion, and in 37% of pure DCIS cases. Within subtypes, CD44-/CD24+ cells were more common in luminal and HER2+ tumors compared with basal-like cases (Table 3).

We analyzed the associations between tumor characteristics and CD24/CD44 expression patterns in two different ways. Specifically, we determined whether the entire tumor has both CD44 and CD24 staining, but possibly in different cells (maybe different areas of those tumors), based on staining for one marker at a time (Table 2). We also analyzed CD24 and CD44 expression within individual cells based on double IHC (Table 3). In both types of analyses, we established four different staining

patterns based on positivity/negativity of the tumors for these two markers, and the distribution of these four possible patterns were analyzed according to tumor subtype and histologic stage. The distributions of the four types of patterns differed significantly by histologic group ($P < 0.001$; Table 2). Considering histologic group within each of the four tumor subtypes, the pattern of distribution was significantly different by histologic type only within luminal A (the tumor type with the most cases).

Table 4. Comparison of the expression of 12 proteins between tumor subtypes in each of the four histologic groups

IDC		IDC alone				IDC with DCIS					
		Luminal A (n = 35)	Basal-like (n = 6)	HER2+ (n = 5)	P	Luminal A (n = 85)	Basal-like (n = 16)	HER2+ (n = 18)	Luminal B (n = 16)	P	
CD44+ cell genes	Vimentin	7 (20)	6 (100)	2 (40)	0.0003	10 (12)	13 (81)	2 (11)	1 (6)	<0.0001	
	Osteonectin	0 (0)	3 (50)	1 (20)	0.001	6 (7)	4 (25)	0 (0)	1 (6)	NS	
	Connexin 43	16 (46)	2 (33)	1 (20)	NS	35 (41)	7 (44)	0 (0)	1 (6)	0.0001	
	ALDH1	0 (0)	2 (33)	0 (0)	NSAA	1 (1)	3 (19)	4 (22)	0 (0)	0.001	
	Caveolin1	1 (3)	3 (50)	0 (0)	NSAA	0 (0)	2 (12)	0 (0)	0 (0)	NSAA	
	CD44	16 (46)	5 (83)	3 (60)	NS	54 (64)	12 (75)	6 (33)	9 (56)	NS	
	CD44 median	14 (40)	5 (83)	2 (40)	NS	44 (52)	11 (69)	5 (28)	6 (38)	NS	
	EPCR	1 (3)	0 (0)	1 (20)	NS	2 (2)	1 (6)	0 (0)	1 (6)	NS	
	CD24+ cell genes	High pos CK18	22 (63)	0 (0)	0 (0)	0.0002	49 (58)	1 (6)	9 (50)	6 (38)	0.0008
		GATA3	29 (83)	0 (0)	1 (20)	<0.0001	78 (92)	1 (6)	6 (33)	11 (69)	<0.0001
GATA3 median		21 (60)	0 (0)	1 (20)	0.004	56 (66)	0 (0)	1 (6)	6 (38)	<0.0001	
High pos MUC1		23 (66)	0 (0)	0 (0)	0.0002	34 (40)	2 (13)	6 (33)	7 (44)	NS	
Claudin7		9 (26)	2 (33)	0 (0)	NS	28 (33)	2 (13)	5 (28)	6 (38)	NS	
CD24		11 (31)	0 (0)	1 (20)	NS	34 (40)	2 (13)	9 (50)	7 (44)	NS	
DCIS		DCIS with microinvasion				Pure DCIS					
		Luminal A (n = 11)	Luminal B (n = 6)	HER2+ (n = 14)	Basal-like (n = 4)	P	Luminal A (n = 41)	Luminal B (n = 7)	HER2+ (n = 9)	P	
CD44+ cell genes	Vimentin	2 (18)	2 (33)	1 (7)	3 (75)	NSAA	4 (10)	1 (14)	4 (44)	NSAA	
	Osteonectin	0 (0)	2 (33)	1 (7)	1 (25)	NS	1 (2)	1 (14)	0 (0)	NS	
	Connexin 43	1 (9)	0 (0)	0 (0)	1 (25)	NS	21 (51)	1 (14)	0 (0)	0.003	
	ALDH1	0 (0)	0 (0)	1 (7)	0 (0)	NS	0 (0)	1 (14)	1 (11)	NS	
	CD44	9 (82)	5 (83)	4 (29)	4 (100)	NSAA	36 (88)	6 (86)	7 (78)	NS	
	CD44 median	4 (36)	4 (67)	4 (29)	3 (75)	NS	24 (59)	3 (43)	4 (44)	NS	
	EPCR	4 (36)	0 (0)	0 (0)	0 (0)	NSAA	4 (10)	0 (0)	0 (0)	NS	
	CD24+ cell genes	High pos CK18	4 (36)	3 (50)	11 (79)	0 (0)	NSAA	32 (78)	5 (71)	4 (44)	NS
		GATA3	9 (82)	3 (50)	5 (36)	0 (0)	NSAA	38 (93)	5 (71)	0 (0)	<0.0001
		GATA3 median	6 (55)	0 (0)	0 (0)	0 (0)	0.0008	29 (71)	3 (43)	0 (0)	0.0001
High pos MUC1		7 (64)	3 (50)	5 (36)	0 (0)	NS	24 (59)	3 (43)	4 (44)	NS	
Claudin7		5 (45)	1 (17)	1 (7)	1 (25)	NS	22 (54)	0 (0)	2 (22)	NSAA	
CD24		6 (55)	4 (67)	10 (71)	0 (0)	NS	19 (46)	4 (57)	3 (33)	NS	

NOTE: Numbers in parentheses indicate percentage of patients who were coded as positive for the binary marker variables (column percentage). *P* values were calculated using Fisher's exact tests. Significance levels were adjusted using Holm's method. Luminal B subtype was omitted in IDC alone group because there was only one case in this group. Basal-like subtype was omitted in pure DCIS group because there was only one case in this group. All DCIS tumors were negative for Caveolin1. Abbreviation: High pos, highly positive.

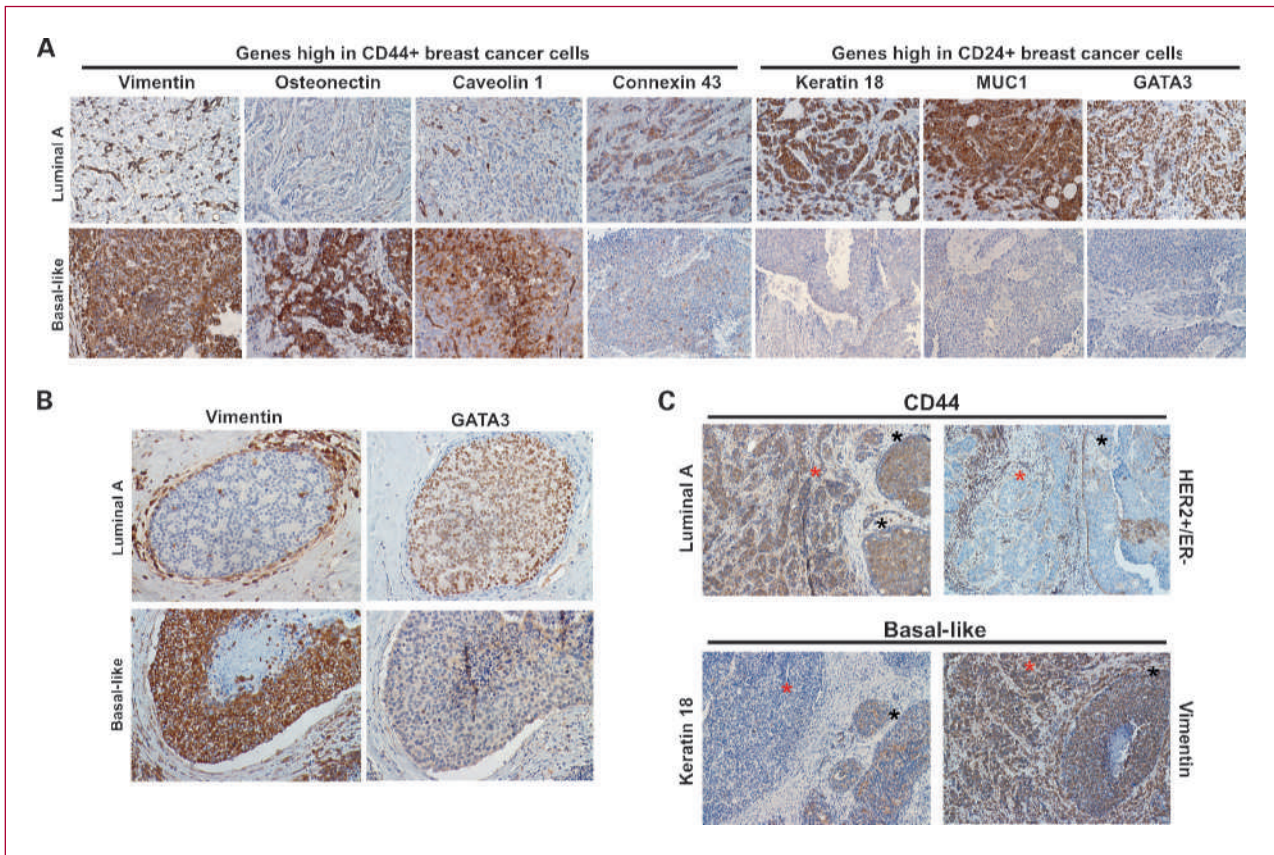


Fig. 2. Immunohistochemical analyses of genes differentially expressed between CD44+ and CD24+ breast cancer cells. A, representative examples of immunohistochemical staining patterns observed in invasive breast tumors using antibodies against the indicated proteins. A subset of markers that were highly expressed in CD44+ compared with CD24+ breast cancer cells based on our prior study are expressed only in basal-like tumors (vimentin, osteonectin, and caveolin 1), whereas Connexin 43 is expressed both in luminal A and basal-like subtypes. Markers more abundantly expressed in CD24+ compared with CD44+ breast cancer cells (Keratin 18, MUC1, and GATA3) are expressed in luminal A tumors. B, representative examples of immunohistochemical staining patterns observed in DCIS using antibodies against the indicated proteins. Vimentin is expressed in basal-like but not in luminal A DCIS. Strong vimentin staining is detected in stromal cells in both tumor subtypes. In contrast, GATA3 is expressed in luminal A but not in basal-like DCIS. C, immunohistochemical analyses of the expression of markers between invasive (*red asterisk*) and *in situ* (*black asterisk*) components of the same tumor. CD44 is expressed in both invasive and *in situ* components of luminal A subtype. HER2+ tumors are negative for CD44; only stromal staining is detected. In basal-like IDC, keratin 18 is expressed only in the *in situ* component whereas vimentin is expressed in both invasive and *in situ* areas.

Overall, the distribution pattern of the four cell types also differed significantly by tumor subtype ($P = 0.0006$; Table 3). Considering tumor subtype within each of the histologic groups, the pattern was significantly different only for IDC with DCIS (the histologic group with the most cases).

Expression of genes differentially expressed between CD44+ and CD24+ breast cancer cells according to histologic stage and tumor subtype. To further investigate intertumor cellular heterogeneity for the expression of stem cell-related markers in breast cancer and to correlate this with tumor subtypes, we selected additional genes that we previously identified as differentially expressed between CD44+ and CD24+ breast cancer cells (19) and analyzed their expression by IHC in IDCs and in DCIS (Supplementary Table S4; Table 4; Fig. 2A and B). The proteins analyzed included the following: vimentin, osteonectin, EPCR, connexin 43, and caveolin 1 (highly expressed in CD44+ breast cancer cells) as well as

CD44 itself and ALDH1; and MUC1, CK18, claudin 7, and GATA3 (highly expressed in more differentiated CD24+ luminal cells) as well as CD24 (any type of staining). Expression of each of these markers was represented by binary (two valued) variables (Supplementary Table S2).

Overall, CD44 ($P = 0.002$) and connexin 43 ($P = 0.003$) staining differed significantly among the four histologic groups; CD44 was higher in pure DCIS tumors whereas connexin 43 was lower in DCIS with microinvasion compared with other histologic groups (Supplementary Table S4). When this analysis was done separately in each of the four tumor subtypes, only the luminal A subtype had any significant associations of positivity of markers and histologic groups. In the luminal A tumor, there was a significant difference in CD44 ($P = 0.0005$) and EPCR ($P = 0.001$) expression; both of them were higher in pure DCIS or DCIS with microinvasion than in IDC only tumors or IDC with DCIS tumors (Supplementary Table S4). When

the definition of positivity for all markers used a cutoff of >10% (instead of the cutoffs in Supplementary Table S2 and the Materials and Methods section), then overall three other markers would be significantly different by histologic group: vimentin ($P = 0.003$), CK18 ($P = 0.0001$), and MUC1 ($P = 0.0002$). In the luminal A group, EPCR was not significant after correction for multiple comparisons if the >10% cutoff was used.

We also explored whether the expression of these proteins differed by tumor subtypes within each of the four histologic groups (Table 4). After adjusting for multiple comparisons (separately within each histologic group), vimentin staining differed significantly by tumor subtype in the IDC alone group ($P = 0.0003$) and in the IDC with DCIS group ($P < 0.0001$); for both of these histologic groups, basal-like tumors were more likely to be positive than the other subtypes (Table 4). Osteonectin differed significantly by tumor subtype in the IDC alone group (50% of basal-like tumors were positive versus 0-20% of the other two subtypes). Connexin 43 differed significantly by tumor subtype in the IDC with DCIS ($P = 0.0001$) and pure DCIS ($P = 0.003$) subgroups, with a larger percent of luminal A tumors being positive in the IDC with DCIS group (41%) and pure DCIS group (51%), and a larger percent of the basal-like tumors being positive in the IDC with DCIS group (44%) than the percent that were positive in luminal B and HER2+ tumors. ALDH1 staining differed significantly ($P = 0.001$) according to subtype in the IDC with DCIS group (19% of basal-like, 22% of HER2+, 1% of luminal A, and 6% of luminal B were positive for ALDH1). Highly positive CK18 differed significantly in the IDC alone and the IDC with DCIS groups ($P = 0.0002$ and $P = 0.0008$, respectively), but there seems to be a difference in the association in these two groups. In both groups, luminal A tumors were very likely to have a highly positive stain for CK18 (63% and 58% of the cases) and basal-like tumors were very unlikely to have a highly positive stain (0% and 6%), but in IDC alone cases, the HER2+ tumors were also unlikely to be highly positive (0%) whereas in the IDC with DCIS cases the HER2+ tumors were likely to be highly positive (50%). GATA3 differed significantly by tumor subtype in three of the histologic groups (IDC alone, IDC with DCIS, and pure DCIS, each $P < 0.0001$). In each case, luminal A tumors were very likely to be positive for GATA3; luminal B tumors were very likely to be positive, and HER2+ tumors had a lower percent of positive cases; and basal-like tumors were very unlikely to be positive (Table 4). All four histologic groups had significant tumor type differences in GATA3 when the IHC scoring was split at the median staining level (all $P \leq 0.004$). For this GATA3 variable, luminal A cases were very likely to have staining above the median (55% to 71%); in two of the groups, luminal B cases were moderately likely to have staining above the median (38% and 43% in IDC with DCIS and in pure DCIS, but 0% in DCIS with microinvasion); HER2+ cases were less likely to have staining above the median (20%, 38%,

0%, and 0%); and basal-like cases never had staining above the median (Table 4). The three tumor subtypes available in the IDC alone group differed significantly in percentage with highly positive MUC1 staining ($P = 0.0002$), with 66% of the luminal A cases being highly positive staining and none of the basal-like tumors or HER2+ tumors being highly positive. If in this group MUC1 was categorized as positive if the staining was >10%, then the three subtypes would have positive staining for 100% of luminal A and HER2+ cases and 83% for basal-like cases, and this was not significant.

Gene expression patterns of invasive and in situ components of the same tumor. To determine if the differences seen in the expression of some proteins between IDCs and DCIS may reflect a potential divergence in expression associated with *in situ* to invasive breast carcinoma progression, we examined the expression of selected markers in tumors with invasive and *in situ* areas present on the same slides. Markers to be included in this analysis were selected based on the trend of their expression pattern when all IDC and all DCIS were combined in each of the tumor subtypes. Thus, CD44 was analyzed in 23 luminal A tumors; CD44 and CK18 were analyzed in 25 HER2+ cases; and ALDH1, caveolin 1, vimentin, and CK18 were analyzed in 25 basal-like tumors (Supplementary Table S5). In luminal A tumors, CD44 had significantly higher expression levels ($P = 0.01$) in the *in situ* than in the invasive components (48% of patients had more expression in the *in situ* component with a median difference in expression scores of 0.40; 17% had more expression in the invasive component with a median difference in expression scores of 0.10; and 35% had the same expression code for both components). This pattern was not seen in HER2+ tumors, in which CD44 expression was higher in the invasive component for 32% of patients and in the *in situ* component for 28% of patients. CK18 in basal-like tumors had significantly higher expression levels ($P = 0.005$) in the *in situ* compared with the invasive components (40% of patients had more expression in the *in situ* component with a median difference of 0.275; 8% had more expression in the invasive component with a median difference of 0.075; and 52% had the same expression code for both components). This pattern was not seen in HER2+ tumors, in which CK18 expression was higher in the invasive component in 12% and higher in the *in situ* component in 24% of cases (with median differences in both cases of 0.20). In contrast, in basal-like tumors, the expression of ALDH 1 and caveolin 1 were highly concordant in the invasive and *in situ* areas (80% of patients had the same expression score) and no tests were done (because the power is zero for a Wilcoxon signed-rank test based on five nonzero differences).

Discussion

Breast tumors display heterogeneity both among tumors in different individuals (e.g., intertumor heterogeneity; refs. 3, 4) and in tumor cell subpopulations

within tumors (e.g., intratumor heterogeneity; ref. 5). The molecular mechanisms underlying intertumor heterogeneity are poorly defined; distinct cell-of-origin and tumor subtype-specific transforming events are potential explanations (1, 32). Intratumor heterogeneity on the other hand can be explained by three potential mechanisms that are not independent of each other: (a) genetic diversity and genomic instability combined with clonal selection, (b) differences in cellular phenotypes including stem cell-like and more differentiated characteristics, and (c) epigenetic diversity and plasticity (1, 12, 32, 33). We and others have been investigating the relative contribution of all three mechanisms to intratumor heterogeneity in breast cancer and the relevance of these to tumor progression and therapeutic response. We previously described the comprehensive gene expression, genetic, and DNA methylation profiles of CD44+ stem cell-like and CD24+ more differentiated luminal epithelial breast cancer cells and found cell type-specific differences for all molecular characteristics analyzed (19, 20). The relative frequency of CD44+ and CD24+ cells in breast tumors (defined based on the presence of cell type-specific gene expression signatures within tumors) was correlated with distant metastasis-free survival (19, 20). Interestingly, CD44+ cell signature in primary invasive tumors was associated with a higher risk of distant metastasis, but distant metastases were enriched for more luminal epithelial CD24+ cells, implying a phenotypic switch during tumor progression or clonal selection for cells with CD24+ phenotype (19, 20). Correlating with this, recent studies described that epithelial-to-mesenchymal transition (EMT) generates cells with CD44+ stem cell-like characteristics (34), whereas in distant metastases, a reversion of this process to mesenchymal-to-epithelial transition have also been reported in bladder (35) and colon (36) carcinomas. However, because distant metastases are frequently detected as recurrences following chemotherapeutic treatment, these observations may not necessarily reflect the natural history of the disease and further studies are needed to define the potential roles of EMT and mesenchymal-to-epithelial transition in tumor progression. To investigate if phenotypic divergence for stem cell-like and more differentiated luminal markers might also be observed between *in situ* and invasive breast carcinomas in patients before systemic treatment, we analyzed the expression of a panel of markers previously associated with CD44+ stem cell-like and more differentiated CD24+ luminal cell characteristics in breast cancer.

We analyzed the expression of each of the markers in a series of IDCs and DCIS in relation to clinicopathologic features, focusing especially on breast tumor subtype. Confirming the results of recent reports (21, 22, 27), the frequency of CD44+/CD24- cells defined based on double immunohistochemistry was the highest in basal-like tumors compared with the other three subtypes. High-grade IDC-only tumors were also more likely to be CD44 positive than lower grade ones. However, the expression of CD44 was lower in IDCs and microinvasive DCIS com-

pared with pure DCIS suggesting a decrease in the number of CD44+ cells as tumors progress.

The expression of ALDH1, another marker associated with stem cell-like features, was significantly more common in ER- tumors in the cases of IDC alone or IDC with DCIS, confirming previous findings (14). We also found that in IDC with DCIS tumors, ALDH1+ cells were the least frequent in luminal A and B tumors compared with basal-like and HER2+ subtypes, correlating with a recent study in breast cancer cell lines (23). CD44+/CD24- cells and ALDH1+ cells were more frequently found in the basal-like than in luminal subtypes, and CD44+/CD24- cells but not ALDH1+ cells were less common in HER2+ than in basal-like cases. In normal breast tissue, CD44+/CD24- cells were limited to the basal layer whereas ALDH1+ cells were found in both basal and luminal compartments, suggesting that ALDH might be a marker of both bipotential mammary epithelial stem cells and luminal lineage committed progenitors. However, other interpretations of these results, including the likelihood that ALDH+ cells defined by enzymatic activity assay and IHC may not completely overlap, are possible. The difference in the relative frequency of CD44+/CD24- and ALDH1+ cells between basal-like and HER2+ tumor subtypes may reflect their distinct cell-of-origin or the alteration of stem cell-like gene expression programs due to tumor subtype-specific transforming events. Correlating with this latter hypothesis, a recent study described that the exogenous expression of HER2 increases the frequency of ALDH+ cells in breast cancer cell lines (37).

We found two different patterns of CD24 expression in breast cancers: apical membranous and circumferential membrano-cytoplasmic, as reported in previous studies (24–26). Whereas apical membranous CD24 localization was found in normal luminal cells and well-differentiated luminal A tumors, circumferential membranous and cytoplasmic CD24 expression was more common in luminal B and HER2+ subtype IDCs, suggesting an association of membrano-cytoplasmic CD24 staining with more aggressive tumor characteristics. We also detected a shift of CD24 staining pattern from apical membranous to membrano-cytoplasmic during the progression of DCIS to IDC. Membrano-cytoplasmic (or cytoplasmic) CD24 expression is associated with tumor progression and poor prognosis (24–26). It has also been reported that ectopic introduction of CD24 into breast carcinoma cells increases tumor cell proliferation and stimulates cell motility and invasion (38). Although the biological significance of the membrano-cytoplasmic localization of CD24 remains unclear, it seems to be associated with invasive and aggressive tumor cell behavior. Thus, it would be important to establish whether cells with membrano-cytoplasmic expression of CD24 retain a differentiated, luminal epithelial cell phenotype.

Among the 12 genes differentially expressed between CD44+ and CD24+ cells analyzed by IHC, the expression of vimentin, GATA3, and EPCR is noteworthy. Vimentin expression was found predominantly in basal-like IDCs. Vimentin expression is one of the characteristics of EMT that may play a role in metastatic progression (39).

Basal-like breast tumors were reported to show high expression of multiple EMT markers including vimentin and were reported to have intrinsic phenotypic plasticity for mesenchymal transition (40). Thus, there seems to be a link among stem cell-like phenotype, EMT, and basal-like subtype of breast cancer. However, not all basal-like tumors were uniformly vimentin positive, suggesting further heterogeneity even within this tumor subtype. Correlating with this, a recent study described the subclassification of basal-like tumors based on claudin expression pattern (41, 42).

The GATA3 transcription factor that was shown to be essential for luminal epithelial cell differentiation (43, 44) in mice was frequently expressed in luminal and rarely detected in basal-like breast tumors, a pattern consistent with previous studies (2–4). Based on these findings, lack of GATA3 expression may be responsible for the decrease of CD24+ more differentiated luminal epithelial cells and the abundance of CD44+ stem cell-like populations in basal-like breast cancers.

EPCR (also called PROCR), a receptor for protein C, has been shown to increase the invasiveness of breast cancer cells (45) and has been identified as a marker of hematopoietic, embryonic, and neural stem cells in mice (46–48). Similar to CD44, we observed an enrichment for EPCR+ cells in basal-like tumors and the number of these cells was lower in invasive compared with *in situ* carcinomas within the luminal A subtype. Thus, EPCR may identify cells with progenitor characteristics in human breast tissue as well.

Although the expression of some markers, such as CD44 and EPCR in luminal subtype, was different between DCIS and IDCs, the expression of the other markers was similar in the two histologic stages. The expression of connexin 43 was lower in microinvasive DCIS compared with IDCs and pure DCIS, potentially implicating connexin 43 in the progression to invasion. The expression CD44 was also lower in the invasive compared with *in situ* areas of the same tumor in the luminal A but not in HER2+ cases. The decreased frequency of CD44+ cells during *in situ* to invasive breast carcinoma transition relates to the decreased number of these cells in distant metastases compared with primary invasive tumors (19, 20). Thus, the frequency of CD44+ cells may gradually decline during breast tumor progression. Similarly, CK18 expression was higher in *in situ* compared with invasive areas in basal-like but not HER2+ subtype tumors. However, there were very few cells positive for CK18 in basal-like tumors;

thus, the possibility of sampling error cannot be excluded. In contrast, the frequency of cells positive for ALDH1 and caveolin 1 seemed to be the same in the *in situ* and invasive areas of the same tumor. The observed differences in the frequency of cells with stem cell-like and more differentiated cell phenotypes may suggest subtype-specific tumor progression pathways.

In summary, our study revealed high degree of diversity for the expression of several selected markers in different tumor subtypes and histologic stages. The expression of CD44 seemed to decrease with tumor progression. The expression of vimentin and osteonectin was more frequent in basal-like subtype, whereas CK18, GATA3, and MUC1 expression was more common in luminal subtypes. CD44+/CD24– cells were most common in basal-like tumors, whereas the frequency of ALDH+ cells was the highest in HER2+ and basal-like tumors. Alterations in the frequency and distribution of cells with stem cell-like and more differentiated luminal cell features among tumors according to subtype and tumor progression stage have implications for therapeutic strategies aimed at the selective targeting of these distinct tumor cell populations.

Disclosure of Potential Conflicts of Interest

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