Long-term cultures of stem/progenitor cells from lobular and ductal breast carcinomas under non-adherent conditions

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Abstract A small subpopulation of stem/progenitor cells can give rise to the diversity of differentiated cells that comprise the bulk of the tumor. Are proliferating cells, within the bulk of tumor, few cells with uncommon features? The cell biological approach provides a limitless model for studying the hierarchical organization of progenitor subpopulation and identifying potential therapeutic targets. Aim of the study was to expand patients' breast cancer cells for evaluating functional cell properties, and to characterize the protein expression profile of selected cells to be compared with that of primary tumors. Breast cancer cells from estrogen receptor (ER α) positive, HER2 negative lobular (LoBS cells) and

ductal (DuBS cells) histotype were cultured under non-adherent conditions to form mammospheres. Sorting of the cells by their surface expression of CD24 and CD44 gave rise to subpopulations which were propagated, enriched and characterized for the expression of epithelial and stromal markers. We found that non-adherent culture conditions generate mammospheres of slowly proliferating cells; single cells, dissociated from mammospheres, grow in soft agar; long-term cultured LoBS and DuBS cells, CD44+/CD24low, express cytokeratin 5 (CK5), α -smooth muscle actin (α -sma) and vimentin, known as markers of basal/myoepithelial cells; and ER α (only DuBS cells), HER1 (EGF-Receptor), activated HER2, and cyclinD1 as markers of luminal epithelial cell. Isolates of cells from breast cancer patients may be a tool for a marker-driven testing of targeted therapies.

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Introduction

Characterization of breast cancer stem cells and identification of their molecular portrait will help to categorize phenotypes to target signaling pathways at cellular level. The design of tailored therapy to treat subtypes of breast cancers requires the analysis of the molecular features of each tumor specimen. One

common feature of solid tumors is the heterogeneity of cells within the tumor mass. Tumor cells differ in morphology, size, protein expression, and in behaviors such as proliferation rate, metastatic potential, and sensitivity to drugs (Campbell and Polyak 2007). Recent data suggest that a small subpopulation of stem/progenitor cells can give rise to the diversity of differentiated cells that comprise the bulk of the tumor (Al-Hajj et al. 2003; Collins et al. 2005). Approximately 75–80% of breast tumors are invasive ductal carcinomas (IDC), less than 15% are invasive lobular carcinomas (ILC) and less than 10% a miscellaneous of rare histological types. The levels of estrogen receptors (ERs) and of epidermal growth factor receptor (HER1)/cerbB2 (HER2) in breast tissue distinguish luminal A and B tumors from basal-like tumors (Perou et al. 2000; Sorlie et al. 2001), and are predictive of response to endocrine treatment (Dowsett et al. 2006). In vitro culture of dissociated tumor cells permit to expand cell populations and overcome the limit of the small size of breast tumors specimen (Nardone et al. 2009), although long-term culture usually leads to selection for basal/myoepithelial cells and all human breast epithelial cell lines established display this phenotype (Petersen and Polyak 2010). Mammosphere generation is a method for culturing mammary gland progenitor cells (Dontu et al. 2003). Cells purified from breast tissues are categorized according to the cell surface expression of CD44 and CD24, which distinguishes CD44[−]/CD24⁺ cells (luminal epithelial cells) from CD44⁺/CD24[−] cells (basal cells) (Shipitsin et al. 2007) however, a considerable heterogeneity in CD44 and CD24 expression was seen both between and within breast tumors (Honeth et al. 2008).

The inter- and intra-tumor heterogeneity of the markers currently used for the categorization of breast tumors into major subtypes (e.g., luminal, HER2⁺, and basal-like) may reflect the lack of methods for the quantitative assessment of intratumor diversity. One critical question is whether different subtypes of breast cancers have common lineage origins, or differing cancer stem cells may explain the different histotype, phenotype, their invasive potential, and, as a consequence, clinical outcome and treatment response.

To address this question, we isolated and characterized cell populations derived from lobular and ductal carcinomas. We established long-term cultures

and generated mammospheres to analyze the progenitor cell properties of different cell isolates. Our results suggest the existence of cancer cells whose features may account for the heterogeneity of cells within the tumor mass; these long-term cultured cells may be a tool for understanding how the molecular features of subtypes of breast cancer may be targeted in breast cancer patients.

Methods

Materials

Non-adherent dishes were 24-well ultra-low binding plates (Corning, NY, USA). Fetal bovine serum (FBS) was purchased from Gibco (Invitrogen, Milan, Italy). The final concentration of the six hormone (6H) mixture was: epidermal growth factor (EGF), 10 ng/ml (E4127); hydrocortisone, 1 µg/ml (H4001); insulin, 5 µg/ml (I5500); estradiol 10 ng/ml (E2758); transferrin, 5 µg/ml (T2252); T3, 10 µM (T5516), all from Sigma–Aldrich. Estradiol and hydrocortisone were dissolved in 70% ethanol. MCF-7 and MDA-MB231 (MDA) were from ATCC. The monoclonal anti-vimentin antibodies were purchased from Sigma–Aldrich (Sigma–Aldrich, Inc., St. Louis, Missouri). Rabbit polyclonal antibodies ER α (HC-20), Cyclin D1 (C-20), EGFR/HER1 (1500, sc03); and mouse monoclonal anti-actin (C-2), cytokeratin 5 (AE14, sc-80606), cytokeratin 19 (BA17, sc53258), α -sma (CGA7, sc53015), p-Neu/HER2 (Tyr 1248) (sc12352-R), antibody were purchased from Santa Cruz (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Ethics and study design

Residual breast cancer tissues were obtained, after informed consent, from patients undergoing surgery for early breast cancer at the Azienda Ospedaliera Universitaria Federico II (Naples, Italy). The samples were anonymously encoded to protect patient confidentiality and used under protocols approved by Azienda Ospedaliera Universitaria Federico II Ethics Committee. Patients did not receive neoadjuvant or adjuvant endocrine therapy. Primary objective of the approved protocol was to expand human breast cancer cells for evaluating functional cell properties

in primary tumors, and to characterize the protein expression profile of selected cells to be compared with that of primary tumors.

Breast tumor specimens: isolation from biopsy and culture conditions of isolated cells

From primary tumor specimens, tumor histotype, size, and markers including ER α , progesterone receptor, grading and HER2 determined by standard procedure (Dako, Carpintera, CA, USA) were retrieved from the pathology report (Table 1). Immediately after surgery, residual fragmented aliquots of fresh specimens were processed as previously reported (Veneziani et al. 2007). The samples were then extensively rinsed with PBS and suspended in standard culture media supplemented with 10% FBS. After three cycles of centrifugation, cells were seeded overnight in minimal essential Dulbecco/Ham F12 (1:1) (DMEM/F12 medium) (Sigma–Aldrich), supplemented with 2 mM glutamine (Sigma–Aldrich), P+S (100 μ g/ml streptomycin, 100 units/ml penicillin), 15 mM HEPES (Sigma–Aldrich) and 5% FBS. After trypsin (0.25% in 1 mM EDTA) (trypsin–EDTA solution, Invitrogen), 2 min at 37 °C, the floating aggregates were transferred to 24 wells and cultured in DMEM/F12 + 0.5% FBS + 6H (Cavaliere et al. 2010) for 21–30 days. Adherent cells, remaining in the primary culture dish after trypsinization, are mainly fibroblasts that can be rescued re-feeding with fresh medium containing 10% FBS. Cells were cultured at 37 °C in a humidified atmosphere of 5% CO₂. The medium was renewed twice weekly.

Cell staining and flow cytometry

Duplicate samples of floating aggregates, cultured in adherent cell culture dishes for 21–30 days, were detached by trypsin–EDTA, pooled, counted in a hemocytometer chamber, and 2.5×10^5 cells/sample were incubated for 5' at RT with 50 μ l of FBS. Four separate experiments were performed per sample. Cells were incubated, in the dark, for 30 min on ice with specific monoclonal antibodies (mAbs) FITC and phycoerythrin(PE)-conjugated directed against CD44 and CD24 cell surface antigens. All conjugated antibodies were purchased from PharMingen (BD Biosciences, Franklin Lakes, NJ). After washing twice with PBS-BSA 0.5%, the cell suspension was centrifuged and the pellet suspended in 300 μ l of PBS-BSA 0.5%. Cell clumps were removed by passing the cell suspension through a 50-micron filter. FACS analysis was performed using the Summit software from DAKO Cytomation. (MoFlo; DakoCytomation, Fort Collins, CO, USA).

Cell counting and cumulative population doubling frequency

LoBS cells were cultured in DMEM/F12 plus 5%FBS (standard medium), while DuBS cells were maintained in DMEM/F12 plus 5%FBS supplemented with 6H (standard medium). For proliferation experiments, growth curves in triplicate were conducted in 24-well plates using 2.5×10^3 cells/well. To this aim, 2.5×10^3 cells/well were plated in low serum (0.5% FBS) for 24 h and then cultured to each well with their own standard medium. At the time indicated, at each passage, after trypsinization, cells,

Table 1 Clinicopathological features of tumour specimens

Histo type	Tumor size grade node	ER α %	PgR%	HER2/c-erbB2 (DAKO score)	Cells ID
Invasive lobular carcinoma	pT1c G3 N0	80	20	Negative (1+)	LoBS
Invasive ductal carcinoma + mucinose carcinoma	pT2 G3 N0	80	80	Negative (0)	DuBS

ER α estrogen receptor, PgR progesterone receptor

maintained in sterile environment, were counted in a hemocytometer chamber, and re-plated 1:2 to new wells. Growth experiments were performed two times per sample. To determine the proliferation rate of the cells, we measured the cumulative population doubling frequency (cpdf) in continuous subculture from a known number of cells. The cpdf at each subcultivation was calculated from the cell count by the formula: $\text{Ln}(\text{No}/\text{Nn})/\text{Ln}2$, where Ln is the natural log and No and Nn are, respectively, initial and final cell numbers. The sum of cpdf of subcultivation periods result in the final total counts.

Sphere formation assay and growth in soft agar

To test the ability of LoBS and DuBS cells to form mammospheres, cells were dissociated and seeded, by serial dilution, in ultra low attachment surface 24-well plates in DMEM/F12 plus 0.5% FCS medium (low serum). The final cell dilutions ranged from 10 to 1,000 cells/well. Sphere formation experiments were carried out at least four times per sample.

For colony growth in soft agar spheres were trypsinized, counted, and 10^4 cells/dish were plated in 60 mm triplicate dishes with 0.3% agar on a 0.5% agar (Type I, Sigma) underlayer DMEM/F12 containing 0.5% FBS. Colonies, cultured for 60 days, were counted in ten fields per dish. The fields to be counted were ID numbered fields on a 7×7 horizontal-vertical transparency grid of 60 mm of diameter. The same ID fields were counted for all the dishes. Results reported are mean $\pm$ SEM of three different experiments performed in triplicate.

Protein extraction and western blot analysis

Western blots were performed, at least three times, on LoBS and DuBS extracts as indicated in the figures. MCF7 cells and MDA-MB231 served as controls. Spheres, transferred for 21–30 days to cell culture dishes, were adherent and proliferating; floating aggregates were detached by trypsin–EDTA, pooled and proteins were extracted. Protein preparations were obtained by lysing samples in 50 mM TRIS pH 7.5, 100 mM NaCl, 1% Nonidet P40, 0.1% Triton, 2 mM EDTA, 10 $\mu\text{g}/\text{ml}$ aprotinin, 100 $\mu\text{g}/\text{ml}$ phenylmethylsulfonyl-fluoride. Protein concentration was measured by the Bio-Rad protein assay (Milan, Italy) and polyacrylamide gels (from 8 to 15%) were

prepared as described (Veneziani et al. 2007). Prestained molecular weight standards were from Biorad (Milan, Italy). Proteins separated on the polyacrylamide gels were blotted on a nitrocellulose membrane (Hybond-C pure, Amersham Italia, Milan, Italy). The membrane was stained with Ponceau S (Sigma) to locate the molecular weight markers. The membranes were stained with secondary antisera, conjugated with horse radish peroxidase (Sigma–Aldrich Corp.) diluted 1:2,000. The luminescent signal was visualized with the ECL Western blotting detection reagent kit (Amersham, Italy) and quantified by scanning with a Discover Pharmacia scanner equipped with a Sun Spark Classic Workstation.

Data analysis

As indicated, flow cytometry, cell counting, sphere formation assay, growth in soft agar, and western blots experiments were carried out 2–4 times and found to be reproducible. Human tissue samples were not pooled, therefore each sample served as its own control. Error bars are presented as standard error of the mean (SEM). We used analysis of variance to identify statistically significant differences among means.

Results

Isolation and propagation of spheres from breast cancer specimens

The cultures of LoBS cells derived from a specimen of breast carcinoma were histologically classified as lobular, pT1c (1–2 cm^2), grade 3, node negative (N0); the cultures of DuBS cells derived from a specimen of breast carcinoma were histologically classified as mixed ductal mucinous, pT2 (2–5 cm^2), grade 3, node negative (N0); both specimens were estrogen receptor positive and *cerbB2*/Neu/HER2-negative (Table 1) (Hammond et al. 2010).

From primary cultures we isolated and cultured human breast cancer sphere-like aggregates of cells in DMEM/F12 plus 0.5%FBS and 6H to disadvantage fibroblasts adhesion and proliferation. Within 3–4 weeks the cultures formed aggregates of growing cells, whose morphology is shown in Fig. 1. We detached these cells with a gentle trypsinization

Fig. 1 Sphere-like aggregates in primary cultures. Primary co-cultures of ductal (*left*) and lobular (*right*) dissociated specimens, maintained in low serum and 6H, to form

aggregates. Aggregates transferred, one per well, to 24-well dishes, within few days, adhere to the well and reach confluence. (Phase-contrast microscopy)

(2 min at 37 °C), and collected only the floating aggregates (300 cells/aggregate) to propagate cells. To this aim we conducted experiments to define culture conditions (data not shown) and found that the cells derived from lobular carcinoma (LoBS cells) adapted to grow in DMEM/F12 plus 5%FBS, and cells derived from ductal carcinoma (DuBS cells) were adapted to grow in DMEM/F12+5%FBS supplemented with 6H. When plated in their own medium, the cells attached and, after 3–4 weeks, formed new aggregates of growing cells. This cell propagating procedure reduced doubling time and generated long-term cultures (30 months, more than 60 passages).

We next evaluated whether the cell aggregates possessed the ability to form spheres. To this aim, we passed, by serial dilution, enzymatically digested aggregates in low-attachment plates with low serum (0.5% FBS); we found that, within few days (2–3), when wells contained 100 cells and more, cells formed floating aggregates that had the characteristic morphology of floating mammospheres (Fig. 2). In these floating conditions, cell counting showed that, during the initial 4–6 weeks of culture, cells proliferate at low rate, with a doubling time of 15 days, while during subsequent long term culture (10–12 weeks) the cells arrested growth. This process is reversible because, as expected, when mammospheres were plated in adherent culture dishes, the cells adhered to the dish and reached confluence (Fig. 3).

Identification of CD44⁺/CD24^{low} cells

In breast cancer CD44⁺/CD24^{low} cells are suggested as a population of cells that contain potential breast cancer stem cells (Al-Hajj et al. 2003; Dontu et al. 2003; Ponti et al. 2005). We evaluated the expression of CD44 and CD24 by flow cytometry. To this aim we collected and analyzed cell floating aggregates which represent a small fraction of all the cells in the dissected tissue (less than 10–15% of the total cell number). As shown (Fig. 4), MCF7 control cells consisted mainly of CD24⁺ expressing cells (luminal-like), whereas LoBS cells and DuBS cells, as indicated, consisted of CD44⁺/CD24^{low} cell populations (myoepithelial and basal cells). This result suggest that the floating aggregates are enriched for CD44⁺/CD24^{low} cells, and that the CD44⁺/CD24^{low} phenotype is not related to the tumor histotype since cells displaying features of stem-like/progenitor can be isolated from either lobular and ductal breast cancer specimens.

Growth properties of CD44⁺/CD24^{low} cells

To evaluate whether the CD44⁺/CD24^{low} cells were able to proliferate in different culture conditions, we measured the growth rate of the cells dissociated from mammospheres in adherent and non-adherent conditions. To this aim cells were enzymatically detached to single-cell suspension and 2×10^4 cells were seeded in DMEM/F12 plus 0.5% FBS medium

Fig. 2 Spheres under non-adherent culture conditions. Cells from confluent wells are transferred, 100 cells/well, to low-attachment multiwells. Within few days DuBS cells (*left panels, a* higher and *b* lower magnification) and LoBS cells (*right panels, c* higher and *d* lower magnification) form floating spheroids. In these conditions, cells initially proliferate at low rate (doubling time of 15 days), then arrest growth. (Phase-contrast microscopy)

Fig. 3 Spheres in adherent culture conditions. Spheres of DuBS (*left panels, a* higher and *b* lower magnification) and LoBS cells (*right panels, c* higher and *d* lower magnification) transferred to cell culture dishes, adhere to the dish and restart proliferation. Cells, detached from adherent wells are pooled and analyzed for flow cytometry and protein expression. (Phase-contrast microscopy)

(low serum) either in ultra low attachment or in adherent 24-well plates for 24 h. The medium was then substituted with standard medium, i.e. 5%FBS

for LoBS cells and 5%FBS+6H for DuBS cells. The effect of adhesion on the long-term growth rates is depicted in Fig. 5. The graph shows the cumulative

Fig. 4 CD44/CD24 flow cytometry of control MCF7, of LoBS and DuBS cells. Sample cells and control cells were detached incubated with specific monoclonal antibodies against CD44 and CD24 cell surface antigens. FACS plots of MCF7 control cells (*upper panel*) are indicative of luminal-like cells, whereas plots of LoBS cells (*intermediate panel*) and DuBS cells (*lower panel*) are indicative of myoepithelial/basal cells

population doubling fraction of the subcultured LoBS cells in low attachment conditions (grey bars) and in adherent plates (black bars) over a period of 105 days and 15 passages. Growth curves for DuBS cells gave

similar results (not shown). The culture of LoBS and DuBS cells, CD44+/CD24^{low}, in low adhesion conditions over long period of time, arrested growth and the ability to propagate.

To determine whether cell growth is anchorage-independent, soft-agar colony assay was done in duplicate, as reported in Fig. 6; the ability to grow in soft-agar corroborates the persistence of repopulating cells after 60 days of sub-cultivation.

Expression of luminal/basal breast cancer markers

To determine whether cultured LoBS and DuBS cells could differentiate into multiple lineages, cells were examined for lineage markers. ER α , and CK19 are categorized as luminal lineage markers (Shipitsin et al. 2007; Sleeman et al. 2006), whereas cytokeratins 5, α -sma and vimentin as basal/myoepithelial lineage markers (Moll et al. 1982; Nagle et al. 1986). We compared LoBS and DuBS cells with MDA-MB231 and MCF-7 reference cell lines (Fig. 7). Cytokeratin 5 and α -sma were overexpressed in LoBS and DuBS cells with respect to MCF7 and MDA-MB231, while cytokeratin 19 was less expressed; vimentin was not expressed in MCF7 epithelial cells and present in MDA-MB231, LoBS and DuBS cells. HER1 (EGFR), frequently co-expressed with HER2, was expressed in MDA-MB231, LoBS and DuBS. Cyclin D1, as marker of cell cycle progression, was present in all the samples tested. Gels for ER α and p-HER2 were done loading 20 μ g of protein for control MCF7 and MDA-MB231 cells and increasing concentrations (10 μ g, 20 μ g, 30 μ g) of cellular extract for LoBS and DuBS cells. ER α were strongly expressed in MCF7 cells, not expressed in LoBS and MDA-MB231, and weakly expressed in DuBS cells. Immunoblotting with anti-phospho-HER2 mAb showed that LoBS and DuBS cells did express p-HER2, and the level of expression was protein-concentration dependent.

Discussion

Studies analyzing the expression of various genes including estrogen receptor, cytokeratins, and HER2 in invasive breast carcinomas demonstrate a high degree of diversity in a subset of tumors (Allred 2008). The heterogeneity of cells within individual

Fig. 5 Long-term growth curve of LoBS cells under non-adherent and adherent conditions. 2.5×10^3 cells/well, were cultured with standard medium for 105 days. At each passage,

cells were enzymatically dissociated to single cells from each triplicate well in a sterile environment, counted, and passaged 1:2 to new wells

Fig. 6 Colony growth in soft agar of LoBS and DuBS cells. *Left*, microphotographs of LoBS and DuBS cells in soft-agar. *Right*, bar-graph of number of colonies; colonies were counted in ten fields per dish on an a horizontal-vertical grid

Fig. 7 LoBS and DuBS cells phenotype. WBs of MCF7, MDA-MB231 (control cells), LoBS and DuBS cells. **a** For CK5, CK19, α -sma, vimentin, HER1, and cycD1, 20 μ g of total protein extract per cell type are loaded on gels. **b** Gels for ER α and p-HER2 are loaded with 20 μ g of protein for control MCF7 and MDA-MB231 cells and increasing concentration (10, 20, 30 μ g) of cellular extract for LoBS and DuBS cells. Actin was used as loading control

tumors may be indicative of a cancer stem cell subpopulation giving rise to more differentiated cell populations. One major problem in breast cancer is whether different subtypes of breast cancers share common lineage origins, or differing cancer stem cells may generate different histotype and phenotype; this critical question for the prediction of clinical outcome and treatment response is still debated.

Although two major subgroups of breast cancer are referred to as ductal and lobular carcinomas, both when invasive and in situ, the majority of breast cancers have a phenotype that supports origin from a cell in the luminal compartment or from a cell that was committed to this lineage.

Putative breast cancer stem cells can be isolated using (i) non-adherent culture conditions to form mammospheres and (ii) identifying the cells by their surface expression of CD24 and CD44 (Clarke et al. 2006). It has been documented that, despite the uniform expression of CD24 or CD44 subsets of tumor cells, these two cell populations are genetically

highly heterogeneous and they display variability for biological and functional traits including tumor-initiating potential and response to therapeutic agents (Park et al. 2010). In the mouse mammary gland, cytokeratin expression and PCR have revealed that CD24[−], CD24^{low}, and CD24^{hi} populations correspond to non-epithelial, basal/myoepithelial, and luminal epithelial cells, respectively (Sleeman et al. 2006). Although not all basal-like tumors contain CD44⁺/CD24[−] cells, an association between basal-like phenotype and CD44⁺/CD24[−] cells has recently been demonstrated (Honeth et al. 2008).

In the breast the term ‘basal’ has acquired two meanings, it has become synonymous with breast myoepithelium, and it defines a specific subpopulation of so-called ‘basal’ cytokeratin expressing cells that may be found in either a luminal or basal location in normal glands (Gusterson et al. 2005). The CD44⁺/CD24^{low} cells that we isolated from lobular and ductal histotype, were yielded from tumor samples that were ER α positive and HER2 negative. ER α positivity

and HER2 negativity are features of tumors defined as “luminal A” according to molecular profiling (Perou et al. 2000), while carcinomas belonging to the basal-like subtype of breast cancers are assumed to be stem cell-derived or to have acquired properties of stem cells during transformation (Sorlie et al. 2001). In general, long-term culture leads to selection for basal/myoepithelial cells. All established human breast epithelial cell lines display a partial loss of the myoepithelial differentiation program along with a partial gain of a luminal differentiation program. Although *in vivo* equivalent of these cells has not been found, this particular culture profile is thought to reflect the existence of human breast epithelial stem cells in the basal compartment (Petersen and Polyak 2010). The cancer stem cell hypothesis postulates that tumors are driven by cells that display the properties of self-renewal and differentiation found in stem cells. Self-renewal is demonstrated by the ability of the enriched stem cell populations to form mammospheres which can grow in an anchorage-independent manner. The long-term culture of LoBS and DuBS cells under non-adherent culture conditions generates mammospheres that initially proliferate, albeit at low rate, then arrest growth; these resting, non proliferating cells, when transferred to adherent dishes, may still be propagated. In addition, their growth is anchorage independent because single cells, dissociated from mammospheres, grow in soft agar. The low growth rate in non-adherent conditions could be due to nutrient and oxygen limitation for the cells within the sphere; indeed these cells, potentially proliferating, restart growth when allowed to form an adherent monolayer.

Myoepithelial cells are cells from the basal layer, defined as cells that express both epithelial characteristics and contractile proteins (Anbazhagan et al. 1998). These cells are distinguished from basal cells in stratified squamous epithelium because they exhibit features of mesenchymal cells, including expression of vimentin (Ronnov-Jessen et al. 1995), smooth muscle actin (α -sma) (Gusterson et al. 1982), the high-molecular-weight cytokeratin CK5 (currently known as basal cytokeratin) (Moll et al. 1982; Nagle et al. 1986). However, studies aimed to define the cell of origin in experimental model systems and in breast tumors show that intermediate filament expression can be modulated in tissue culture and expression of CK5 is not restricted to

myoepithelial cells (Gusterson et al. 2005). Recent studies showed that these cells may represent early progenitors and are candidates for precursor cells of basal-like breast cancer (Ginestier et al. 2007; Villadsen et al. 2007; Lim et al. 2009; Pece et al. 2010). As expected, our long-term cultured LoBS and DuBS cells express vimentin, CK5, and smooth muscle actin (α -sma), all markers of basal/myoepithelial cells.

The majority of human breast cancer cell lines, as well as MCF7 and MDA-MB231, are cytokeratin 19 (CK19) positive. All established breast epithelial cell lines from long-term, primary-cell-derived cultures of reductive mammaplasties are completely cytokeratin CK19-negative (Bartek et al. 1985), and CK19 staining of luminal cells in the fetal breast is homogeneously positive (Anbazhagan et al. 1998). However, recent developments in cell culture technology have allowed long-term expansion of CK19-positive cells from primary tissues in the absence of immortalization (Garbe et al. 2009). With respect to MCF7 and MDA-MB231, LoBS and DuBS cells are weakly CK19-positive thus reinforcing the idea that these cells are likely to be originated from the basal layer.

The lack of the CK19 lineage differentiation is consistent with the lack of estrogen receptor (ER α) in the nonmalignant precursor cells (Petersen and Polyak 2010). In the adult virgin mouse mammary epithelium the majority of stem/progenitor cell activity is located in the basal compartment (Shackleton et al. 2006; Sleeman et al. 2006; Stingl et al. 2006) and the ER α -positive cells are distinct from the mammary stem cell population (Asselin-Labat et al. 2008; Sleeman et al. 2007). In the adult mammary gland only a subset of cells express ER α ; these cells can either form a stem cell compartment that is directly stimulated by circulating hormones (Clarke et al. 2005) or ER α -positive cells may stimulate proliferation of a separate stem cell compartment in a paracrine manner (Briskin and Duss 2007). Pulse-chase experiments suggest that ER α -positive cells form a slowly cycling cell compartment (Booth and Smith 2006) and the stimulation with estrogens down-regulates ER α before the proliferative response (Chen et al. 2008), indeed ER α -positive cells do not express markers of proliferation (Clarke et al. 2005). ER α -expressing luminal epithelial subpopulation contains little *in vivo* stem cell activity. The hormone

susceptibility of mammary epithelial stem cells has been extensively investigated. Notwithstanding, there are conflicting reports regarding the hormone receptor status of these cells in both mouse and human experiments. In mouse, pulse-chase experiments (Booth and Smith 2006) predicted that the putative mouse mammary epithelial stem cells were ER α -positive, while other studies (Asselin-Labat et al. 2008) reported that stem cells identified by specific cell surface markers were negative for both ER α and HER2. Similar divergent results have been described for human mammary epithelial stem cells (Oliveira et al. 2010). To ascertain that the lack of ER α was due to the lack of expression we performed WB experiments loading increasing concentration of cellular extract. We show that, with respect to MCF7, LoBS cells do not express ER α , while DuBS cells do express the receptor in a concentration-dependent fashion. Breast cancer is not a single disease, but rather a group of diseases displaying heterogeneous features at both the molecular and clinical level, and each subtype has its own stable phenotype maintained during tumor evolution (Dontu et al. 2003; Shipitsin et al. 2007; Sorlie et al. 2001). Our data confirm the divergence, that may be presumed to reflect the well-established heterogeneity of breast cancer.

Epidermal growth factor receptor (HER1) and erbB2 (HER2) are two RTKs with oncogenic properties, and two of the most targeted oncoproteins in cancer. HER1 and HER2 overexpression is generally associated with more aggressive tumors and poor prognosis (Sorlie et al. 2003; Sotiriou et al. 2003; Suzuki et al. 2008). Overexpression of HER2 occurs in about 20% of breast carcinomas, and is more frequent in estrogen receptor-negative than in estrogen receptor-positive cases (Sotiriou et al. 2003). In patients with ER α -positive tumors and active growth factor receptor signaling there is growing evidence that crosstalk between ER α and growth factor receptor signaling pathways, especially the HER family, is one of the mechanisms for resistance to endocrine therapy in breast cancer (Knowlden et al. 2003; Osborne et al. 2005). In ER α -positive and HER2-positive metastatic breast cancer two trials demonstrated that the strategy of combining hormone deprivation with antiHER therapy, i.e., anastrozole and herceptin (Kaufman et al. 2009) or letrozole and lapatinib (Johnston et al. 2009), significantly prolonged PFS (Progression Free Survival, defined as

time from random assignment until the earliest date of disease progression or death as a result of any cause in the HER2-positive population). In experimental models of ER α -positive breast cancer cells, initially HER2 negative and hormone-responsive, EGFR and HER2 pathways may become upregulated on development of endocrine resistance over time and a combined hormonal-and growth factor receptor-targeted treatment may delay acquired resistance (Schiff and Osborne 2005; Massarweh et al. 2008). It has been reported that activated phospho-HER2 is expressed also in HER2-negative tumors (Frogne et al. 2009; Singer et al. 2009), and expression of activated HER2 is associated with poor prognosis in the series of hormone receptor-positive breast cancer patients (Frogne et al. 2009). We report data demonstrating the expression of HER1 and activated HER2 in the LoBS and DuBS cells; this supports the idea that subpopulations of cells expressing activated RTK, do exist within the context of a tumor mass that exhibits features of luminal A breast cancer, i.e., ER α -positivity and HER2-negativity. This observation confirms other reports that HER2 expression increases the stem cell population of normal and malignant mammary cells and that the effects of HER2 overexpression on mammary tumorigenesis and invasion are mainly due to its effects on the stem cells population (Korkaya et al. 2008). Our results are also in agreement with studies reporting that HER2-negative tumors express an amount of activated HER2 sufficient to elicit signal transduction, and may explain why some patients with HER2-negative tumors are responsive to herceptin (Paik et al. 2008).

The expression profiling classification of breast cancer into five main molecular subtypes (basal, luminal A and B, HER2-positive/ER α -negative, and normal breast-like) (Sorlie et al. 2001) correlates with clinical outcome, is predictive of response to treatment and prognostic (Sorlie et al. 2001; van de Vijver et al. 2002; Shipitsin et al. 2007). Our approach suggests that the molecular classification can be enriched of intermediate or new subtypes as new methods for the quantitative assessment of intratumor diversity are introduced in clinical practice.

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References

- Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF (2003) Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci USA* 100:3983–3988
- Allred DC (2008) The utility of conventional and molecular pathology in managing breast cancer. *Breast Cancer Res* 10 (suppl 4):S4
- Anbazhagan R, Osin PP, Bartkova J, Nathan B, Lane EB, Gusterson BA (1998) The development of epithelial phenotypes in the human fetal and infant breast. *J Pathol* 184:197–206
- Asselin-Labat ML, Vaillant F, Shackleton M, Bouras T, Lindeman GJ, Visvader JE (2008) Delineating the epithelial hierarchy in the mouse mammary gland. *Cold Spring Harb Symp Quant Biol* 73:469–478
- Bartek J, Taylor-Papadimitriou J, Miller N, Millis R (1985) Patterns of expression of keratin 19 as detected with monoclonal antibodies in human breast tissues and tumours. *Int J Cancer* 36:299–306
- Booth BW, Smith GH (2006) Estrogen receptor- α and progesterone receptor are expressed in label-retaining mammary epithelial cells that divide asymmetrically and retain their template DNA strands. *Breast Cancer Res* 8:R49
- Briskin C, Duss S (2007) Stem cells and the stem cell niche in the breast: an integrated hormonal and developmental perspective. *Stem Cell Rev* 3:147–156
- Campbell LL, Polyak K (2007) Breast tumor heterogeneity: cancer stem cells or clonal evolution? *Cell Cycle* 37:2332–2338
- Cavaliere C, Corvigno S, Galgani M, Limite G, Nardone A, Veneziani BM (2010) Combined inhibitory effect of formestane and herceptin on a subpopulation of CD44+/CD24low breast cancer cells. *Cancer Sci* 107:1661–1669
- Chen ST, Dou J, Temple R, Agarwal R, Wu KM, Walker S (2008) New therapies from old medicines. *Nat Biotechnol* 26:1077–1083
- Clarke RB, Spence K, Anderson E, Howell A, Okano H, Potten CS (2005) A putative human breast stem cell population is enriched for steroid receptor-positive cells. *Dev Biol* 277:443–456
- Clarke MF, Dick JE, Dirks PB, Eaves CJ, Jamieson CHM, Jones DL, Visvader J, Weissman IL, Wahl GM (2006) Cancer stem cells-perspectives on current status and future directions: AACR workshop on cancer stem cells. *Cancer Res* 66:9339–9344
- Collins AT, Berry PA, Hyde C, Stower MJ, Maitland NJ (2005) Prospective identification of tumorigenic prostate cancer stem cells. *Cancer Res* 65:10946–10951
- Dontu G, Abdallah WM, Foley JM, Jackson KW, Clarke MF, Kawamura MJ, Wicha MS (2003) In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. *Genes Dev* 17:1253–1270
- Dowsett M, Houghton J, Iden C, Salter J, Farndon J, A'Hern R, Sainsbury R, Baum M (2006) Benefit from adjuvant tamoxifen therapy in primary breast cancer patients according oestrogen receptor, progesterone receptor, EGF receptor and HER2 status. *Ann Oncol* 17:818–826
- Frogne T, Laenkholm AV, Lyng MB, Henriksen KL, Lykkesfeldt AE (2009) Determination of HER2 phosphorylation at tyrosine 1221/1222 improves prediction of poor survival for breast cancer patients with hormone receptor-positive tumors. *Breast Cancer Res* 11:R11
- Garbe JC, Bhattacharya S, Merchant B, Bassett E, Swisshelm K, Feiler HS, Wyrobek AJ, Stampfer MR (2009) Molecular distinctions between stasis and telomere attrition senescence barriers shown by long-term culture of normal human mammary epithelial cells. *Cancer Res* 69:7557–7568
- Ginestier C, Hur MH, Charafe-Jauffret E, Monville F, Dutcher J, Brown M, Jacquemier J, Viens P, Kleer CG, Liu S, Schott A, Hayes D, Birnbaum D, Wicha MS, Dontu G (2007) ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. *Cell Stem Cell* 1:555–567
- Gusterson BA, Warburton MJ, Mitchell D, Ellison M, Neville AM, Rudland PS (1982) Distribution of myoepithelial cells and basement membrane proteins in the normal breast and in benign and malignant breast disease. *Cancer Res* 42:4763–4770
- Gusterson BA, Ross DT, Heath VJ, Stein T (2005) Basal cytokeratins and their relationship to the cellular origin and functional classification of breast cancer. *Breast Cancer Res* 7:143–148
- Hammond ME, Hayes DF, Dowsett M, Allred DC, Hagerty KL, Badve S, Fitzgibbons PL, Francis G, Goldstein NS, Hayes M, Hicks DG, Lester S, Love R, Mangu PB, McShane L, Miller K, Osborne CK, Paik S, Perlmutter J, Rhodes A, Sasano H, Schwartz JN, Sweep FC, Taube S, Torlakovic EE, Valenstein P, Viale G, Visscher D, Wheeler T, Williams RB, Wittliff JL, Wolff AC (2010) American society of clinical oncology/College of American pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer. *Arch Pathol Lab Med* 134:907–922
- Honeth G, Bendahl PO, Ringnér M, Saal LH, Gruvberger-Saal SK, Lövgren K, Grabau D, Fernö M, Borg A, Hegardt C (2008) The CD44+/CD24- phenotype is enriched in basal-like breast tumors. *Breast Cancer Res* 10:R53
- Johnston S, Pippen J Jr, Pivot X, Lichinitser M, Sadeghi S, Dieras V, Gomez HL, Romieu G, Manikhas A, Kennedy MJ, Press MF, Maltzman J, Florance A, O'Rourke L, Oliva C, Stein S, Pegram M (2009) Lapatinib combined with letrozole versus letrozole and placebo as first-line therapy for postmenopausal hormone receptor-positive metastatic breast cancer. *J Clin Oncol* 27:5538–5546
- Kaufman B, Mackey JR, Clemens MR, Bapsy PP, Vaid A, Wardley A, Tjulandin S, Jahn M, Lehle M, Feyereislova A, Révil C, Jones A (2009) Trastuzumab plus anastrozole versus anastrozole alone for the treatment of postmenopausal women with human epidermal growth factor receptor 2-positive, hormone receptor-positive metastatic breast cancer: results from the randomized phase III TAndEM study. *J Clin Oncol* 27:5529–5537
- Knowlden JM, Hutcheson IR, Jones HE, Madden T, Gee JM, Harper ME, Barrow D, Wakeling AE, Nicholson RI

- (2003) Elevated levels of epidermal growth factor receptor/c-erbB2 heterodimers mediate an autocrine growth regulatory pathway in tamoxifen-resistant MCF-7 cells. *Endocrinology* 144:1032–1044
- Korkaya H, Paulson A, Iovino F, Wicha MS (2008) HER2 regulates the mammary stem/progenitor cell population driving tumorigenesis and invasion. *Oncogene* 27:6120–6130
- Lim E, Vaillant F, Wu D, Forrest NC, Pal B, Hart AH, Asselin-Labat ML, Gyorki DE, Ward T, Partanen A, Feleppa F, Huschtscha LI, Thorne HJ, ConFab FoxSB, Yan M, French JD, Brown MA, Smyth GK, Visvader JE, Lindeman GJ (2009) Aberrant luminal progenitors as the candidate target population for basal tumor development in BRCA1 mutation carriers. *Nat Med* 15:907–913
- Massarweh S, Osborne CK, Creighton CJ, Qin L, Tsimelzon A, Huang S, Weiss H, Rimawi M, Schiff R (2008) Tamoxifen resistance in breast tumors is driven by growth factor receptor signaling with repression of classic estrogen receptor genomic function. *Cancer Res* 68:826–833
- Moll R, Franke WW, Schiller DL (1982) The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. *Cell* 31:11–24
- Nagle RB, Bocker W, Davis JR, Heid HW, Kaufmann M, Lucas DO, Jarasch ED (1986) Characterization of breast carcinomas by two monoclonal antibodies distinguishing myoepithelial from luminal epithelial cells. *J Histochem Cytochem* 34:869–881
- Nardone A, Cavaliere C, Corvigno S, Limite G, De Placido S, Veneziani BM (2009) A banking strategy toward customized therapy in breast cancer. *Cell Tissue Bank* 10:301–308
- Oliveira LR, Jeffrey SS, Ribeiro-Silva A (2010) Stem cells in human breast cancer. *Histol Histopathol* 25:371–385
- Osborne CK, Shou J, Massarweh S, Schiff R (2005) Crosstalk between estrogen receptor and growth factor receptor pathways as a cause for endocrine therapy resistance in breast cancer. *Clin Cancer Res* 11:865s–870s
- Paik S, Kim C, Wolmark N (2008) HER2 status and benefit from adjuvant trastuzumab in breast cancer. *N Engl J Med* 358:1409–1411
- Park SY, Gönen M, Kim HJ, Michor F, Polyak K (2010) Cellular genetic diversity in the progression of in situ human breast carcinomas to an invasive phenotype. *J Clin Invest* 120:636–644
- Pece S, Tosoni D, Confalonieri S, Mazzarol G, Vecchi M, Ronzoni S, Bernard L, Viale G, Pelicci PG, Di Fiore PP (2010) Biological and molecular heterogeneity of breast cancers correlates with their cancer stem cell content. *Cell* 140:62–73
- Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, Fluge O, Pergamenschikov A, Williams C, Zhu SX, Lonning PE, Borresen-Dale AL, Brown PO, Botstein D (2000) Molecular portraits of human breast tumours. *Nature* 406:747–752
- Petersen OW, Polyak K (2010) Stem cells in the human breast. *Cold Spring Harb Perspect Biol* 2. doi: [10.1101/cshperspect.a003160](https://doi.org/10.1101/cshperspect.a003160)
- Ponti D, Costa A, Zaffaroni N, Pratesi G, Petrangolini G, Coradini D, Pilotti S, Pierotti MA, Daidone MG (2005) Isolation and in vitro propagation of tumorigenic breast cancer cells with stem/progenitor cell properties. *Cancer Res* 65:5506–5511
- Ronnov-Jessen L, Petersen OW, Kotliansky VE, Bissell MJ (1995) The origin of the myofibroblasts in breast cancer. Recapitulation of tumor environment in culture unravels diversity and implicates converted fibroblasts and recruited smooth muscle cells. *J Clin Invest* 95:859–873
- Schiff R, Osborne CK (2005) Endocrinology and hormone therapy in breast cancer: new insight into estrogen receptor- α function and its implication for endocrine therapy resistance in breast cancer. *Breast Cancer Res* 7:205–211
- Shackleton M, Vaillant F, Simpson KJ, Stingl J, Smyth GK, Asselin-Labat ML, Wu L, Lindeman GJ, Visvader JE (2006) Generation of a functional mammary gland from a single stem cell. *Nature* 439:84–88
- Shipitsin M, Campbell LL, Argani P, Weremowicz S, Bloushtain-Qimron N, Yao J, Nikolskaya T, Serebryskaya T, Beroukhim R, Hu M, Halushka MK, Sukumar S, Parker LM, Anderson KS, Harris LN, Garber JE, Richardson AL, Schnitt SJ, Nikolsky Y, Gelman RS, Polyak K (2007) Molecular definition of breast tumor heterogeneity. *Cancer Cell* 11:259–273
- Singer CF, Gschwanner-Kaulich D, Fink-Retter A, Pfeiler G, Walter I, Hudelist G, Helmy S, Spiess AC, Lamm W, Kubista E (2009) HER2 overexpression and activation, and tamoxifen efficacy in receptor-positive early breast cancer. *J Cancer Res Clin Oncol* 135:807–813
- Sleeman KE, Kendrick H, Ashworth A, Isacke CM, Smalley MJ (2006) CD24 staining of mouse mammary gland cells defines luminal epithelial, myoepithelial/basal and non-epithelial cells. *Breast Cancer Res* 8:R7
- Sleeman KE, Kendrick H, Robertson D, Isacke CM, Ashworth A, Smalley MJ (2007) Dissociation of estrogen receptor expression and in vivo stem cell activity in the mammary gland. *J Cell Biol* 176:19–26
- Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, Hastie T, Eisen MB, van de Rijn M, Jeffrey SS, Thorsen T, Quist H, Matese JC, Brown PO, Botstein D, Eystein Lonning P, Borresen-Dale AL (2001) Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci USA* 98:10869–10874
- Sorlie T, Tibshirani R, Parker J, Hastie T, Marron JS, Nobel A, Deng S, Johnsen H, Pesich R, Geisler S, Demeter J, Perou CM, Lønning PE, Brown PO, Børresen-Dale AL, Botstein D (2003) Repeated observation of breast tumor subtypes in independent gene expression data sets. *Proc Natl Acad Sci USA* 100:8418–8423
- Sotiriou C, Neo SY, McShane LM, Korn EL, Long PM, Jazaeri A, Martiat P, Fox SB, Harris AL, Liu ET (2003) Breast cancer classification and prognosis based on gene expression profiles from a population-based study. *Proc Natl Acad Sci USA* 100:10393–10398
- Stingl J, Eirew P, Ricketson I, Shackleton M, Vaillant F, Choi D, Li HI, Eaves CJ (2006) Purification and unique properties of mammary epithelial stem cells. *Nature* 439:993–997
- Suzuki T, Miki Y, Ohuchi N, Sasano H (2008) Intratumoral estrogen production in breast carcinoma: significance of aromatase. *Breast Cancer* 15:270–277

- van de Vijver MJ, He YD, van't Veer LJ, Dai H, Hart AA, Voskuil DW, Schreiber GJ, Peterse JL, Roberts C, Marton MJ, Parrish M, Atsma D, Witteveen A, Glas A, Delahaye L, van der Velde T, Bartelink H, Rodenhuis S, Rutgers ET, Friend SH, Bernards R (2002) A gene-expression signature as a predictor of survival in breast cancer. *N Engl J Med* 347:1999–2009
- Veneziani BM, Criniti V, Cavaliere C, Corvigno S, Nardone A, Picarelli S, Tortora G, Ciardiello F, Limite G, De Placido S (2007) In vitro expansion of human breast cancer epithelial and mesenchymal stromal cells: optimization of a coculture model for personalized therapy approaches. *Mol Cancer Ther* 6:3091–3100
- Villadsen R, Fridriksdottir AJ, Rønnov-Jessen L, Gudjonsson T, Rank F, Labarge MA, Bissell MJ, Petersen OW (2007) Evidence for a stem cell hierarchy in the adult human breast. *J Cell Biol* 177:87–101