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

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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 up-regulation 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). More recently, the claudin-low subtype has been identified among triple-negative breast cancers. These distinct tumor subtypes present different prognosis, with the shortest survival time observed among HER2-positive and basal-like/claudin-low breast cancer patients. 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. 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 and in vivo by the ability of mammospheres to generate tumors when injected at limiting dilutions in immunocompromised mice. 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 and, indeed, these genes and their targets are frequently overexpressed in poorly differentiated breast cancer. Furthermore, breast CSC have been reported to display the CD44+/CD24low antigenic profile that correlates with resistance to conventional therapeutics.

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. The major players of the EMT program include members of the SNAIL, 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.
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.\textsuperscript{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 (standard condition) or 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; and, instead, MDA-436 and MDA-231 grew as clusters or grape-like spheres (Fig. 1B).

The CD44\textsuperscript{+}/CD24\textsuperscript{-}low phenotype recognizes a subpopulation of breast cancer stem cells endowed with tumor-initiating ability and mesenchymal phenotype.\textsuperscript{16} In all but HBL100 claudin-low cell lines, a large fraction of cells displayed a CD44\textsuperscript{+}/CD24\textsuperscript{-}low phenotype (Fig. 1C and Table 1). In HBL100, both CD44\textsuperscript{+}/CD24\textsuperscript{-}low and CD133\textsuperscript{+} profiles were detected (Fig. 1C and Table 1). A CD133\textsuperscript{+} phenotype was displayed by MDA-468 basal-like cells (Fig. 1D). Luminal and the HER2-positive SKBR3 cells were almost negative for CD44\textsuperscript{+}/CD24\textsuperscript{-}low and CD133\textsuperscript{+} phenotypes (Fig. 1C and Table 1). The growth as mammospheres resulted in an expansion of the CD44\textsuperscript{+}/CD24\textsuperscript{-}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 ALDH1A1 and ALDH1A3 isofoms of the ALDH1 enzyme, whose activity has been reported to characterize stem cells.\textsuperscript{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

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

| Cell line | Tumor subtype | % CD24\textsuperscript{*} % CD44\textsuperscript{*} % CD44\textsuperscript{+}/CD24\textsuperscript{*} % CD133\textsuperscript{*} | ESR1\textsuperscript{§} | CDH1\textsuperscript{§} | KRT18\textsuperscript{§} | KRT19\textsuperscript{§} | VIM\textsuperscript{§} |
|-----------|--------------|-------------------------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| 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.
cell lines used tested negative for the expression of TWIST2 that was instead expressed in the control cell line FADU (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 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 (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
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
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 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. On the other hand, it has been shown that that three-dimensional cultures may affect cell signaling pathways, and loss of cell polarity and cell-cell interactions may promote epithelial-mesenchymal transition.

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 reported for the cell lines analyzed, decreased in the long-term 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.

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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 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 efficiency (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 μg/ml heparin (Sigma), 0.6% glucose, 0.1% NaHCO3, 100 μg/ml apotransferrin, 25 μg/ml insulin, 9 μg/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 replated 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 spheres/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 accordance 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 TT TTT TTT TTT TTT TTT TTVV 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 PCR cycles performed. White boxes signify absence of measurable transcript expression.

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, ESRI (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.

CD10-PECy5 (BD, Biosciences), CD44-FITC (BD, Biosciences), CD133/1-PE (BioLegend), CD24-PE (BD, Biosciences), CD24-PE/Cy7 (BioLegend), 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.

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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 mM EDTA, 0.1% P-40, complete protease inhibitor cocktail Roche Molecular Biochemicals) using mouse antibodies reacting to human SOX2 (R&B Systems), actin (Santa Cruz) and rabbit antibodies to human SNAI1 (Cell Signaling) and β-tubulin (Santa Cruz). 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.

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