

Isolation and *In vitro* Propagation of Tumorigenic Breast Cancer Cells with Stem/Progenitor Cell Properties

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Abstract

Breast cancer–initiating cells have been recently identified in breast carcinoma as CD44⁺/CD24^{-low} cells, which exclusively retain tumorigenic activity and display stem cell–like properties. However, at present, direct evidence that breast cancer–initiating cells can be propagated *in vitro* is still lacking. We report here the isolation and *in vitro* propagation of breast cancer–initiating cells from three breast cancer lesions and from an established breast carcinoma cell line. Our breast carcinoma–derived cultures encompassed undifferentiated cells capable of self-renewal, extensive proliferation as clonal nonadherent spherical clusters, and differentiation along different mammary epithelial lineages (ductal and myoepithelial). Interestingly, cultured cells were CD44⁺/CD24⁻ and Cx43⁻, overexpressed neoangiogenic and cytoprotective factors, expressed the putative stem cell marker Oct-4, and gave rise to new tumors when as few as 10³ cells were injected into the mammary fat pad of SCID mice. Long-term cultures of breast tumorigenic cells with stem/progenitor cell properties represent a suitable *in vitro* model to study breast cancer–initiating cells and to develop therapeutic strategies aimed at eradicating the tumorigenic subpopulation within breast cancer. (Cancer Res 2005; 65(13): 5506-11)

Introduction

In the last years, a growing body of evidence has been reported supporting the notion that tumors are organized in a hierarchy of heterogeneous cell populations with different biological properties and that the capability to sustain tumor formation and growth exclusively resides in a small proportion of tumor cells, termed *cancer stem cells* or *tumor-initiating cells* (1–3). Tumor-initiating cells have been identified in blood, brain, and breast cancers (4–6) through an experimental strategy that combines sorting of tumor cell subpopulations, identified on the basis of the different expression of surface markers, with functional transplantation into appropriate animal models. These studies have shown that tumor-initiating cells are responsible for tumor formation and progression and, interestingly, that they are endowed with stem/progenitor cell properties; in particular, tumor-initiating cells share with stem cells the key feature of self-renewal.

The stem cell–like phenotype of tumor-initiating cells and their limited number within the bulk of the tumor may account for their capability to escape conventional therapies, thus leading to disease relapse although the primary lesion is eradicated; hence, the

importance to develop therapeutic strategies capable of affecting tumor-initiating as well as nontumorigenic cells survival. To this extent, the restricted number of tumor-initiating cells may represent a limiting factor that could be overcome by propagating tumor-initiating cells *in vitro*. Accordingly, the establishment of long-term cultures of tumor-initiating cells would represent a step of crucial importance, providing a suitable *in vitro* model for these cells to be studied. On this basis and referring to the work of Al-Hajj et al. (6), who prospectively identified putative breast cancer tumorigenic cells as CD44⁺/CD24^{-low} cells capable to drive tumor formation when a few hundreds cells were injected into the mammary fat pad of NOD/SCID mice, we tried to isolate and propagate *in vitro* breast cancer–initiating cells. By applying a previously described procedure for the culturing of mammary gland stem/progenitor cells, we obtained cultures of CD44⁺/CD24⁻ cells with stem/progenitor cells properties, which were able to form new tumors when as few as 10³ cells were injected into SCID mice.

Materials and Methods

Isolation and *in vitro* expansion of progenitor cells from breast tumor specimens. Tumor specimens were obtained from consenting patients according to the Internal Review and the Ethics Boards of the Istituto Nazionale Tumori of Milan, Italy. Sixteen breast lesions, from the histologic diagnostic assessment and sampled by pathologists, were received in the Laboratory within 30 minutes of surgery and immediately mechanically disaggregated. Occasionally, enzymatic digestion was also required and tissue fragments were incubated at 37°C for 2 hours in a 1:1 solution of collagenase/hyaluronidase (Roche Diagnostics GmbH, Mannheim, Germany and Sigma-Aldrich Corp., St. Louis, MO, respectively). After filtration through a 30 μm pore filter, single cells were plated at 1,000 cells/mL in serum-free DMEM-F12 (Cambrex BioScience, Veniers, Belgium), supplemented with 10 ng/mL basic fibroblast growth factor (bFGF), 20 ng/mL epidermal growth factor (EGF), 5 μg/mL insulin, and 0.4% bovine serum albumin (all from Sigma). Cells grown in these conditions as nonadherent spherical clusters of cells (usually named “spheres” or “mammospheres”) were enzymatically dissociated every 3 days by incubation in a trypsin-EDTA solution (Cambrex) for 2 minutes at 37°C. Conversely, differentiation was induced by culturing mammosphere-derived cells for 8 days on collagen-coated dishes in DMEM-F12 supplemented with 5% fetal bovine serum (Cambrex) without growth factors.

Viable, floating single cells were collected from the supernatant of confluent MCF7 cells by centrifugation at 1,200 rpm for 5 minutes and plated at 1,000 cells/mL in the growth medium described above.

Sphere formation assay. Primary spheres were dissociated as described above and 100 cells per well were plated in 96-well culture dishes in 200 μL of growth medium; 25 μL of medium per well were added every 2 days. The number of spheres for each well was evaluated after 7 days of culture.

Flow-cytometric analysis. By using a FACScan (Becton Dickinson, San José, CA), the expression of a panel of differentiation markers was distinctly evaluated on cells obtained from mammospheres or from cells

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cultured in differentiating conditions. The antibodies used were anti-CK14, anti-CK18, anti-epithelial-specific antigen (ESA), anti- α -smooth muscle actin (α -SMA), anti-CD44, anti-MUC1 (directly or indirectly conjugated with fluorescein, FITC), anti-CD10, and anti-CD24 (directly or indirectly conjugated with phycoerythrin). Antibodies against CK14, CK18, CD10, ESA, and α SMA were obtained from Neomarkers (Fremont, CA); anti-MUC1 from Abcam (Cambridge, United Kingdom); and anti-CD24 from PharMingen (BD Biosciences, Franklin Lakes, NJ). Staining was done according to the instructions of the manufacturer. For staining of cytoplasmic antigens (CK14, CK18, and α SMA), single cells were fixed in 70% ethanol at -20°C for 10 minutes. Conversely, before staining of membrane antigens (CD10, ESA, MUC1, CD44, and CD24), unfixed cells were allowed to recover in fresh medium for 1 hour at 37°C in gentle agitation after dissociation.

In vivo injection of mammosphere cells. Spheres were collected, enzymatically dissociated, washed in PBS, and kept at 4°C until injection into the mammary fat pad of 5-week-old SCID mice. Mice were maintained in laminar flow rooms under constant temperature and humidity and received an estradiol supplementation (0.4 mg/kg s.c., Prodyn Depot, Schering-Plough, Kenilworth, NY) every 10 days for 40 days after cell injection. Mice were inspected for tumor appearance, by observation and palpation, for 15 weeks following cell injection; after this time interval, all mice were sacrificed by cervical dislocation and the presence of each tumor nodule was confirmed by necropsy. Experimental protocols were approved by the Ethics Committee for Animal Experimentation of the Istituto Nazionale Tumori according to the United Kingdom Co-ordinating Committee on Cancer Research Guidelines.

Immunohistochemistry. At sacrifice, nodules grown in mice were immediately removed, fixed in 10% phosphate-buffered formalin, and embedded in paraffin. Four-micrometer-thick formalin-fixed, paraffin-embedded sections were cut, mounted on poly-L-lysine-coated slides (Sigma), dried overnight at 37°C , dewaxed in xylene, rehydrated according to histopathologic procedures, and stained with H&E. For determining factor VIII immunoreactivity, additional slides were placed in a pressure cooker containing 0.1 mol/L sodium citrate (pH 6.5) for 15 minutes. Sections were incubated overnight at 4°C with anti-human factor VIII primary antibody (DakoCytomation, Fort Collins, CO). Immunodetection was done using the ChemMate Detection Kit (peroxidase/3,3'-diaminobenzidine, rabbit/mouse, DakoCytomation).

Biological characterization of mammosphere-derived cells. One million cells were plated into 75 cm^2 flasks in 10 mL of culture medium; 48 hours after seeding, cells were harvested and centrifuged for 5 minutes at 1,200 rpm. Vascular endothelial growth factor (VEGF)-A (secreted isoform 165) and VEGF-C concentrations in culture medium were determined by commercial quantitative immunoassay kits (from R&D Systems, Minneapolis, MN and Bender MedSystem, San Bruno, CA, respectively) according to the instructions of the manufacturer. Total mRNA was extracted by means of Trizol (Life Technologies, Frederick, MD) according to the instructions of the manufacturer. Reverse transcription-PCR amplification of VEGF-A and VEGF-C mRNA was carried out using the GeneAmp Gold RNA PCR reagent kit (Perkin-Elmer Biosystems, Foster City, CA) and the human VEGF-C PCR Primer Pair kit, respectively; samples were processed as described elsewhere (7). β -Actin was used as housekeeping gene and results were expressed as normalized densitometric units.

To determine telomerase activity, the PCR-based telomerase repeat amplification protocol assay was carried out by means of TRAPeze kit (Intergen Co., Oxford, United Kingdom) on 1 μg of protein extract according to the instructions of the manufacturer. To measure telomere length, total DNA was isolated by Quick Pick gDNA (Bionobile Oy, Turku, Finland), digested and separated by pulse field-gel electrophoresis (8), transferred to a nylon membrane, and hybridized with a 5'-end [γ - ^{32}P]dATP-labeled telomeric oligonucleotide probe (TTAGGG) $_4$ by a standard protocol. Filter autoradiographs were scanned and digitalized by Image Quant (Molecular Dynamics, Sunnyvale, CA); the mean terminal restriction fragment length was calculated as previously reported (9).

Expression of survivin, connexin-43 (Cx43), and Oct-4 was evaluated by Western immunoblotting. Total cellular lysates were separated on a 12%

SDS-polyacrylamide gel and transferred to nitrocellulose. Filters were blocked in PBS with 5% skim milk and incubated overnight with the primary antibody anti-survivin (Abcam), anti-Oct-4, and anti-Cx43 (Chemicon Int., Temecula, CA). Filters were then incubated with secondary peroxidase-linked antibody (Amersham Bioscience Europe, Freiburg, Germany). Detection was done by enhanced chemoluminescence Western blotting detection system (Amersham Biosciences). Anti-proliferating cell nuclear antigen or anti- β -actin was used to ensure equal loading of protein on the gel.

Results and Discussion

Establishment of primary cultures of mammary gland precursors from breast tumors. We applied a previously described method for the culturing of both neural and mammary gland stem/progenitor cells (10, 11) to pathologic specimens obtained from 16 breast lesions (13 primary invasive carcinomas, 1 recurrent carcinoma, and 2 fibroadenomas; among the 14 cancer lesions, 8 were estrogen receptor positive and 6 were estrogen receptor negative). Briefly, tumor specimens were mechanically/enzymatically disaggregated and the resulting single-cell suspension was plated at clonal density (1,000 cells/mL) in serum-free medium supplemented with EGF, bFGF, and insulin. In 7 of 16 cases (3 of 8 estrogen receptor-positive cancers, 2 of 6 estrogen receptor-negative cancers, and 2 fibroadenomas), 10 to 15 days after plating the formation of mammospheres could be observed in culture (Fig. 1A and B). Primary spheres could be enzymatically dissociated to single cells, which in turn gave rise to secondary spheres; this procedure could be repeated, leading to an extensive amplification in cell number. However, four of the seven primary cultures adhered and terminally differentiated within a few passages *in vitro* (i.e., four to eight passages), suggesting they did not contain long-term self-renewing cells. Conversely, from the three remaining cases (including one recurrent and two primary cancers), long-term primary cultures were established (i.e., B3R, B16, and BP1), which could be expanded as floating spheres for more than 40 passages *in vitro*; notably, the long-term cultures derived only from estrogen receptor-positive lesions. These data should indicate a correlation between estrogen receptor expression and availability of long-term cultures that need to be further investigated. An additional long-term culture (i.e., MCF-S) was derived from the MCF7 cell line where a side population of Hoechst-excluding cells has been recently shown to persist (12).

Because the ability of growing as spherical clusters does not show per se the presence in culture of self-renewing cells, one single cell per well was plated into 96-well culture dishes; clonal, nonadherent mammospheres formed, which in turn gave rise to long-term cultures, thus providing definitive evidence for the presence of self-renewing cells in the four established cultures. Mammosphere cells extensively proliferated although differences could be observed (Fig. 1C) according to the histologic grade of the corresponding primary tumor and in correlation with the sphere forming efficiency (Fig. 1D), suggesting that tumors characterized by aggressiveness features may encompass a higher percentage of self-renewing precursors than the putatively less aggressive counterparts, in agreement with data obtained on brain tumors (13).

Mammosphere cells were undifferentiated because they failed to express lineage-specific markers of the mammary epithelium, such as CK14 and 18, ESA (or epithelial cellular adhesion molecule), and CD10 (or common acute lymphoblastic leukemia

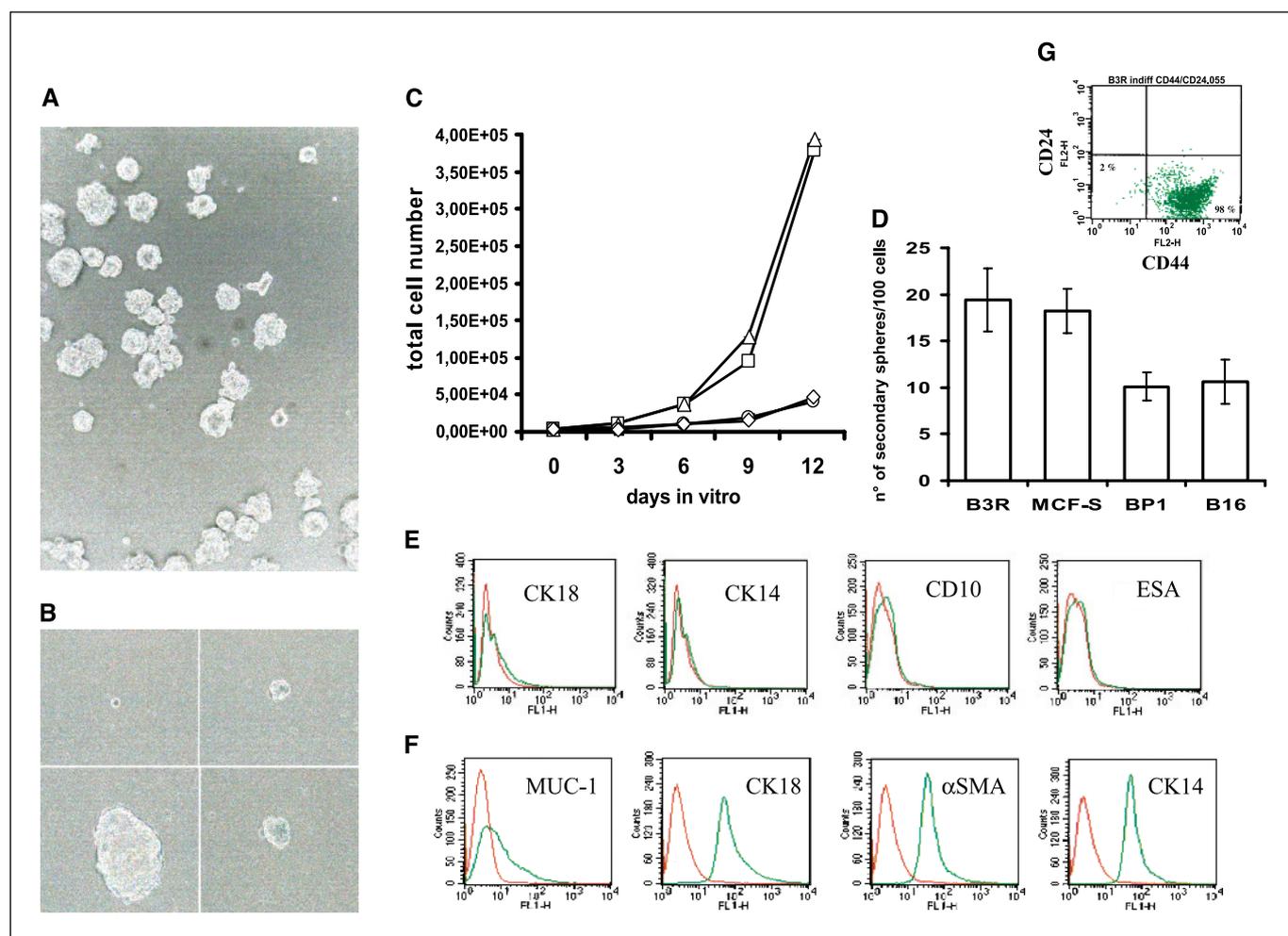


Figure 1. A, culture of isolated breast cancer cells growing as nonadherent mammospheres. B, generation of a mammosphere from a single cell. C, proliferation of isolated breast cancer precursor cell lines: \square , MCF-S, from MCF7 breast carcinoma cell line; \triangle , B3R, primary culture from a ductal infiltrating breast carcinoma, grade 3 (recurrent lesion); \circ , B16, primary culture from a ductal infiltrating primary breast carcinoma, grade 2. D, sphere formation analysis on isolated cell lines. E, fluorescence-activated cell sorting (FACS) analysis of CK18, CK14, CD10, and ESA expression in breast cancer cells grown in serum-free medium as nonadherent mammospheres. F, serum supplementation and withdrawal of growth factors induce the expression of lineage specific markers of the mature mammary epithelium in cultured breast cancer precursors cells. G, FACS analysis of CD44/CD24 expression in breast cancer cells grown in serum-free medium as nonadherent mammospheres.

antigen; Fig. 1E). Under differentiating conditions (i.e., after withdrawal of growth factors and addition of 5% fetal bovine serum), floating cells could adhere and differentiate. They acquired an epithelial-like morphology and expressed mature markers associated to myoepithelial cells (CK14 and α -SMA) and luminal/ductal cells (CK18 and MUC-1; Fig. 1F).

Self-renewing activity and extensive proliferation on mitogen stimulation, as well as undifferentiated status and capability to differentiate into heterogeneous mature cell types, represent hallmarks of stem/progenitor cells in culture. Our breast carcinoma-derived cell cultures display stem/progenitor cell properties and resemble previously described normal breast epithelial cells with similar features (14, 15), which have been shown to belong to the luminal lineage and to reside in an intermediate position between the basal layer and the ductal lumen. These normal breast stem/progenitor cells express estrogen receptor (16, 17) and were found to be highly susceptible to immortalization and transformation (18), suggesting they could be primary targets for breast carcinogenesis; all together, our results

seem to support this hypothesis. Nonetheless, we cannot state at present whether these cultures encompass an actual stem cell population or, rather, downstream progenitors which have regained stem cell-like properties because of genetic alterations. Because breast cancer-initiating cells have been identified as CD44⁺/CD24^{low} cells (6), we evaluated the expression of CD44 and CD24 by flow cytometry; the large majority of cells in culture (95-98%) stained positively for CD44 and negatively for CD24 (Fig. 1G). As in our hands self-renewing cells accounted for 10% to 20% of the total cell number (Fig. 1D), one would conclude that only 1 to 2 of 10 CD44⁺/CD24⁻ cells retain the ability to self-renew. This assumption could be explained considering that the CD44⁺/CD24⁻ subpopulation, which is enriched in tumor-initiating capability, may encompass self-renewing cells together with other cell types lacking this property. If this is the case, one or more additional markers should be able to univocally identify the self-renewing subpopulation among CD44⁺/CD24⁻ tumor-initiating cells, in accord with the enrichment in tumor-initiating capability of CD44⁺/CD24⁻/ESA⁺ cells as compared with CD44⁺/CD24⁻/ESA⁻ (6). However, also the

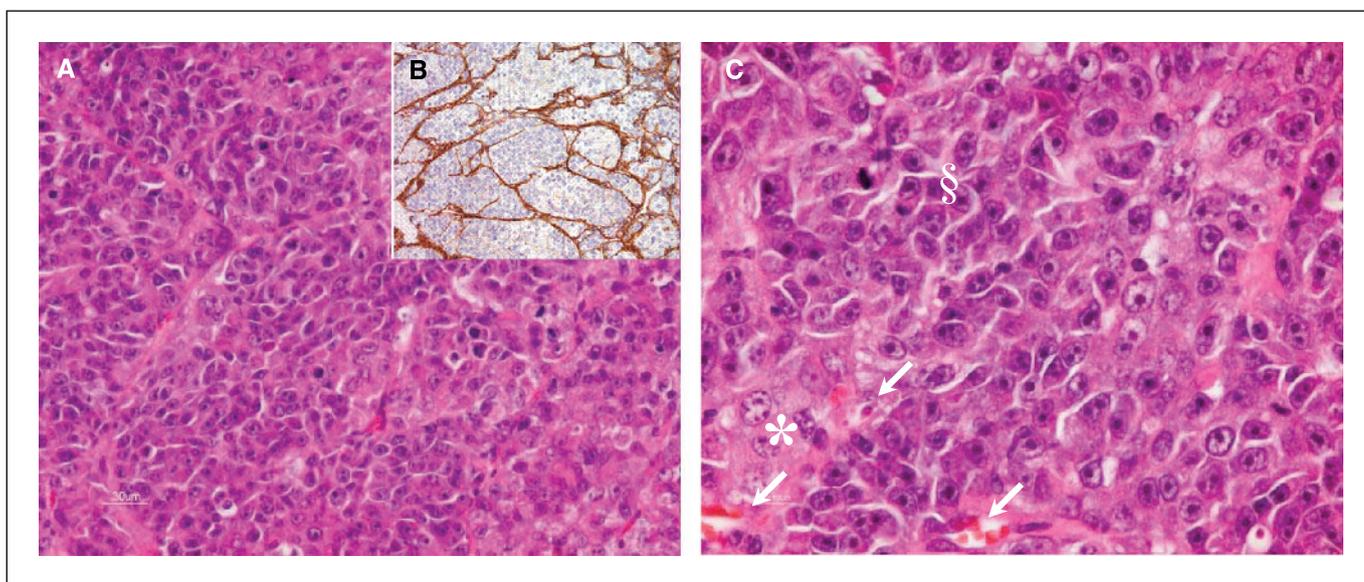


Figure 2. *A* and *B*, a representative section (20× objective) and its higher magnification (60× objective) of tumor grown in SCID mice following the injection of isolated CD44⁺/CD24⁻ cells (H&E). Macronucleated malignant cells showed dark basophilic (s) or pale and vacuolated (*) cytoplasm. In some areas, erythrocytes are visible within tumor cell-lined cavities (arrows). *C*, tumor sections display high vessel density as shown by anti-factor VIII staining (10× objective).

possibility that *in vitro* environmental conditions may induce some changes in antigen expression cannot be excluded.

Tumor-initiating capability of isolated breast cancer precursor cells. To test the hypothesis that CD44⁺/CD24⁻ cells propagated *in vitro* retained tumor-initiating capability, we injected isolated CD44⁺/CD24⁻ cells (from MCF-S and B3R cultures) and MCF7 cells (as control) into the mammary fat pad of SCID mice. After 15 weeks, MCF7 cells gave rise to new

tumors when at least 1 million cells per animal were injected, but failed at lower doses (10⁵ cells/animal). On the contrary, CD44⁺/CD24⁻ cells from MCF-S could form tumors in four of five, three of five, and three of five animals when 10⁵, 10⁴, and 10³ cells/animal were injected, respectively. Superimposable results were obtained with the B3R cell line, as the injection of 10⁵, 10⁴, and 10³ CD44⁺/CD24⁻ cells/animal allowed the development of five of five, four of five, and three of five tumors,

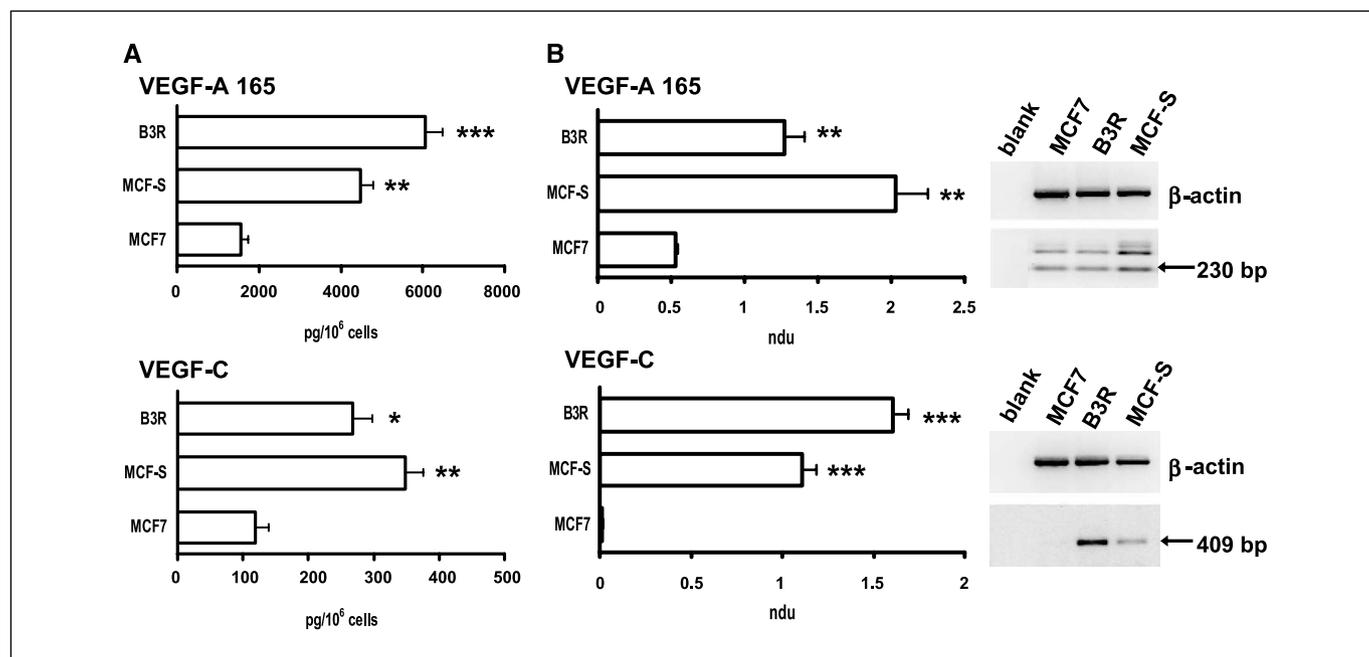


Figure 3. Determination of VEGF-A (isoform 165) and VEGF-C concentration in culture medium (*A*) and measurement of the corresponding mRNAs expression (*B*). Concentration in culture medium of both VEGF-A and VEGF-C is significantly higher for CD44⁺/CD24⁻ breast cancer-initiating cells (B3R and MCF-S) as compared with MCF7 breast carcinoma cells; accordingly, significant differences can be observed in mRNA expression. Columns, mean of three independent measures; bars, SD (values are expressed as normalized densitometric units). Statistical analysis was done by two-tailed Student's *t* test (*, *P* < 0.01; **, *P* < 0.001; ***, *P* < 0.0001).

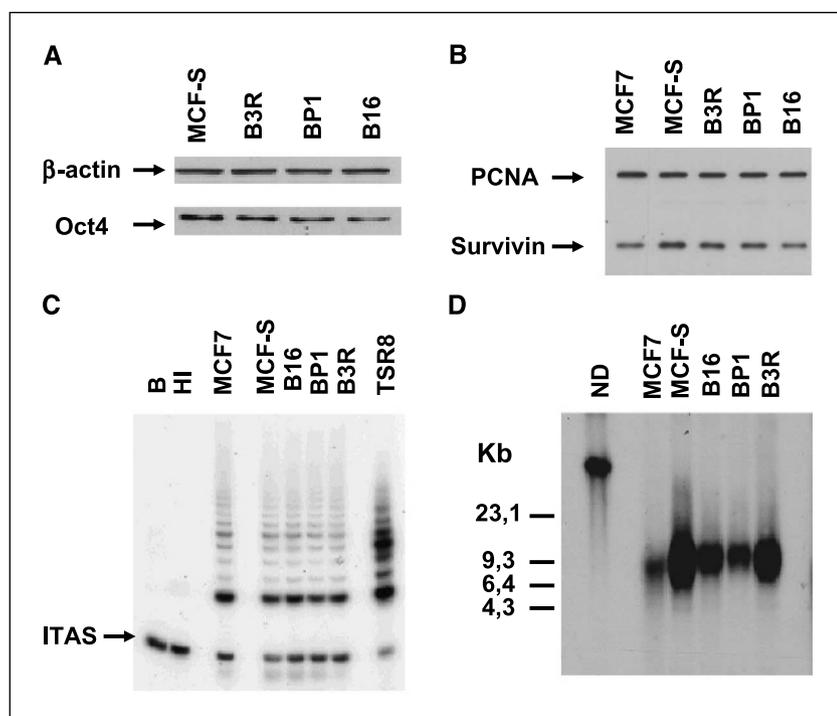


Figure 4. A, expression of the putative stem cell marker Oct-4 in CD44⁺/CD24⁻ breast cancer-initiating cells. B, analysis by Western blotting of the expression of the antiapoptotic protein survivin. C, evaluation of telomerase activity by telomerase repeat amplification protocol assay in cells obtained from breast cancer-initiating cells cultures; no significant difference could be observed in the enzymatic activity among the different cell lines (B, blank; HI, heat inactivated). D, breast cancer-initiating cells displayed homogeneous mean terminal restriction fragments as measured by Southern blotting.

respectively. Taken together, these data show that CD44⁺/CD24⁻ isolated cells are tumorigenic and they are up to 1,000-fold enriched in tumor-initiating capability in comparison with breast carcinoma cells (MCF7). Thus, our isolated cell lines represent *in vitro* cultures of breast cancer-initiating cells with stem/progenitor properties.

Analysis of tumors grown *in vivo*. H&E staining of tumors grown in mice after injection of breast cancer-initiating cells revealed the presence of malignant cells, with large nuclei and prominent nucleoli; some cells showed a dark basophilic cytoplasm, whereas others seemed pale and vacuolated (Fig. 2A and B). Interestingly, high vessel density was observed, as nodules of epithelioid malignant cells were surrounded by back-to-back loops of blood vessels (Fig. 2C). Accordingly, higher amounts of VEGF-A (isoform 165) and VEGF-C were detected in culture medium, as well as at mRNA level, of breast cancer-initiating cells in comparison with MCF7 (Fig. 3). These data provide evidence that breast cancer-initiating cells have a huge angiogenic activity. However, it remains to be defined whether breast cancer-initiating cells contribute to tumor neovascularization by merely secreting soluble factors or by a direct involvement through the formation of vessel-like structures lined by tumor cells, as suggested by the presence of back-to-back loops of vessels as well as of erythrocyte-containing cavities lined by tumor cells (Fig. 2B; ref. 19).

Further characterization of breast cancer-initiating cells. The stem/progenitor cell phenotype of our isolated breast cancer-initiating cells was further confirmed by the expression of the putative stem cell marker Oct-4 (Fig. 4A) and by the absence of Cx43 (data not shown). In addition to stem/progenitor cell properties, isolated breast cancer-initiating cells exhibited features related to the activation of cytoprotective mechanisms and to immortalization. The antiapoptotic protein survivin resulted to be overexpressed in MCF-S as compared with MCF7 cells and

expressed at high levels in the remaining breast cancer-initiating cells cultures (Fig. 4B). Breast cancer-initiating cells displayed a similar extent of telomerase activity (Fig. 4C) and comparable telomere length, with mean terminal restriction fragments ranging from 8 to 10 kb (Fig. 4D). Taken together, these data show that cultured breast cancer-initiating cells display specific tumor cell properties. Reactivation of cytoprotective pathways may provide breast cancer-initiating cells with the ability to escape conventional anticancer treatments; at the same time, however, they might be sensitive to selectively targeted inhibition of the aforementioned pathways, as well as to the switching off of self-renewal apparatus.

Conclusions

In this study, we retrospectively confirmed that CD44⁺/CD24⁻ breast cancer cells, which have been prospectively identified as tumorigenic cells (6), display stem/progenitor cell properties. To our knowledge, for the first time we showed that breast tumorigenic cells with stem/progenitor cell properties can be propagated *in vitro* as nonadherent mammospheres, in keeping with similar findings obtained with normal mammary stem/progenitor cells (11). This experimental system may represent a suitable *in vitro* model to study breast cancer-initiating cells and to challenge them with molecularly targeted agents specifically interfering with breast cancer-initiating cells self-renewal and survival.

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