Cell Cycle 11:22, 4242–4251; November 15, 2012; © 2012 Landes Bioscience

Mesenchymal traits are selected along with stem features in breast cancer cells grown as mammospheres

Silvia Borgna, Michela Armellin, Alessandra di Gennaro, Roberta Maestro* and Manuela Santarosa

Experimental Oncology 1; CRO IRCCS; National Cancer Institute; Aviano, Pordenone, Italy

Keywords: breast cancer, cancer stem cells, epithelial-to-mesenchymal transition, mammospheres, cell lines

Abbreviations: EMT, epithelial-to-mesenchymal transition; CSC, cancer stem cells; MS-proficient conditions, mammospheres-proficient conditions; ER, estrogen receptor

Increasing evidence indicates that invasive properties of breast cancers rely on gain of mesenchymal and stem features, which has suggested that the dual targeting of these phenotypes may represent an appealing therapeutic strategy. It is known that the fraction of stem cells can be enriched by culturing breast cancer cells as mammospheres (MS), but whether these pro-stem conditions favor also the expansion of cells provided of mesenchymal features is still undefined.

In the attempt to shed light on this issue, we compared the phenotypes of a panel of 10 breast cancer cell lines representative of distinct subtypes (luminal, HER2-positive, basal-like and claudin-low), grown in adherent conditions and as mammospheres. Under MS-proficient conditions, the increment in the fraction of stem-like cells was associated to upregulation of the mesenchymal marker Vimentin and downregulation of the epithelial markers expressed by luminal cells (E-cadherin, KRT18, KRT19, ESR1). Luminal cells tended also to upregulate the myoepithelial marker CD10. Taken together, our data indicate that MS-proficient conditions do favor mesenchymal/myoepithelial features, and indicate that the use of mammospheres as an in vitro tumor model may efficiently allow the exploitation of therapeutic approaches aimed at targeting aggressive tumors that have undergone epithelial-to-mesenchymal transition.

Introduction

Breast cancer is a heterogeneous disease, with distinct subtypes characterized by different biology and response to therapy. At least four subtypes of breast tumors have been identified by initial molecular profiling: luminal A and luminal B, which are positive for both estrogen and progesterone receptors (ER and PGR); HER2-positive and basal-like/triple-negative (lacking ER, PGR and HER2).[1-3] More recently, the claudin-low subtype has been identified among triple-negative breast cancers.[4] These distinct tumor subtypes present different prognosis, with the shortest survival time observed among HER2-positive and basal-like/claudin-low breast cancer patients.[5] Interestingly, both basal-like/claudin-low and HER2-positive tumors have proven to be particularly enriched in cancer stem cells (CSC), which are cells endowed with self-renewal and pluripotency potential.[6,7] Stem-like features may be functionally demonstrated in vitro by the ability of CSC to grow as mammospheres (MS) in non-adherent/serum-free stem conditions[8] and in vivo by the ability of mammospheres to generate tumors when injected at limiting dilutions in immunocompromised mice.[9-12] Indeed, the capability to generate primary mammospheres (M1) in stem conditions, and to perpetrate as secondary (M2) and tertiary (M3) spheres, is considered as an in vitro surrogate of the in vivo evaluation of self-renewal potential. The coordinated expression of pluripotency transcription factors, including POU5F1 (OCT3/4), NANOG and SOX2, has emerged as a regulatory mechanism of stem cell pluripotency and differentiation[13] and, indeed, these genes and their targets are frequently overexpressed in poorly differentiated breast cancer.[14,15] Furthermore, breast CSC have been reported to display the $CD44^{+}/CD24^{-/low}$ antigenic profile that correlates with resistance to conventional therapeutics.[16-18]

Beside stem-like features, basal-like and especially claudin-low tumors express mesenchymal markers typical of tumors that have undergone epithelial-to-mesenchymal transition (EMT), an embryonic trans-differentiation program reactivated in several carcinomas and correlated with tumor progression and invasiveness.[4,19] The major players of the EMT program include members of the SNAI, ZEB and TWIST family of embryonic transcription factors that, through the establishment of a complex network of interactions only partially elucidated, induce the expression of mesenchymal markers (Vimentin and N-cadherin) and the repression of epithelial molecules such as E-cadherin.[20,21]

*Correspondence to: Roberta Maestro; Email: rmaestro@cro.it
Submitted: 09/05/12; Revised: 10/11/12; Accepted: 10/12/12
http://dx.doi.org/10.4161/cc.22543

Table 1. Stem and epithelial/mesenchymal features of the breast cancer cell lines used in this study

Cell line	Tumor subtype	% CD24 *	% CD44 *	% $CD44^+/CD24^-$ *	% CD133 *	ESR1§	CDH1§	KRT18§	KRT19§	VIM§
HBL100	Claudin-low	50 ± 15	99 ± 1	50 ± 10	10 ± 1	-	-	+	-	+
MDA-157	Claudin-low	1 ± 1	98 ± 2	98 ± 2	1 ± 1	-	-	++	-	+
MDA-231	Claudin-low	3 ± 3	98 ± 2	97 ± 3	1 ± 1	-	-	-	-	+
MDA-436	Claudin-low	8 ± 8	98 ± 2	95 ± 5	2 ± 1	-	-	+	++	+
Hs578T	Claudin-low	1 ± 1	98 ± 2	98 ± 2	2 ± 1	-	-	++	-	+
MDA-468	Basal-like	98 ± 2	98 ± 2	2 ± 1	80 ± 4	-	+	+	-	+
SKBR3	HER2-pos	99 ± 1	5 ± 2	0	2 ± 1	-	-	++	++	-
BT474	Luminal	99 ± 1	1 ± 1	0	1 ± 1	+	+	+	+	+
MCF7	Luminal	97 ± 2	92 ± 5	0	2 ± 1	++	+	++	++	-
T47D	Luminal	97 ± 2	5 ± 2	0	2 ± 1	++	+	++	++	-

*As assessed by flow cytometry analyses. §As assessed by semi-quantitative PCR.

Both EMT and elevated content of cells endowed with stem-like properties characterize aggressive undifferentiated tumors, and data suggest that certain components of the EMT program may also play a critical role in tissue homeostasis, thus linking stemness to EMT.[9,22,23] Indeed, growing evidence indicates that the induction of EMT results in expanded CSC populations (reviewed in ref. 24). Whether, instead, the expansion of cells endowed with stem-like properties selects also for cells provided with EMT features is less established.

Based on this information, we sought to investigate whether the growth of breast cancer cells in MS-proficient conditions affects the fraction of cells displaying EMT features. To this end, we compared EMT and stem features of a series of 10 breast cancer cell lines, representative of distinct subtypes (luminal; HER2-positive; basal-like and claudin-low), grown in adhesion and in MS-proficient conditions. We found that, along with selection of stem properties, a shift toward a mesenchymal phenotype was observed for all mammosphere-forming cell lines, indicating that CSC-proficient culture conditions co-select for mesenchymal properties, irrespectively of the parental breast cancer subtype.

Results

Stem features in breast cancer cell lines grown as mammospheres. Ten breast cancer cell lines, representative of different subtypes, were used in this study. The analysis of the expression of estrogen-receptor, Vimentin, E-cadherin and cytokeratin 18 and 19 under standard growth condition (adhesion in standard medium plus 10% serum) confirmed the lineage-specific expression pattern (**Table 1**).

The expression pattern remained unaffected when cells were grown in adhesion in standard medium or in stem medium, indicating that the growth factors present in the stem medium do not significantly impinge on the expression of these genes. Nevertheless, since we could not exclude that the shift from one medium to another may transiently perturb the expression pattern, we sought to compare the features of cells grown in adhesion (control) vs. cells grown in suspension as mammospheres, all cultured in the same stem medium.

In suspension (MS-proficient conditions), all cell lines were able to proliferate. However, MDA-157 and MDA-468 gave rise to loose aggregates and SKBR3 to tubular structures, which prevented accurate assessment of sphere-forming efficiency (SFE), and, therefore, these cell lines were excluded from the SFE analysis. High and progressively increasing SFE of secondary (M2) and tertiary (M3) spheres were observed among claudin-low cell lines. Luminal MCF7 and T47D cells formed M1 sphere at high efficiency, but the SFE decreased with passages (**Fig. 1A**). Noteworthy, the morphology of the spheres differed among cell lines: HBL100, Hs578T, MCF7 and BT474 generated clear round spheres; T47D formed tiny round spheres; instead, MDA-436 and MDA-231 grew as clusters or grape-like spheres (**Fig. 1B**).

The $CD44^+/CD24^{-low}$ phenotype recognizes a subpopulation of breast cancer stem cells endowed with tumor-initiating ability and mesenchymal phenotype.[16] In all but HBL100 claudin-low cell lines, a large fraction of cells displayed a $CD44^+/CD24^{-low}$ phenotype (**Fig. 1C** and **Table 1**). In HBL100, both $CD44^+/CD24^{-low}$ and $CD133^+$ profiles were detected (**Fig. 1C** and **Table 1**). A $CD133^+$ phenotype was displayed by MDA-468 basal-like cells (**Fig. 1D**). Luminal and the HER2-positive SKBR3 cells were almost negative for $CD44^+/CD24^{-low}$ and $CD133^+$ phenotypes (**Fig. 1C** and **Table 1**). The growth as mammospheres resulted in an expansion of the $CD44^+/CD24^{low}$ fraction solely in the HER2-positive SKBR3 cell line (**Fig. 1E**).

To better characterize the stem component of the cell lines analyzed, we evaluated the expression of ALDH1A and ALDH1A3 isoforms of the ALDH1 enzyme, whose activity has been reported to characterize stem cells.[25,26] In adhesion, the ALDH1A isoform was mostly expressed by MDA-468 and BT474 (**Fig. 2A**); the ALDH1A3 isoform was instead detected in the claudin-low HBL100 and MDA-436, the basal-like MDA-468 and the HER2-positive SKBR3 cells (**Fig. 2B**). In mammospheres, a progressive increase in the expression of ALDH1A1 in MDA-468 and SKBR3 was detected (**Fig. 3A**). Intriguingly, we observed that the expression of ALDH1A3 decreased with passages in seven out of 10 cell lines, although a transient increase was observed in HBL100 and BT474 (**Fig. 3B**).

None of the cell lines analyzed expressed the embryonic pluripotency-associated genes POU5F1 and NANOG. SOX2 was

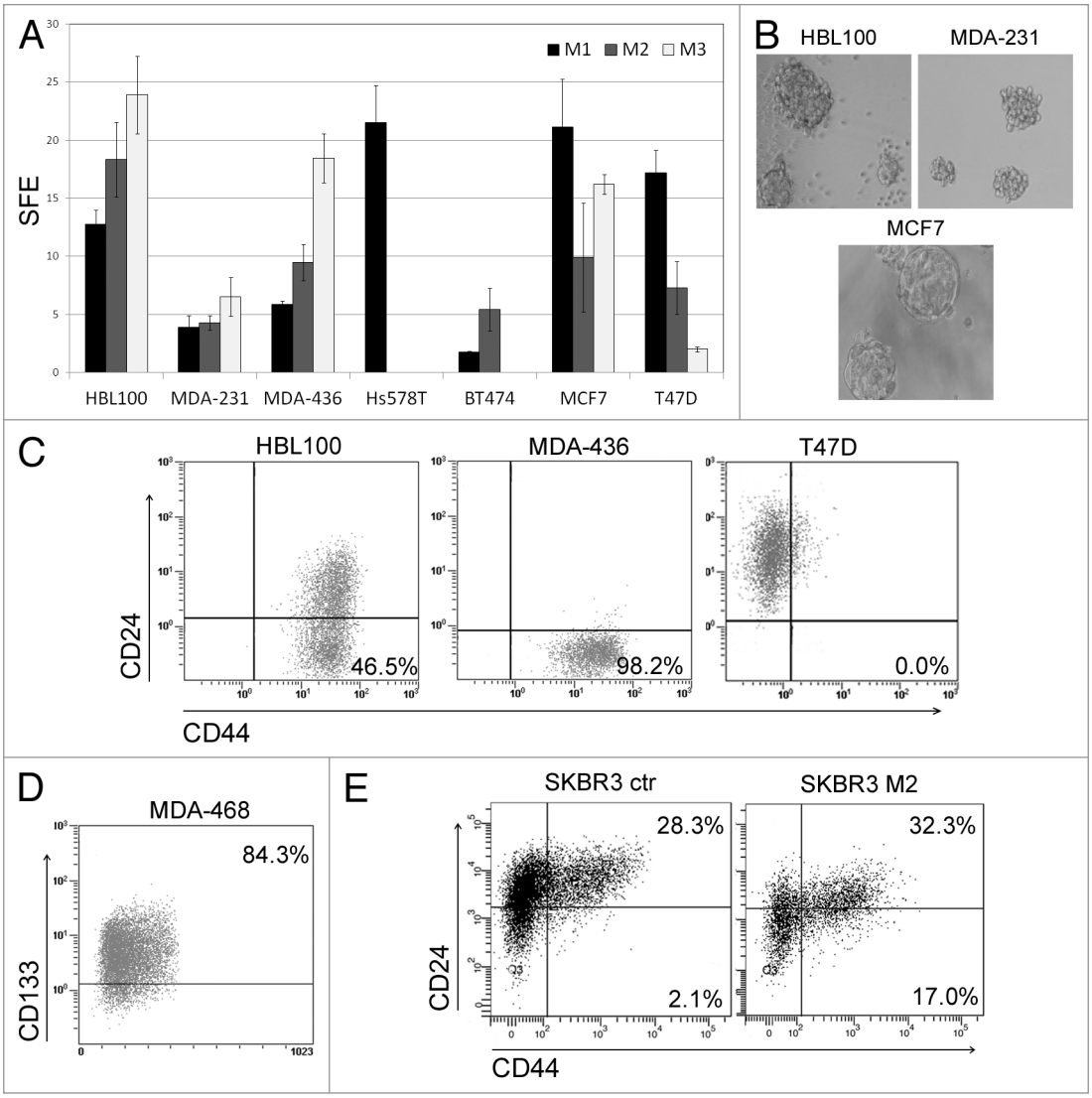

Figure 1. Stem properties of breast cancer cell lines. (**A**) Sphere-forming efficiency (SFE) of seven cell lines determined as described in Materials and Methods. Primary (M1, black colums), secondary (M2, gray columns) and tertiary (M3, light gray columns) SFE are reported. (**B**) Representative images of secondary spheres of HBL100, MDA-231 and MCF7. (**C**) Typical CD44/CD24 flow cytometry analyses observed in the 10 cell lines. $CD44^{-}/CD24^{+}$ phenotypes of HBL100, MDA436 and T47D cell lines were reported as examples of mixed, almost pure $CD44^{+}/CD24^{-low}$ and $CD44^{-}/CD24^{+}$ phenotypes, respectively. Percentages, referring to $CD44^{+}/CD24^{-low}$-positive cells, were determined by setting the gate on the isotype control. (**D**) CD133 flow cytometry analysis of MDA-468 cell line. Again, percentage refers to CD133-positive cells and was determined by setting the gate on the isotype control. (**E**) CD44/CD24 analysis of SKBR3 cells grown in MS-proficient conditions (M2) and in adherence (ctr) in the same-stem medium. Percentages, determined by setting the gate on CD24 high-positive parental cells, refer to $CD44^{+}/CD24^{+}$ and $CD44^{+}/CD24^{-low}$-positive cells.

expressed by MCF7 under standard conditions (**Fig. 2C**) and was further induced in mammospheres (**Fig. 3C**).

Mesenchymal traits in mammospheres of breast cancer cell lines. Intrinsic mesenchymal or epithelial features of the 10 breast cancer cell lines analyzed paralleled the expression pattern of the EMT-associated transcription factors. In fact, under standard growth conditions, ZEB1/2, SNAI2 and SNAI1 were all globally higher in claudin-low cells compared with the other cell types (**Fig. 4A and B**), although each cell line expressed a distinct set of these transcription factors. TWIST1, in addition to be expressed by the claudin-low HBL100 and MDA-436 cell lines, was expressed by the luminal cell line BT474 (**Fig. 4A**). All cell lines used tested negative for the expression of TWIST2 that was instead expressed in the control cell line FADU[27] (data not shown). The global low levels of expression of EMT-associated transcription factors displayed by basal-like, luminal and HER2-positive cell lines under standard conditions correlated with high levels of expression of miR200c (**Fig. 4C**), a non-coding RNA targeting ZEB1/2 and SNAI1 mRNAs.[28]

We then examined whether mesenchymal and epithelial features were modulated when cells were grown in suspension as mammospheres. In these culture conditions, seven out of 10 cell lines displayed increased expression of the mesenchymal marker Vimentin (VIM; **Fig. 5A**). Alongside, a significant decrease in the

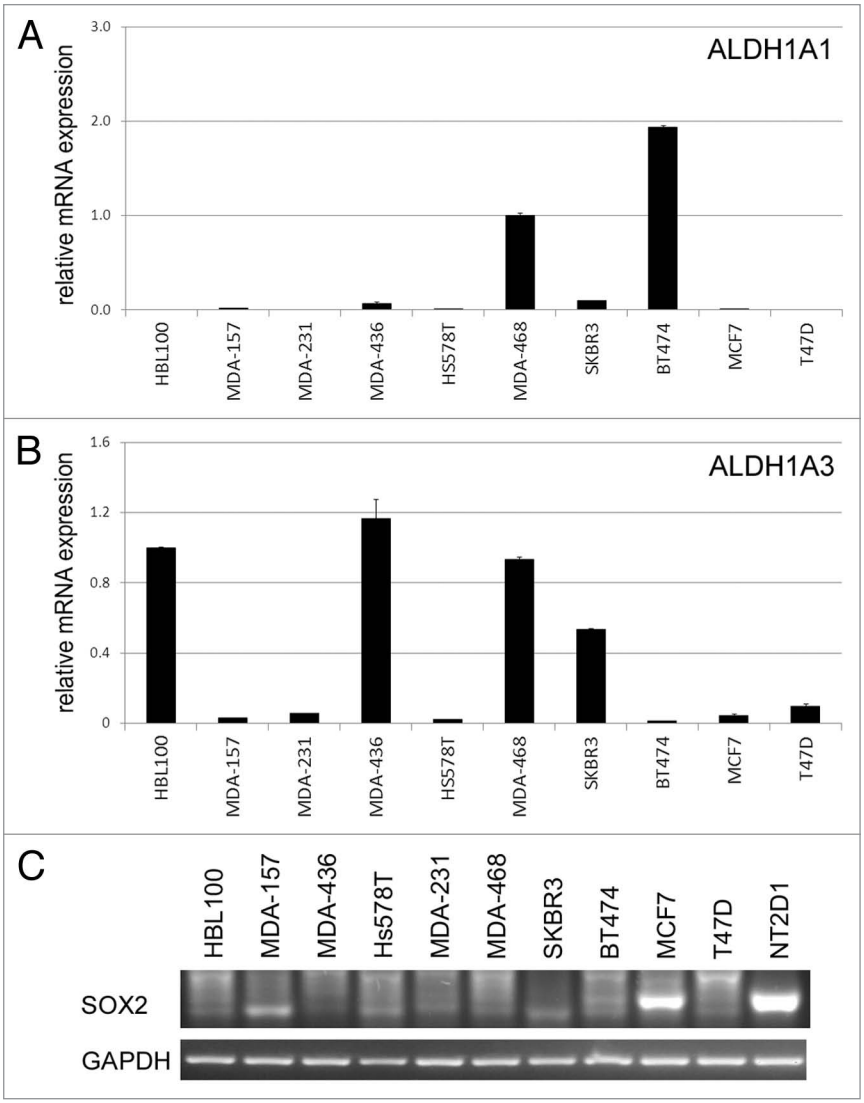

Figure 2. ALDH1 expression in the breast cancer cell lines analyzed. (**A**) ALDH1A1 and (**B**) ALDH1A3 mRNA-relative expression determined by qRT-PCR. Data were normalized to three reference genes as described in Materials and Methods. Bars represent standard deviation. (**C**) SOX2 transcript determined by RT-PCR. GAPDH is reported as RNA control. NT2D1, NTera D1 cell line was used as a positive control for SOX2.

expression of Keratin 18 (KRT18), 19 (KRT19) and E-cadherin epithelial markers (CDH1) was observed (**Fig. 5B–D**). Furthermore, luminal cell lines displayed a decrease in the expression of estrogen receptor-α (ESR1) and an increment in the fraction expressing CD10 (membrane metalloendopeptidase, MME), suggesting a shift toward an ER-α-negative/myoepithelial phenotype (**Fig. 5E and F**).

These changes were paralleled by a global increment in the expression of EMT transcription factors (**Fig. 6**). The pattern of expression of these genes was independent of the cell subtype, and sometimes a shift from one set of EMT transcription factors to another was observed. For instance, in MDA-157 and MDA-231, the marked induction of the ZEB members was associated with a progressive shutoff of SNAI2, particularly evident in M3 passage. A marked increment in SNAI2 and TWIST1 transcripts was instead observed in HBL100, SKBR3, MCF7 and T47D, and MDA-468 showed an increment of all but ZEB2 transcription factors (**Fig. 6**). Taken together, these data suggest that the growth in suspension as mammospheres promotes the expansion of a population provided of mesenchymal features and characterized by the expression of a combination of EMT transcription factors, mainly SNAI2 and TWIST1.

Discussion

An increasing amount of evidence suggests that in breast carcinomas, stemness and EMT phenotypes may be related phenomena. In fact, not only basal-like/claudin-low tumors, which frequently show mesenchymal features, display a high percentage of $CD44^{+}/CD24^{-low}$ phenotype,[6,29] but the ectopic expression of EMT-transcription factors in vitro has been proven to induce an expansion of the CSC fraction.[30] Instead, it remains unclear whether the reciprocal is also true, i.e., whether culture conditions that favor the selection of CSC favor also the expansion of cells undergoing epithelial to mesenchymal transition.

Here, we provide evidence that mesenchymal traits are selected along with stem features in breast cancer cells grown as mammospheres. The enrichment in stem-like cells under MS-proficient conditions was demonstrated by the increment in markers associated to stemness. In agreement with the literature data indicating that claudin-low tumors display high percentage of $CD44^{+}/CD24^{-low}$ phenotype,[29] we observed a clear association between $CD44^{+}/CD24^{-low}$ phenotype and mesenchymal features of the claudin-low cell lines analyzed. If the $CD44^{+}/CD24^{-low}$ population increased only in the mammospheres of the HER2-positive SKBR3 cells, in MCF7 and in BT474 cell lines an increase of the myoepithelial and stem marker CD10 was observed. The expression of CD10 characterizes CSC that give rise to metaplastic/claudin-low tumors when injected into mouse models.[31,32] Also, ALDH activity has been associated to CSC, and, accordingly, we observed that ALDH1A1, whose expression has been reported to correlate with disease-free survival, high-grade and lymph node positivity,[33-35] increased in the mammospheres of two cell lines. In contrast to what was originally proposed, ALDH1A3 expression inversely correlates with stemness, as it peaks during the commitment of breast stem cells to the luminal lineage and in the luminal progenitors.[36] Accordingly, we observed that ALDH1A3 transcript levels, which well-reflected the aldefluor activity previously reported for the cell lines analyzed,[26,37,38] decreased in the long-term

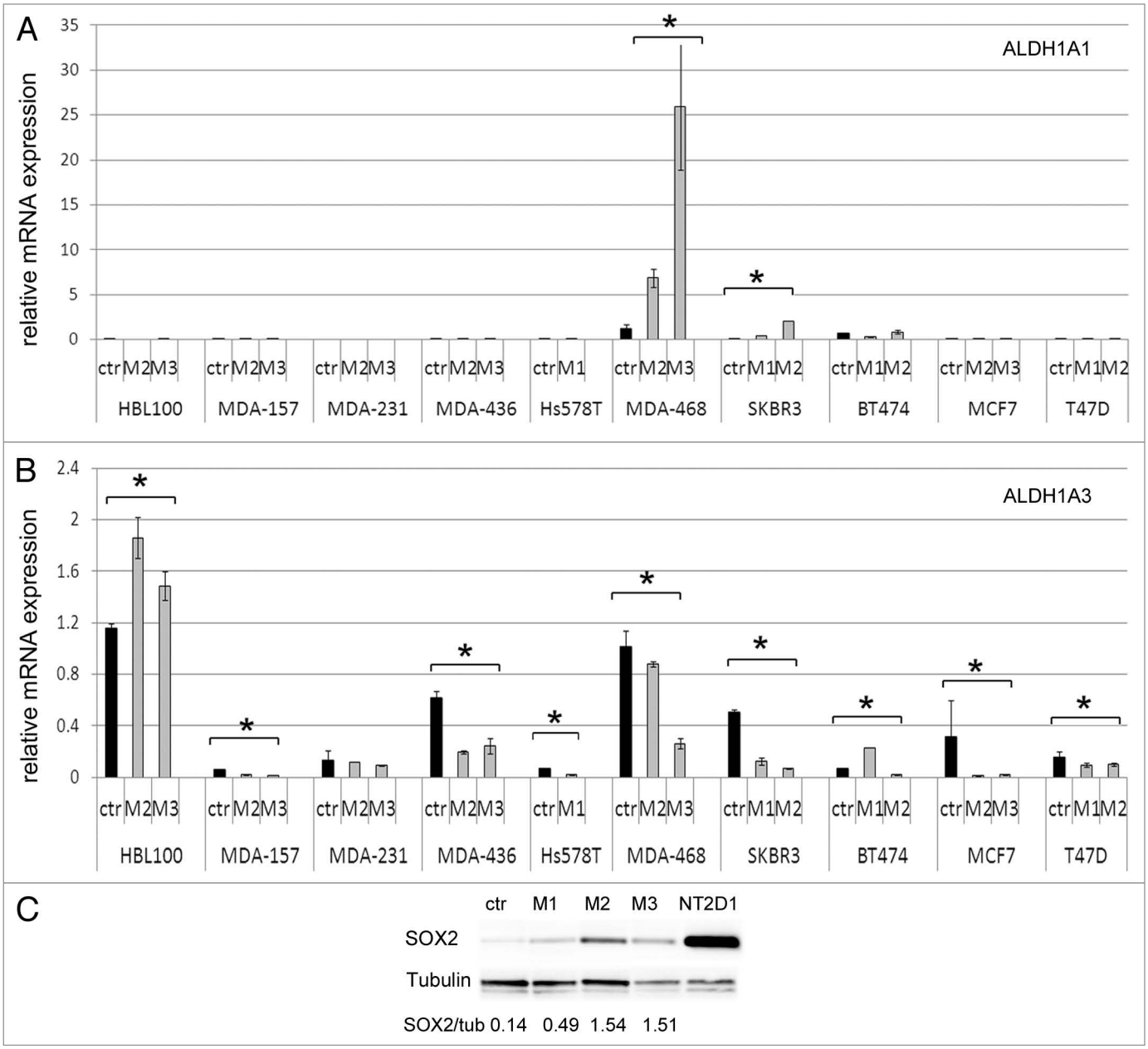

Figure 3. Stem properties were modulated by MS-proficient conditions. (**A**) ALDH1A1 and (**B**) ALDH1A3 transcript levels in cells grown as primary (M1), secondary (M2) and tertiary (M3) mammospheres (gray columns) or in adherence (ctr, black columns) in the same-stem medium. Data, obtained by qRT-PCR, were normalized to three reference genes as described in Materials and Methods. Bars represent standard deviation; asterisks $p < 0.05$ in one-way Anova analysis. (**C**) SOX2 protein levels, determined by western blotting, in MCF7 cells grown as primary (M1), secondary (M2) and tertiary (M3) mammospheres or in adherence (ctr) in the same-stem medium. NT2D1 is reported as a positive control. The increment of SOX2, determined by the ratio of SOX2 signal over tubulin signal (used as a loading control), is reported.

mammospheres irrespective of the breast cancer subtype of origin.

Finally, although the analysis of pluripotency-associated genes indicated a minor role for these factors in the stem phenotype of the breast cancer cell lines, SOX2 seemed to be involved in the promotion of stem features in MCF7 cells.

Under MS-proficient conditions, we observed upregulation of mesenchymal markers and downregulation epithelial factors and estrogen-receptor. In cells grown as mammospheres, the gain of mesenchymal traits was paralleled by the rise in EMT-transcription factors, SNAI2 and TWIST1 in particular. We can rule out that the observed selection of EMT features may be the result of a shift induced by the stem-cell culture cytokines included in the stem medium, because all analyses were done by comparing cells grown in different conditions (in adhesion vs. in suspension as mammospheres) but in the very same medium.

The enrichment in mesenchymal traits along with stem features may be explained by either a selection of a pre-existing

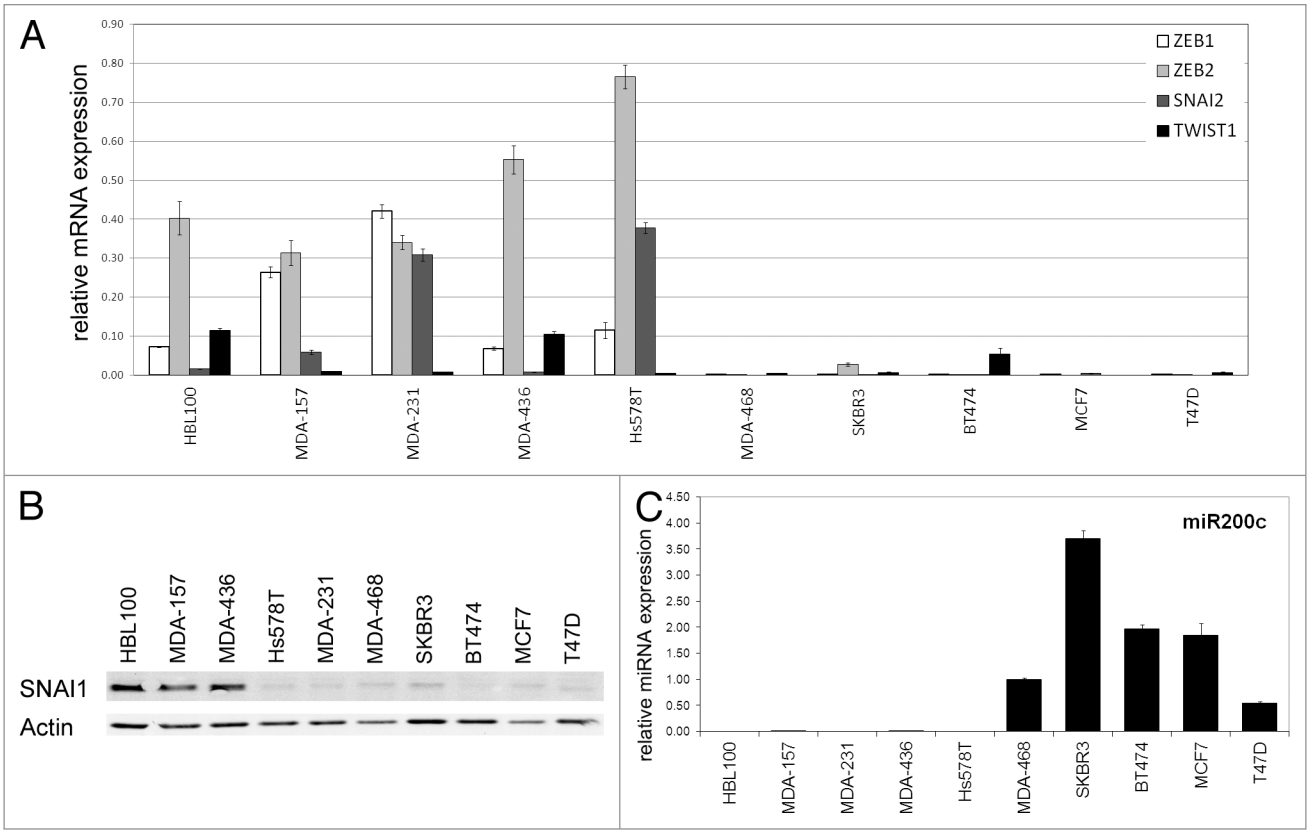

Figure 4. EMT transcription factors and miR200c are distinctively expressed in claudin-low, basal-like, HER2-positive and luminal breast cancer cell lines. (**A**) Quantitative-RT-PCR of ZEB1 □, ZEB2 ■, SNAI2 ■, TWIST1 ■ in the 10 breast cancer cell lines analyzed. Relative expression of transcripts were normalized to three reference genes as described in Materials and Methods. Bars represent standard deviation. (**B**) Representative western blot analysis of SNAI1 protein levels. Blots were probed with actin for normalization. (**C**) miR200c relative levels measured by qRT-PCR as described in Materials and Methods and normalized to RNU48 as a reference control. Bars represent standard deviation.

minute subpopulation of cells with mesenchymal features or by de novo induction of EMT. It has been recently demonstrated that breast cell lines, including luminal cell lines, contain a small fraction of cells expressing basal-like and claudin-low markers and EMT transcription factors.[39] On the other hand, it has been shown that that three-dimensional cultures may affect cell signaling pathways,[40] and loss of cell polarity and cell-cell interactions may promote epithelial-mesenchymal transition.[41,42] Intriguingly, we observed that short-term mammospheres of MCF7 cells display gain of mesenchymal features but fail to enrich in the $CD44^{+}/CD24^{-/low}$ fraction, which Gutilla and colleagues reported to occur in long-term mammospheres.[43] This result suggests that the shift toward a mesenchymal phenotype may precede the gain of stem features. In this scenario, the gain of stem features would be the outcome, rather than the cause, of an EMT reprogramming, which is somehow in agreement with what observed in BRCA1-mutated tumors (reviewed in ref. 44).

Taken together, our data suggest that MS-proficient conditions select for cells with mesenchymal traits. This observation is not trivial. In fact, phenomena of transdifferentiation may result in chemoresistance and tumor progression. For instance, the gain of mesenchymal features in mammospheres derived from luminal tumors correlates with downregulation of ER-α and, hence, with resistance to anti-ER therapies.[39] Thus, the screening for new compounds should ideally be performed in culture conditions that well-represent these phenomena, and mammosphere culture conditions have the potential to allow the exploitation of novel therapeutic approaches.[45,46]

In summary, the demonstration that MS-proficient conditions allows the expansion of cell populations displaying the two major features of highly aggressive breast tumors (stemness and EMT) supports the adoption of mammospheres as in vitro model for the development and validation of novel and more effective therapeutic approaches.

Materials and Methods

Cell lines and standard culture conditions. A panel of 10 human breast cancer cell lines (MDAMB-157, MDAMB-231, MDAMB-436, MDAMB-468, MCF7, T47D, BT474, SKBR3) was purchased from the American Type Culture Collection (ATCC) and Interlab Cell Line Collection-Genova (Hs578T, HBL100). All cell lines were validated for short tandem repeat profiling.

MDAMB-157, MDAMB-231, MDAMB-436, MDAMB-468, Hs578T, HBL100, T47D, SKBR3 cell lines were cultured in EMEM (Lonza) supplemented with 10% fetal bovine

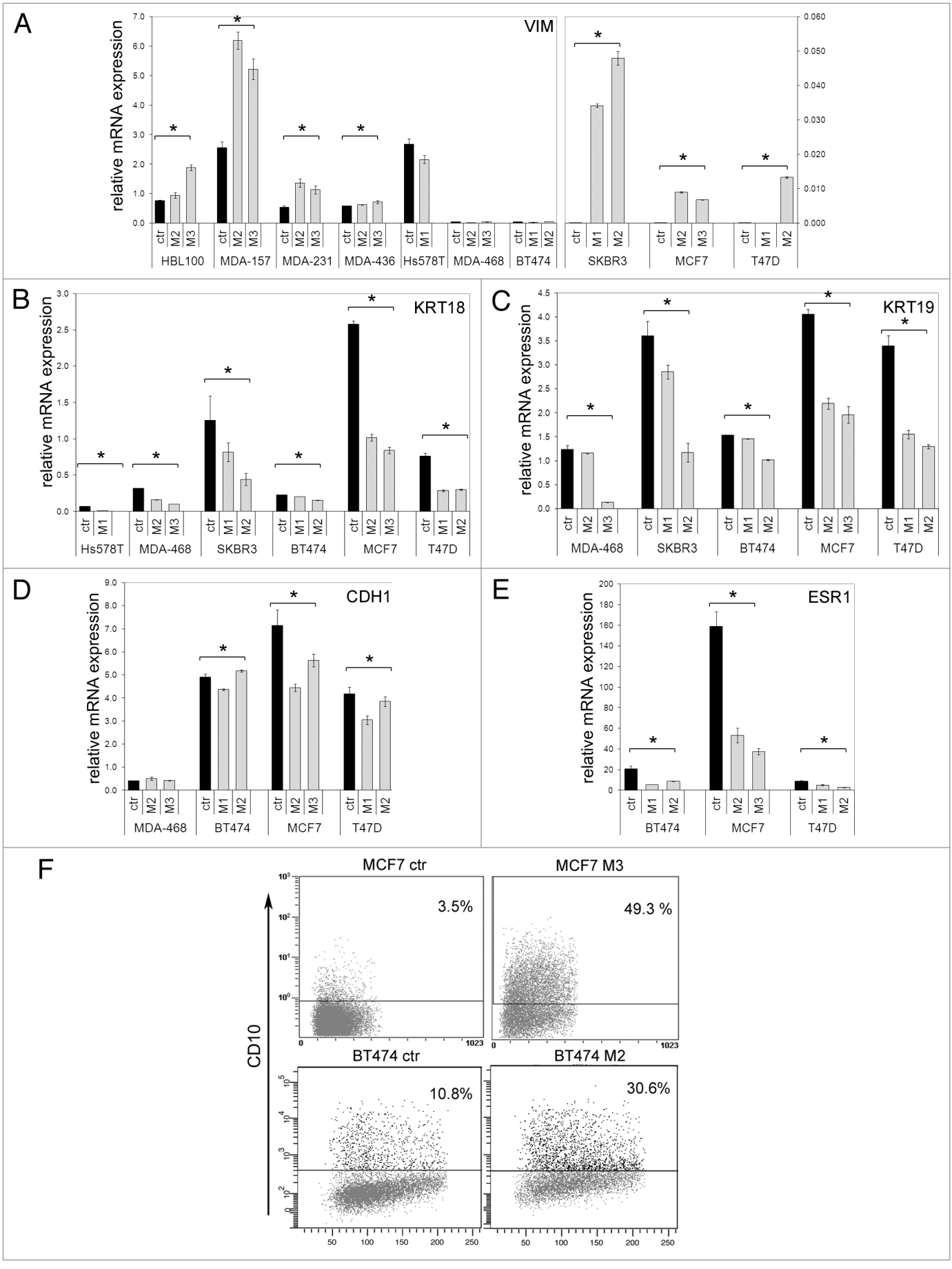
A
VIM
relative mRNA expression
HBL100
MDA-157
MDA-231
MDA-436
Hs578T
MDA-468
BT474
SKBR3
MCF7
T47D
B
KRT18
C
KRT19
D
CDH1
E
ESR1
F
MCF7 ctr
MCF7 M3
3.5%
49.3 %
BT474 ctr
BT474 M2
10.8%
30.6%
CD10

Figure 5 (See previous page). Enhanced mesenchymal features in mammospheres derived from breast cancer cell lines. Relative expression of vimentin, VIM (**A**), Keratin 18, KRT18 (**B**), Keratin 19, KRT19 (**C**), E-cadherin, CDH1 (**D**) and estrogen receptor-α, ESR1 (**E**) transcripts determined by qRT-PCR. Black columns represent control cells (ctr) grown in adherence in the stem medium; gray columns represent cells grown as primary M1, secondary M2 and tertiary M3 spheres. Data were normalized to three reference genes as described in Materials and Methods. Bars represent standard deviation, asterisks $p < 0.05$ in one-way Anova analysis. (**F**) Flow cytometry analysis of CD10-expressing cells in MCF7 and BT474 cell lines grown in adherence (ctr) or as mammospheres (M3 and M2, respectively). Percentages were determined setting the gate on the isotype control.

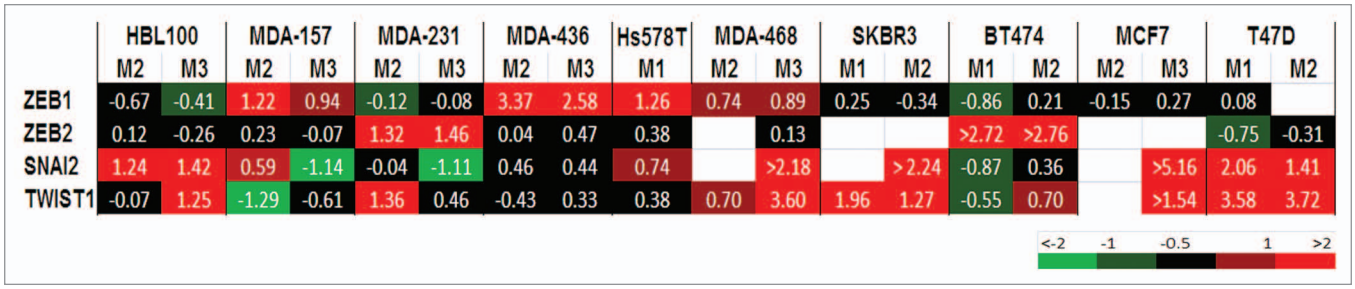

Figure 6. Modulation of ZEB1, ZEB2, SNAI2 and TWIST1 transcript levels in cells grown as primary (M1), secondary (M2) and tertiary (M3) mammospheres compared with control (cells grown in adherence in the stem medium). Heat-map represents the logarithm of the relative mRNA levels, obtained by qRT-PCR, normalized to three reference genes, as described in Materials and Methods, and referred to the control value. Red means upregulation and green downregulation as reported in the legend. Values preceded by > indicate that the control adherent cells did not express the transcription factor and, thus, calculation was performed setting their cycle threshold value at 40 cycles which were the maximum number of PCR cycles performed. White boxes signify absence of measurable transcript expression.

serum (Gibco, Invitrogen), non-essential amino acids (Gibco, Invitrogen) and antibiotics. MCF7 and BT474 were cultered in RPMI (Lonza) supplemented with 10% FBS and antibiotics.

MDAMB-157, MDAMB-231, MDAMB-436 and MDAMB-468 are referred in this paper as MDA-157, MDA-231, MDA-436 and MDA-468, respectively.

Mammosphere culture conditions (MS-proficient condition) and Sphere-forming eficiency (SFE). Single-cell suspensions derived from monolayer cultures were maintained in a serum-free "stem medium" consisting of DMEM F-12 medium (Gibco) supplemented with 4 ug/ml heparin (Sigma), 0.6% glucose, 0.1% NaHCO3, 100 ug/ml apotransferrin, 25 ug/ml insulin, 9 ug/ml putrescine (1–4 diaminobutane dihydrochloride) (Sigma) and 3 × 10–4 M sodium selenite (Sigma) in ultra-low attachment plates (Corning). Every other day, culture medium was enriched with fresh 20 ng/ml EGF and 10 ng/ml bFGF (Peprotech). Mammospheres were collected by centrifugation every 6–12 d and dissociated enzymatically (5 min in 0.05% Trypsin/EDTA) or mechanically by pipetting. The obtained single cells were re-plated for secondary and tertiary cultures and collected for RNA and protein.

For SFE analysis, single-cell suspensions derived from monolayer cultures were plated at a density of 2,500 cells/well into 96-well ultra-low attachment plates (Corning). Cells were maintained in the medium conditions mentioned above and were allowed to form spheres up to 10 d. The number of spheres (> 50 cells/sphere) per well was then counted and expressed as ratio of speres/1,000 plated cells (SFE). Mammospheres were collected by centrifugation and mechanically or enzymatically dissociated. Single cells obtained were counted and re-seeded for secondary and tertiary cultures.

Flow cytometry. Monolayer cultured cells and mammospheres were detached in PBS 2 mM EDTA, washed in cold PBS, counted and resuspended at 1×10^6 cells/100 μl in blocking solution (PBS 10% Rabbit Serum, Dako). Cells were labeled with anti-human CD24-PE/Cy7 (BioLegend), CD24-PE (BD, Biosciences), CD44-FITC (BD, Biosciences), CD133/1-PE (Miltenyi Biotec), CD10-PECy5 (BD, Biosciences) or isotype controls according to the suppliers' protocols. Then, cell samples were washed, resuspended in cold PBS 2 mM EDTA, examined on FACSCanto (BD, Biosciences) or Cytomics FC500 (Beckman Coulter) and analyzed by FACSDiva software (BD, Biosciences) or CXP Software (Beckman Coulter), respectively.

RNA extraction, RT-PCR and qRT-PCR. Total RNA was extracted from cells using Trizol (Invitrogen). mRNAs were reverse transcribed into cDNA with SuperScript II Reverse Transcriptase (Invitrogen) and random primers in according with the supplier's protocol. For SOX2 analysis, total RNA was reverse transcribed using a modified poly T oligo (5'-GAA CGA GAC GAC GAC AGA CTT TTT TTT TTT TTT TTT TVV N).[47] RT-PCR was performed using cDNA in a 35 cycles of PCR amplification using Taq Polymerase kit (Promega).

Quantitative RT-PCR (qRT-PCR), using gene-specific primers, was performed in triplicate with SsoFast EvaGreen Supermix (BioRad) and the CFX96 Real-Time System (Bio-Rad). Relative expression levels were normalized to controls by using the comparative Ct (ΔΔCt) method and the geometric average of a set of three housekeeping genes (GAPDH, B2M and RN18S1) by the Bio-Rad CFX manager software. Specific primer pairs have been reported in the **Table S1**. For each gene, three experiments were performed.

Variation in EMT-transcription factor levels was performed using ΔΔCt method reported to the control cells grown in adherence in the same stem medium. Logarithm of relative mRNA levels was then performed. In the case control cells did

not express an EMT-transcription factor, calculation was performed, setting their Ct value at 40 cycles, which is the maximum number of cycles for PCR, and values were reported as major of (>).

For miR200c analysis, RNA was converted into cDNA and amplified in the qRT-PCR by using TaqMan-specific kits for miR200c or for RNU48, as a normalizer, (Applied Biosystems). Again, relative miRNA expression was determined by the ΔΔCt method. Statistical differences between means were evaluated using one-way analysis of variance (Anova) test.

Western blot analysis. Western blotting was performed on cell protein lysates (50 mM Tris, pH 7.6, 250 mM NaCl, 0.2% Triton X-100, 0.3% NP40, 2 nM EGTA, 2 nM EDTA, 0.1% P-40, complete protease inhibitor cocktail Roche Molecular Biochemicals) using mouse antibodies reacting to human SOX2 (R&D Systems), actin (Santacruz) and rabbit antibodies to human SNAI1 (Cell Signaling) and β-tubulin (Santacruz). Immunoreactivity was detected with anti-mouse and anti-rabbit secondary antibodies HRP-labeled (PerkinElmer) using Western Lightning™ Chemiluminescence Reagent Plus (PerkinElmer) by the imaging analyzer Chemidoc XRS+ (Biorad). Protein expression was analyzed using the ImageLab imaging software (Bio-Rad).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

This work was supported by grants from Regione Friuli Venezia Giulia, Italian Ministry of Health (J31J11000480001), Associazione Via di Natale, and Associazione Italiana per la Ricerca sul Cancro (AIRC, MCO-10016).

Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/cc/article/22543/

References

1. Perou CM, Sørlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, et al. Molecular portraits of human breast tumours. Nature 2000; 406:747-52; PMID:10963602; http://dx.doi.org/10.1038/35021093.
2. Sørlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci USA 2001; 98:10869-74; PMID:11553815; http://dx.doi.org/10.1073/pnas.191367098.
3. Perou CM. Molecular stratification of triple-negative breast cancers. Oncologist 2010; 15(Suppl 5):39-48; PMID:21138954; http://dx.doi.org/10.1634/theoncologist.2010-S5-39.
4. Prat A, Parker JS, Karginova O, Fan C, Livasy C, Herschkowitz JI, et al. Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer. Breast Cancer Res 2010; 12:R68; PMID:20813035; http://dx.doi.org/10.1186/bcr2635.
5. Nguyen PL, Taghian AG, Katz MS, Niemierko A, Abi Raad RF, Boon WL, et al. Breast cancer subtype approximated by estrogen receptor, progesterone receptor, and HER-2 is associated with local and distant recurrence after breast-conserving therapy. J Clin Oncol 2008; 26:2373-8; PMID:18413639; http://dx.doi.org/10.1200/JCO.2007.14.4287.
6. Honeth G, Bendahl PO, Ringnér M, Saal LH, Gruvberger-Saal SK, Lövgren K, et al. The CD44+/CD24- phenotype is enriched in basal-like breast tumors. Breast Cancer Res 2008; 10:R53; PMID:18559090; http://dx.doi.org/10.1186/bcr2108.
7. Park SY, Lee HE, Li H, Shipitsin M, Gelman R, Polyak K. Heterogeneity for stem cell-related markers according to tumor subtype and histologic stage in breast cancer. Clin Cancer Res 2010; 16:876-87; PMID:20103682; http://dx.doi.org/10.1158/1078-0432.CCR-09-1532.
8. Dontu G, Abdallah WM, Foley JM, Jackson KW, Clarke MF, Kawamura MJ, et al. In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. Genes Dev 2003; 17:1253-70; PMID:12756227; http://dx.doi.org/10.1101/gad.1061803.
9. Liao MJ, Zhang CC, Zhou B, Zimonjic DB, Mani SA, Kaba M, et al. Enrichment of a population of mammary gland cells that form mammospheres and have in vivo repopulating activity. Cancer Res 2007; 67:8131-8; PMID:17804725; http://dx.doi.org/10.1158/0008-5472.CAN-06-4493.
10. Grimshaw MJ, Cooper L, Papazisis K, Coleman JA, Bohnenkamp HR, Chiapero-Stanke L, et al. Mammosphere culture of metastatic breast cancer cells enriches for tumorigenic breast cancer cells. Breast Cancer Res 2008; 10:R52; PMID:18541018; http://dx.doi.org/10.1186/bcr2106.
11. Cicalese A, Bonizzi G, Pasi CE, Faretta M, Ronzoni S, Giulini B, et al. The tumor suppressor p53 regulates polarity of self-renewing divisions in mammary stem cells. Cell 2009; 138:1083-95; PMID:19766563; http://dx.doi.org/10.1016/j.cell.2009.06.048.
12. Eguiara A, Holgado O, Beloqui I, Abalde L, Sanchez Y, Callol C, et al. Xenografts in zebrafish embryos as a rapid functional assay for breast cancer stem-like cell identification. Cell Cycle 2011; 10:3751-7; PMID:22033190; http://dx.doi.org/10.4161/cc.10.21.17921.
13. Hu X, Ghisolfi L, Keates AC, Zhang J, Xiang S, Lee DK, et al. Induction of cancer cell stemness by chemotherapy. Cell Cycle 2012; 11:2691-8; PMID:22732500; http://dx.doi.org/10.4161/cc.21021.
14. Ben-Porath I, Thomson MW, Carey VJ, Ge R, Bell GW, Regev A, et al. An embryonic stem cell-like gene expression signature in poorly differentiated aggressive human tumors. Nat Genet 2008; 40:499-507; PMID:18443585; http://dx.doi.org/10.1038/ng.127.
15. Wong DJ, Segal E, Chang HY. Stemness, cancer and cancer stem cells. Cell Cycle 2008; 7:3622-4; PMID:19029796; http://dx.doi.org/10.4161/cc.7.23.7104.
16. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci USA 2003; 100:3983-8; PMID:12629218; http://dx.doi.org/10.1073/pnas.0530291100.
17. Phillips TM, McBride WH, Pajonk F. The response of CD24(-/low)/CD44+ breast cancer-initiating cells to radiation. J Natl Cancer Inst 2006; 98:1777-85; PMID:17179479; http://dx.doi.org/10.1093/jnci/djj495.
18. Calcagno AM, Salcido CD, Gillet JP, Wu CP, Fostel JM, Mumau MD, et al. Prolonged drug selection of breast cancer cells and enrichment of cancer stem cell characteristics. J Natl Cancer Inst 2010; 102:1637-52; PMID:20935265; http://dx.doi.org/10.1093/jnci/djq361.
19. Smit MA, Peeper DS. Epithelial-mesenchymal transition and senescence: two cancer-related processes are crossing paths. Aging (Albany NY) 2010; 2:735-41; PMID:20975209.
20. Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. J Clin Invest 2009; 119:1420-8; PMID:19487818; http://dx.doi.org/10.1172/JCI39104.
21. Browne G, Sayan AE, Tulchinsky E. ZEB proteins link cell motility with cell cycle control and cell survival in cancer. Cell Cycle 2010; 9:886-91; PMID:20160487; http://dx.doi.org/10.4161/cc.9.5.10839.
22. Brabletz T, Jung A, Spaderna S, Hlubek F, Kirchner T. Opinion: migrating cancer stem cells - an integrated concept of malignant tumour progression. Nat Rev Cancer 2005; 5:744-9; PMID:16148886; http://dx.doi.org/10.1038/nrc1694.
23. Scheel C, Weinberg RA. Phenotypic plasticity and epithelial-mesenchymal transitions in cancer and normal stem cells? Int J Cancer 2011; 129:2310-4; PMID:21792896; http://dx.doi.org/10.1002/ijc.26311.
24. Ansieau S. EMT in breast cancer stem cell generation. [Epub ahead of print]. Cancer Lett 2012; PMID:22634497; http://dx.doi.org/10.1016/j.canlet.2012.05.014.
25. Ginestier C, Hur MH, Charafe-Jauffret E, Monville F, Dutcher J, Brown M, et al. ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. Cell Stem Cell 2007; 1:555-67; PMID:18371393; http://dx.doi.org/10.1016/j.stem.2007.08.014.
26. Charafe-Jauffret E, Ginestier C, Iovino F, Wicinski J, Cervera N, Finetti P, et al. Breast cancer cell lines contain functional cancer stem cells with metastatic capacity and a distinct molecular signature. Cancer Res 2009; 69:1302-13; PMID:19190339; http://dx.doi.org/10.1158/0008-5472.CAN-08-2741.
27. Gasparotto D, Polesel J, Marzotto A, Colladel R, Piccinin S, Modena P, et al. Overexpression of TWIST2 correlates with poor prognosis in head and neck squamous cell carcinomas. Oncotarget 2011; 2:1165-75; PMID:22201613.
28. Gregory PA, Bracken CP, Bert AG, Goodall GJ. MicroRNAs as regulators of epithelial-mesenchymal transition. Cell Cycle 2008; 7:3112-8; PMID:18927505; http://dx.doi.org/10.4161/cc.7.20.6851.
29. Blick T, Hugo H, Widodo E, Waltham M, Pinto C, Mani SA, et al. Epithelial mesenchymal transition traits in human breast cancer cell lines parallel the CD44(hi/)CD24 (lo/-) stem cell phenotype in human breast cancer. J Mammary Gland Biol Neoplasia 2010; 15:235-52; PMID:20521089; http://dx.doi.org/10.1007/s10911-010-9175-z.

30. Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, et al. The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell 2008; 133:704-15; PMID:18485877; http://dx.doi.org/10.1016/j.cell.2008.03.027.
31. Bachelard-Cascales E, Chapellier M, Delay E, Pochon G, Voeltzel T, Puisieux A, et al. The CD10 enzyme is a key player to identify and regulate human mammary stem cells. Stem Cells 2010; 28:1081-8; PMID:20506111; http://dx.doi.org/10.1002/stem.435.
32. Keller PJ, Arendt LM, Skibinski A, Logvinenko T, Klebba I, Dong S, et al. Defining the cellular precursors to human breast cancer. Proc Natl Acad Sci USA 2012; 109:2772-7; PMID:21940501; http://dx.doi.org/10.1073/pnas.1017626108.
33. Ali HR, Dawson SJ, Blows FM, Provenzano E, Pharoah PD, Caldas C. Cancer stem cell markers in breast cancer: pathological, clinical and prognostic significance. Breast Cancer Res 2011; 13:R118; PMID:22112299; http://dx.doi.org/10.1186/bcr3061.
34. Ricardo S, Vieira AF, Gerhard R, Leitão D, Pinto R, Cameselle-Teijeiro JF, et al. Breast cancer stem cell markers CD44, CD24 and ALDH1: expression distribution within intrinsic molecular subtype. J Clin Pathol 2011; 64:937-46; PMID:21680574; http://dx.doi.org/10.1136/jcp.2011.090456.
35. Khoury T, Ademuyiwa FO, Chandrasekhar R, Jabbour M, Deleo A, Ferrone S, et al. Aldehyde dehydrogenase 1A1 expression in breast cancer is associated with stage, triple negativity, and outcome to neoadjuvant chemotherapy. Mod Pathol 2012; 25:388-97; PMID:22080062; http://dx.doi.org/10.1038/modpathol.2011.172.
36. Eirew P, Kannan N, Knapp DJ, Vaillant F, Emerman JT, Lindeman GJ, et al. Aldehyde dehydrogenase activity is a biomarker of primitive normal human mammary luminal cells. Stem Cells 2012; 30:344-8; PMID:22131125; http://dx.doi.org/10.1002/stem.1001.
37. Marcato P, Dean CA, Pan D, Araslanova R, Gillis M, Joshi M, et al. Aldehyde dehydrogenase activity of breast cancer stem cells is primarily due to isoform ALDH1A3 and its expression is predictive of metastasis. Stem Cells 2011; 29:32-45; PMID:21280157; http://dx.doi.org/10.1002/stem.563.
38. Marcato P, Dean CA, Giacomantonio CA, Lee PW. Aldehyde dehydrogenase: its role as a cancer stem cell marker comes down to the specific isoform. Cell Cycle 2011; 10:1378-84; PMID:21552008; http://dx.doi.org/10.4161/cc.10.9.15486.
39. Haughian JM, Pinto MP, Harrell JC, Bliesner BS, Joensuu KM, Dye WW, et al. Maintenance of hormone responsiveness in luminal breast cancers by suppression of Notch. Proc Natl Acad Sci USA 2012; 109:2742-7; PMID:21969591; http://dx.doi.org/10.1073/pnas.1106509108.
40. Kenny PA, Lee GY, Myers CA, Neve RM, Semeiks JR, Spellman PT, et al. The morphologies of breast cancer cell lines in three-dimensional assays correlate with their profiles of gene expression. Mol Oncol 2007; 1:84-96; PMID:18516279; http://dx.doi.org/10.1016/j.molonc.2007.02.004.
41. Royer C, Lu X. Epithelial cell polarity: a major gatekeeper against cancer? Cell Death Differ 2011; 18:1470-7; PMID:21617693; http://dx.doi.org/10.1038/cdd.2011.60.
42. Viloria-Petit AM, Wrana JL. The TGFbeta-Par6 polarity pathway: linking the Par complex to EMT and breast cancer progression. Cell Cycle 2010; 9:623-4; PMID:20107326; http://dx.doi.org/10.4161/cc.9.4.10779.
43. Guttilla IK, Phoenix KN, Hong X, Tirnauer JS, Claffey KP, White BA. Prolonged mammosphere culture of MCF-7 cells induces an EMT and repression of the estrogen receptor by microRNAs. Breast Cancer Res Treat 2012; 132:75-85; PMID:21553120; http://dx.doi.org/10.1007/s10549-011-1534-y.
44. Santarosa M, Maestro R. BRACking news on triple-negative/basal-like breast cancers: how BRCA1 deficiency may result in the development of a selective tumor subtype. Cancer Metastasis Rev 2012; 31:131-42; PMID:22101651; http://dx.doi.org/10.1007/s10555-011-9336-6.
45. Cioce M, Gherardi S, Viglietto G, Strano S, Blandino G, Muti P, et al. Mammosphere-forming cells from breast cancer cell lines as a tool for the identification of CSC-like- and early progenitor-targeting drugs. Cell Cycle 2010; 9:2878-87; PMID:20581442; http://dx.doi.org/10.4161/cc.9.14.12371.
46. Eckert MA, Yang J. Targeting invadopodia to block breast cancer metastasis. Oncotarget 2011; 2:562-8; PMID:21725138.
47. Hurteau GJ, Spivack SD. mRNA-specific reverse transcription-polymerase chain reaction from human tissue extracts. Anal Biochem 2002; 307:304-15; PMID:12202248; http://dx.doi.org/10.1016/S0003-2697(02)00058-1.