Biological and Molecular Heterogeneity of Breast Cancers Correlates with Their Cancer Stem Cell Content

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SUMMARY

Pathways that govern stem cell (SC) function are often subverted in cancer. Here, we report the isolation to near purity of human normal mammary SCs (hNMSCs), from cultured mammospheres, on the basis of their ability to retain the lipophilic dye PKH26 as a consequence of their quiescent nature. PKH26-positive cells possess all the characteristics of hNMSCs. The transcriptional profile of PKH26-positive cells (hNMSC signature) was able to predict biological and molecular features of breast cancers. By using markers of the hNMSC signature, we prospectively isolated SCs from the normal gland and from breast tumors. Poorly differentiated (G3) cancers displayed higher content of prospectively isolated cancer SCs (CSCs) than did well-differentiated (G1) cancers. By comparing G3 and G1 tumors in xenotransplantation experiments, we directly demonstrated that G3s are enriched in CSCs. Our data support the notion that the heterogeneous phenotypical and molecular traits of human breast cancers are a function of their CSC content.

INTRODUCTION

Cancer is frequently characterized by the alteration of pathways that control the homeostasis of normal stem cells (SCs) (Visvader and Lindeman, 2008). The elucidation of the molecular mechanisms that govern normal SC function might, therefore, advance our understanding of tumorigenesis.

In the mammary gland, resident multipotent mammary SCs (MSCs) orchestrate the development of the gland during embryogenesis, and its modifications in postnatal life (Williams and Daniel, 1983). Since MSCs are rare, their purification constitutes a major hurdle to their characterization. A number of approaches, based on the exploitation of MSC surface markers, have allowed the prospective isolation of mouse and human MSCs (Eirew et al., 2008; Liao et al., 2007; Lim et al., 2009; Raouf et al., 2008; Shackleton et al., 2006; Stingl et al., 2006). However, the relative promiscuity of these markers (Carter et al., 1990; Jones et al., 2004; Stingl et al., 1998; Stingl et al., 2006) limits their usefulness when highly purified MSCs are needed.

At the onset of the present study, we devised a strategy to obtain highly pure populations of MSCs, based on their functional, rather than immunophenotypical, characteristics, and relying on the “mammosphere” technology (Dontu et al., 2003). Since MSCs can withstand anoikis, they proliferate/differentiate in anchorage-independent conditions, giving rise to clonal spheroids, which can in part recapitulate the mammary morphogenetic program. MSCs, however, constitute less than 1% of all cells in a mammosphere (Dontu et al., 2003). To identify this fraction of cells, we used a lipophilic fluorescent dye, PKH26, which labels relatively quiescent cells within a proliferating population (Huang et al., 1999; Lanzkron et al., 1999). During the growth of a mammosphere, the rare quiescent/slowly dividing MSCs retain PKH26 epifluorescence, while the bulk population, derived from the proliferation of progenitors of the transit-amplifying compartment, progressively lose it by dilution. We were able to purify, by fluorescence-activated cell sorting (FACS), a minority of PKH-positive (PKHPOS) cells from human mammospheres to near homogeneity and to show that they represent human normal MSCs (hNMSCs). We then obtained the transcriptional profile of PKHPOS/hNMSCs and compared it to that of their immediate progeny, thus identifying a hNMSC signature.

The apparently simple cytoarchitecture of the mammary gland, composed of an internal layer of luminal epithelial cells and an external layer of myoepithelial cells, is difficult to reconcile with the diversity of breast cancer phenotypes (Stingl and Caldas, 2007). This has led to the hypothesis that, despite its morphological simplicity, the mammary gland is functionally complex and molecularly heterogeneous (Stingl and Caldas, 2007). This hypothesis has stimulated much study and debate regarding the cellular origin of breast cancer subtypes, as it affects our ability to predict tumor behavior and responsiveness to therapy.
With this background in mind, we used the hNMSC signature to interrogate expression data sets of breast cancers. We discovered that the signature can stratify breast cancers according to their biological and molecular features. We further showed that this is due to the different number of cancer SCs (CSCs) (or cancer-initiating cells) present within different types of breast tumors. Based on our data, we propose a model of breast tumorigenesis that might resolve the incongruence between the complexity of breast cancer phenotypes and the simplicity of the normal breast parenchyma.

RESULTS

Isolation of hNMSCs

Cells from normal human mammary glands were labeled with PKH26 and plated in suspension to allow mammosphere growth (Figure S1A available online). As expected, very few cells within mammospheres retained strong epifluorescence (Figure 1 A). The sphere-forming efficiency (SFE) of cells from the mammary gland was 0.003%–0.01% (depending on whether bulk mammary cells or pre-enriched mammary epithelial cells were employed, Figure S1B). The SFE of cells obtained from dissociated mammospheres was 0.1% (F2 in Figure S1B). Normal mammospheres could be propagated for at least four generations (Figure 1B), and their clonogenic ability decreased exponentially (Figure 1B, Figure S1C): at every generation, the SFE was 23% of that measured in the preceding generation (Figure S1C). Mammospheres reproducibly contained ~300 cells per sphere (Figure S1D).

We estimated that in the normal mammary gland there is one mammosphere-initiating cell per ~7500 total cells or ~2000 epithelial cells (Figure S1D), in good agreement with previous reports in mouse and human (Shackleton et al., 2006; Stingl et al., 2006). In addition, we calculated that approximately one mammosphere-initiating cell is present per mammosphere, and that this cell is likely found within the PKH26-positive (PKHPOS) cell fraction (Figure S1D). This was confirmed by monitoring of the ability of single cells, obtained from dissociated mammospheres, to form second-generation mammospheres, as a function of their PKH positivity. Only PKHPOS cells formed...
mammospheres, with a SFE of ~23% (PKH^{POS} vs-PRE-FACS in Figure S1B; note that this number is in agreement with the maximum expected SFE, Figures S1B and S1C).

We used FACS to isolate PKH^{POS} cells. Based on the typical PKH26 distribution in mammospheres and the SFE of cells from mammospheres, we isolated the most epifluorescent 0.2%–0.4% of the total cell population (PKH^{POS} cells, gated at 10^3–10^4 fluorescence units), as well as a "dull" population (gated at 10^1–10^2 fluorescence units, PKH^{NEG} cells) (Figure S1A, inset). Only the PKH^{POS} population gave rise to mammospheres (Figure S1A, inset), with a SFE of 11% (Figure S1B; note that on the basis of this value and a series of normalizations, we estimate that our PKH^{POS} population is 90% pure, Figure S1B). Of note, PKH^{POS} cells could support at least four additional generations of mammosphere growth (Figure 1C, Figure S1C).

By monitoring microscopically the growth of PKH^{POS} cells embedded in methylcellulose, we established that mammospheres are truly the result of the clonal expansion of single PKH^{POS} cells (Figure 1D). PKH^{POS} cells, but not PKH^{NEG} cells showed features of the quiescent state, as evidenced by retention of BrdU and absence of expression of the proliferation marker Ki-67 (Figure 1E). Moreover, PKH^{POS} cells divided asymmetrically, as demonstrated by the unequal partitioning at mitosis of the cell fate determinant Numb (Figures 1F and 1G) (Gönczy, 2008).

Characterization of PKH^{POS} Cells

We analyzed PKH^{POS} cells, by immunofluorescence (IF), with a panel of markers (Experimental Procedures). PKH^{POS} cells shared features with both epithelial (CD24+/EpCAM+) and myoepithelial (CD49F+/CK5+/TP63+) cells (Figure 2A, Figure S2A); however, they did not express detectable terminal differentiation markers, such as Muc1, E-cadherin, and ASMA (data not shown), suggesting that lineage specification has not yet
occurred in these cells. PKH\textsuperscript{NEG} cells, conversely, expressed little or no CD24, CD49F, and TP63; compared to PKH\textsuperscript{POS} cells, they displayed similar levels of EpCAM and lower levels of CK5 (Figure 2A, Figure S2A). Finally, PKH\textsuperscript{NEG} cells did not display detectable levels of Muc1 or ASMA (data not shown).

These findings suggest that, as expected of SCs, PKH\textsuperscript{POS} cells are bipotent. This was confirmed in 2D-differentiation assays on Matrigel. The majority (~87%) of colonies generated by PKH\textsuperscript{POS} cells expressed both epithelial (EpCAM) and myoepithelial (ASMA) markers (Figure 2B, Figure S2B). Within these colonies, individual cells expressed either EpCAM or ASMA, but never both, indicating terminal differentiation of cells into either the epithelial or the myoepithelial lineage (Figure 2B, Figure S2B). By contrast, PKH\textsuperscript{NEG} cells originated either pure epithelial colonies (97%) or, in rarer cases, pure myoepithelial colonies (Figure 2B, Figure S2B). When individual colonies were picked and replated in suspension, bipotent colonies, originating from PKH\textsuperscript{POS} cells, formed mammospheres at high efficiency, whereas lineage-restricted colonies (either epithelial—originating from PKH\textsuperscript{POS} or PKH\textsuperscript{NEG} cells—or myoepithelial—originating from PKH\textsuperscript{NEG} cells) were unable to do so (data not shown).

We also used 3D Matrigel cultures, in which hNMSCs can generate mammary gland organotypic outgrowths (Donut et al., 2003). PKH\textsuperscript{POS} cells generated two types of outgrowths, which recapitulated several aspects of the mammary gland in vivo: hollow, branched lobuloalveolar, and cavitated, acinar-like structures (Figure 2C). Both epithelial (E-cadherin\textsuperscript{+}) and myoepithelial (TP63\textsuperscript{+}) cells could be detected in the acinar-like structures (Figure 2D) by immunohistochemistry (IHC). In addition, these structures accumulated β-casein in the lumen, upon prolactin stimulation (Figure 2E). PKH\textsuperscript{NEG} cells did not form 3D structures, but either grew as monolayers of terminally differentiated cells (Figure 2C) or formed small structures that never reached the dimension or acquired the differentiation-specific features detected in the outgrowths generated by PKH\textsuperscript{POS} cells (data not shown).

Finally, we analyzed the ability of PKH\textsuperscript{POS} cells to reconstitute a normal mammary epithelium when transplanted into humanized epithelium-cleared fat pads of immunocompromised NOD/SCID mice. PKH\textsuperscript{POS} cells were able to do so with an efficiency of around 4% (one in 26 cells, range 1:10–66), while PKH\textsuperscript{NEG} cells could not reconstitute the mammary gland, even when injected at concentrations as high as 10\textsuperscript{5} cells/transplant (Figure 2F). Of note, this value is compatible with the maximum expected value of reconstitution, estimated on the basis of the replicative kinetics of PKH\textsuperscript{POS} cells (Figure S1E). The outgrowths generated by PKH\textsuperscript{POS} cells displayed the normal mammary gland cytoarchitecture (Figure 2G, Figure S2C) and were derived unequivocally from transplanted human cells (Figure 2G, Figure S2D).

Transcriptomic Analysis of hNMSCs

We performed expression profile analysis of PKH\textsuperscript{POS} and PKH\textsuperscript{NEG} cells. We extracted RNAs from FACS-sorted cells derived from three independent pools of mammospheres, each from five to six individuals. We compared profiles of PKH\textsuperscript{POS} and PKH\textsuperscript{NEG} cells for each pool and obtained three separate gene lists of differentially expressed genes (see the Experimental Procedures). Considering the heterogeneity of the samples (see above), the three lists overlapped significantly (Figure 3A). In particular, 2306 probesets (psets) (the “hNMSC signature”) showed a concordant trend of regulation (increased/decreased in two pools, and unchanged in the third one), and significant enrichment (p < 1 × 10\textsuperscript{-8}, Figure 3A). Within this group, 377 psets (the “3/3 signature”) were consistently up- or downregulated in all the three pools (expected 8.35, actual 377, enrichment factor 45.11, p < 1 × 10\textsuperscript{-8}, Figure 3A). The genes of the hNMSC signature and of the 3/3 signature are listed in Table S1, together with their functional annotation and other characteristics that will be discussed later (see the Discussion).

The hNMSC signature, which readily distinguished PKH\textsuperscript{POS} from PKH\textsuperscript{NEG} cells (Figure 3B), was validated by testing the expression of 69 transcripts on three independent RNA preparations of PKH\textsuperscript{POS} and PKH\textsuperscript{NEG} cells, with 64% concordance with the GeneChip data (83% if one considers only those genes, 53, for which a reliable trend of regulation could be obtained, Table S1). Similar results were obtained if only transcripts present in the 3/3 signature were considered (Table S1).

We also used IF to validate the expression of selected genes (based on the antibody availability) upregulated in PKH\textsuperscript{POS} (JAG1, SOX4, DNER, and DLL1/DELT(A) or PKH\textsuperscript{NEG} (HEY1) cells. JAG1, SOX4, DNER, and DLL1 were significantly enriched in PKH\textsuperscript{POS} cells, whereas HEY1 was enriched in PKH\textsuperscript{NEG} cells (Figures 3C and 3D). Finally, we selected markers (CK5, TP63, and SERPINB5/Maspin, for which antibodies suitable for IHC are available), from those upregulated in PKH\textsuperscript{POS} cells, to test whether they could identify hNMSCs within mammospheres. All three markers were expressed in rare (frequently single) cells within the mammosphere (Figure S3A).

Identification of hNMSCs In Vivo Using Markers from the hNMSC Signature

hNMSCs are thought to reside in the basal layer of ducts and/or at the level of the duct-branch points (Chepko and Smith, 1997; Shackleton et al., 2006; Stingl et al., 2006). We analyzed the expression of markers from the hNMSC signature (DLL1, CK5, CD49F, JAG1, and TOP2A) by IF (Figure 3E, Figure S3B) or IHC (Figure S3B) on sections of human mammary glands. In IF experiments, we used double labeling with EpCAM, which allows the visualization of the epithelial layer. Since we have shown that PKH\textsuperscript{POS} cells are EpCAM\textsuperscript{+}, double-positive staining for EpCAM and the selected hNMSC marker should identify those rare cells displaying an EpCAM\textsuperscript{+}/hNMSC-marker\textsuperscript{+} phenotype as putative hNMSCs. Indeed, such rare double-positive cells were found to reside in close proximity to the basal/myoepithelial layer in ducts, or at the duct-branch point (Figure 3E, Figure S3B).

Next, we used surface markers, derived from the hNMSC signature, to isolate cells endowed with mammosphere-forming ability, directly from the mammary gland. We used markers (CD24, CD49F, DNER, and DLL1) for which we had confirmed the differential protein expression between PKH\textsuperscript{POS} and PKH\textsuperscript{NEG} cells (Figures 2A and 3C). In monoparametric FACS, all markers were able to select populations enriched in mammosphere-forming cells, as witnessed by SFEs 15- to 50-fold higher than that of the bulk mammary population (Table 1, Figure S4A). Of note, DNER and DLL1, which were not previously known as hNMSC markers, were the most efficient ones (Table 1). We then tested
our markers in three combinations (CD24/CD49F/DNER, CD24/CD49F/DLL1, and CD49F/DNER/DLL1) in triparametric sorting. In all cases, only cells positive for the expression of all three markers were able to support mammosphere growth (Table 1, Figures S4 B, S4C, and S4D), with efficiencies significantly increased with respect to the monoparametric sorting (additional data on biparametric FACS experiments are in Figure S4E). In particular, the combination CD49F/DNER/DLL1 was the most effective one and yielded cells displaying a SFE >500-fold higher than that of the bulk mammary population (Table 1). Finally, we showed that CD24H/CD49FH/DNERH cells, but not CD24H/CD49FH/DNERL cells, could reconstitute a normal mammary epithelium upon transplant (Figures S4F, S4G, and S4H).

We note that some of results reported herein on markers of hNMSC are at variance with recently published ones (Lim et al., 2009; Shipitsin et al., 2007). While these differences, and possible explanations for them, are discussed in details in the legend to Figure S4 (and in the corresponding section of Extended Experimental Procedures), the sum of our results shows that markers of our hNMSC signature reliably allow the identification and the prospective isolation in vivo of hNMSCs.

The hNMSC Signature Predicts Biological and Molecular Features in Breast Cancer

We used the hNMSC signature (and the 3/3 signature) to perform a meta-analysis of published breast cancer expression data sets. We initially investigated whether the hNMSC signature could stratify breast cancers according to their biological characteristics, exemplified by tumor grade. We employed two data sets published by Ivshina et al. (2006) and Pawitan et al. (2005), from which we extracted the information relative to poorly differentiated (G3) and well-differentiated (G1) tumors (Extended Experimental Procedures). We tested, by gene set enrichment analysis (GSEA), whether the genes of the hNMSC signature were coordinately expressed in G3 versus G1 tumors. There was a significant enrichment of genes upregulated in PKHPOS cells among the genes upregulated in G3 tumors, and a mirroring enrichment of concordantly downregulated genes (Figure 4A, and Table S2, “Specificity controls” sheet). From the GSEA of the Ivshina and Pawitan data sets, we identified 792 and 842 “core enriched genes,” respectively, with 595 genes in common (Figure 4C, Table S2). Comparable results were obtained when we used, as a starting point for GSEA, the more stringent 3/3 signature of 377 psets (Figures 4B and 4D).
We then investigated whether the hNMSC signature could distinguish among the molecular subtypes of breast cancer identified by Perou et al. (2000) and Sørlie et al. (2001). The signature could separate basal-type tumors from other molecular types of breast cancers (ErbB2-type, luminal-A or -B), in a manner that was apparently independent of their histological grade (Figure S5). Furthermore, GSEA showed enrichment of genes concordantly upregulated in PKH POS cells and in basal-type tumors, despite the fact that 63% of the nonbasal tumors were G3s, as compared to 16% G1s in the same group (Figure S5).

Cancer-Initiating Cells in G3 and G1 Tumors

The above results show that the hNMSC signature can stratify breast cancers on the basis of their biological and—at least in part—molecular characteristics. One possible interpretation of these results is that the heterogeneity of breast cancers (ErbB2-type, luminal-A or -B), in a manner that was apparently independent of their histological grade (Figure S5). Furthermore, GSEA showed enrichment of genes concordantly upregulated in PKH POS cells and in basal-type tumors, despite the fact that 63% of the nonbasal tumors were G3s, as compared to 16% G1s in the same group (Figure S5).

Table 1. Sphere-Forming Potential of Purified Mammary Cell Fractions

<table>
<thead>
<tr>
<th>Type of Sorting</th>
<th>Cell Fraction</th>
<th>Gate</th>
<th>SFE (%)</th>
<th>Enrichment (Fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonsorted</td>
<td>Bulk mammary</td>
<td>Fr. 0</td>
<td>0.003 ± 0.001</td>
<td></td>
</tr>
<tr>
<td>Monoparametric Sorting</td>
<td>CD24 HIGH</td>
<td>Fr. 1</td>
<td>0.100 ± 0.007</td>
<td>33</td>
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<tr>
<td></td>
<td>CD24 MEDIUM</td>
<td>Fr. 2</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CD24 LOW</td>
<td>Fr. 3</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CD49F HIGH</td>
<td>Fr. 4</td>
<td>0.049 ± 0.015</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>CD49F MEDIUM</td>
<td>Fr. 5</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CD49F LOW</td>
<td>Fr. 6</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DNER HIGH</td>
<td>Fr. 7</td>
<td>0.146 ± 0.005</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>DNER LOW</td>
<td>Fr. 8</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DLL1 HIGH</td>
<td>Fr. 9</td>
<td>0.121 ± 0.004</td>
<td>40</td>
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<td></td>
<td>DLL1 LOW</td>
<td>Fr. 10</td>
<td>&lt;0.01</td>
<td></td>
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<td></td>
<td>CD24/CD49F/DNER Multiparametric Sorting</td>
<td>Fr. 17</td>
<td>0.659 ± 0.040</td>
<td>220</td>
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<tr>
<td></td>
<td>CD24/CD49F/DLL1 Multiparametric Sorting</td>
<td>Fr. 18</td>
<td>&lt;0.01</td>
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<td>CD49F/DLL1/DNER Multiparametric Sorting</td>
<td>Fr. 19</td>
<td>0.512 ± 0.058</td>
<td>171</td>
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<tr>
<td></td>
<td>CD49F POS (MED+HIGH) , DLL1* (MED+HIGH) , DNER* (HIGH)</td>
<td>Fr. 21</td>
<td>1.591 ± 0.330</td>
<td>530</td>
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<tr>
<td></td>
<td>CD49F POS (MED+HIGH) , DLL1* (HIGH) , DNER* (LOW)</td>
<td>Fr. 22</td>
<td>&lt;0.01</td>
<td></td>
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<td></td>
<td>CD49F POS (MED+HIGH) , DLL1* (LOW) , DNER* (LOW)</td>
<td>Fr. 23</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CD49F NEG (LOW) , DLL1* (HIGH) , DNER* (LOW)</td>
<td>Fr. 24</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CD49F NEG (LOW) , DLL1* (LOW) , DNER* (LOW)</td>
<td>Fr. 25</td>
<td>&lt;0.01</td>
<td></td>
</tr>
</tbody>
</table>

Cells from the indicated FACS experiments (see also Figure S4) were tested for their mammosphere-forming ability. The column “gate” shows the cellular fractions as from Figure S4. The fold enrichment was calculated with respect to the bulk mammary population.

a In these experiments CD49F-sorted cells were divided into a “positive” fraction (corresponding to the medium + high fractions of the monoparametric sorting experiments) and in a “negative” fraction (corresponding to the low fraction of the monoparametric sorting experiments).

b CD49F POS/DLL1 LOW, CD49F NEG/DLL1 HIGH, and CD49F NEG/DLL1 LOW cells were also tested for DNER and found to be DNER LOW (data not shown).

We then investigated whether the hNMSC signature could distinguish among the molecular subtypes of breast cancer identified by Perou et al. (2000) and Sørlie et al. (2001). The signature could separate basal-type tumors from other molecular types of breast cancers (ErbB2-type, luminal-A or -B), in a manner that was apparently independent of their histological grade (Figure S5). Furthermore, GSEA showed enrichment of genes concordantly upregulated in PKH POS cells and in basal-type tumors, despite the fact that 63% of the nonbasal tumors were G3s, as compared to 16% G1s in the same group (Figure S5).

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The precise correspondence between the data obtained in IHC/IF (Figures 5A and 5B), and those obtained by prospective isolation of CSCs (Figures 5D–5F) strongly argues in favor of the possibility that G3 tumors indeed display a higher CSC content than G1 tumors. If so, this should be reflected in different abilities of unsorted cells from the two types of tumors to form mammosphere-like structures in vitro and to give rise to tumors, when transplanted in vivo. To test this, we initially employed a cohort of 28 patients (eight normal, five G1, and 15 G3). Cells from all patients grew as spheroids, when plated in suspension culture (Figure 6A). G1 tumors formed mammosphere-like structures with an efficiency slightly, but significantly, higher than hNMSCs, both at the first and second generations (Figure 6A). Conversely, G3 tumors displayed ~3-fold higher SFE than normal or G1-derived cells (Figure 6A), in good agreement with the 3- to 4-fold increase in putative CSCs evidenced in the IHC/IF analysis (Figure 5A). In addition, G3 tumors gave rise to spheroids that were almost twice as big as those generated by normal or G1 cells (Figures 6A and 6B). Of note, in PKH26-labeling experiments, spheroids from G3 tumors displayed ~4-fold more PKHPOS cells than spheroids from G1 tumors (Figure 6B, Figure S7A).

Finally, in xenotransplantation experiments, cells from both G1 and G3 tumors gave rise to tumors. In G1s (seven tumors tested), the frequency of CSCs was comparable to that of hNMSCs present in the normal gland (Figure 6C, quantitations in Figure S7B), in agreement with the mammosphere data of Figure 6A. In G3 tumors (eight tumors tested), we detected an ~4-fold increase in CSCs (Figure 6C, Figure S7B). In addition, G3 tumors grew to a larger size with respect to G1 tumors (Figure 6D, top), again in agreement with data on mammosphere size, shown in Figure 6A. Tumors obtained in mice retained the G1 and G3 characteristics of the original human tumors (Figure 6D, bottom). Finally, comparable results were obtained when cells derived from G1 or G3 mammospheres were used to induce tumors in mice (Figure S7C), thus formally linking the data obtained in the mammosphere assay and in the tumor transplantation experiments.

**DISCUSSION**

We report here a method, based on the functional labeling of hNMSCs, that enables the purification of hNMSCs to near homogeneity. We demonstrated that cells prospectively isolated from...
cultured mammospheres through this methodology (PKHPOPCellassociated markers from the hNMSC signature by FACS or IHC were validated for authentic hNMSCs. This allowed functional and molecular studies of hNMSCs, leading to a number of conclusions relevant to the homeostasis of the hNMSC compartment and its subversion in cancer.

**Molecular Features of hNMSCs and of the Progenitors of the TA Compartment**

By exploiting the high degree of purity of PKHPOPCells, we obtained comparative molecular profiles of hNMSCs and of their immediate progeny, which were validated by several molecular and cellular criteria. In principle, molecular determinants and pathways of the hNMSC signature should reflect circuitries that are relevant to the maintenance of the hNMSC compartment, and to the molecular strategies enacted by progenitors to exit this compartment. Many such circuitries could be readily identified (Table S1, “Functional classification” sheet), including:

- (1) regulators of cell survival, cell cycle, and telomerase activity, which seemingly underlie the hNMSC quiescent state and their refractoriness to apoptosis;
- (2) growth factor and chemokine

**Figure 5. Cells Expressing Markers of the hNMSC Signature Are Enriched in G3 versus G1 Tumors**

(A) Paraffin-embedded or frozen OCT-embedded sections were analyzed with markers derived from the hNMSC signature in IHC (CK5, TP63, SERPINB5, TOPO2A, SOX4, and DLL1) or IF (CD24, JAG1, ADRM1, and DNER). Examples of staining are on the left (original magnification ×40). Scale bars represent 10 μm. Magnifications of the boxed regions are shown on the right. Scale bars represent 10 μm.

(B) Paraffin-embedded sections (3 μm thick) from the indicated type of preinvasive DCIS lesions were analyzed in IHC with the indicated antibodies. Results were confirmed on multiple sections from three different DCIS for each subtype. Original magnification ×40.

(C) Representative image of a fresh section of a G3 tumor analyzed in IF with the indicated antibodies (DNER, CD49F, and DLL1). Magnifications of the boxed regions are shown on the right. Scale bars represent 10 μm.

(D) Bar graph depicting the frequency of different cell populations identified by FACS analysis with antibodies against the indicated markers, in G1 (n = 6) and G3 (n = 10) tumors (FACS profiles are in Figure S6D). Data are expressed as the percentages of the total number of epithelial cells. Data are expressed as the percentages of positive tumor cells (mean ± SD) in the total epithelial population.

(E) FACS-sorted CD49F+/DLL1+/DNER+ cells (fractions 5 and 6 in Figure S6D) from G1 (n = 2) and G3 (n = 2) tumors were tested for their mammosphere-forming ability. Data are from two independent experiments, each performed in duplicate.

(F) FACS-sorted (shown on the left, see Figure S6D) or unsorted cells, from three G1 and three G3 tumors, were transplanted at the indicated numbers into mammary fat pads of 21-day-old immunocompromised NOD/SCID mice. The number of tumors/injections is shown. See also Figure S6.
receptors, and molecules involved in cell-to-cell and cell-to-extracellular matrix contacts, which suggest the ability of SCs to organize their “niche” by interacting with neighboring cells, and to respond to their special environment; (3) transcription and chromatin remodeling factors, possibly required to regulate transcriptional programs; and (4) molecules involved in oxidative stress/drug response and in DNA damage checkpoint/repair, in line with the notion that SCs are uniquely programmed to preserve their homeostasis, and in particular their genome integrity (see Table S1, where a more detailed discussion is also provided).

While a comprehensive analysis of the molecular characteristics of the hNMSC signature will be impossible here, one feature is worth mentioning. We have previously shown that p53 critically controls the binary fate decision of NMSCs in the mouse mammary gland by influencing the rate of symmetric versus asymmetric self-renewing cell divisions (Cicalese et al., 2009). While some caution is due when extrapolating results from mice to humans, it is nevertheless tempting to speculate that similar regulatory mechanisms might also exist in hNMSCs. Indeed, we found that genes annotated as putative p53 targets on the basis of chromatin immunoprecipitation experiments...
SCs and the Heterogeneity of Human Breast Cancers

When the hNMSC signature was applied to the meta-analysis of breast cancer expression data sets, we found out that it was predictive of biological and molecular features of human breast cancers. Thus, breast cancers can be distinguished based on their degree of resemblance to the hNMSC molecular phenotype.

Recently, Ben-Porath et al. described an embryonic stem-like signature, which could predict, in breast cancers, tumor grade and several additional features, including clinical outcome (Ben-Porath et al., 2008). A direct comparison of our results with those of Ben-Porath et al. is not straightforward, since they meta-analyzed several gene sets associated with human embryonic stem cell identity and used different tools for the analysis of gene set enrichment patterns. In general, however there is no significant overlap between the hNMSC signature and signatures of the ES state. For instance, the “ES exp1” signature of Ben-Porath et al., which includes 380 genes and separates clearly G3 from G1 tumors, shows an overlap of only 16 concordantly regulated genes with the hNMSC signature.

There are plausible reasons to explain the lack of overlap. First, the starting points of the signatures are not the same. In our case, adult mammary SCs were analyzed, while the study of Ben-Porath et al. meta-analyzed signatures representative of various embryonic SC states. Second, signatures do not portray the “absolute” molecular picture of a given condition, but only the comparative picture with respect to another condition. Thus, a “SC signature” can be different according to whether it was derived by comparison of the SC to a pluripotent progenitor, a committed progenitor, or a differentiated cell—a consideration to bear in mind also when comparing our data to other signatures reported for mammary SCs (see for instance Raouf et al. [2008] and discussion in the legend to Figure S7A) that a different rate of skipping of asymmetric division might determine the different number of cancer stem cells in G3 versus G1 tumors, and might therefore sit at the heart of the biological and clinical heterogeneity of breast cancers.

Together, data in our present and previous (Cicalese et al., 2009) studies suggest a scenario for mammary tumorogenesis. In this model, normal SCs are the targets of different oncogenic events. The nature of the transforming event(s) determines the frequency with which the transformed SCs will skip asymmetric self-renewing division. This, in turn, will determine the final number of CSCs within the tumor tissue, and a number of biological and clinical features of the tumor. This model does not preclude additional differential effects of the transforming events, which might, for instance, only allow differentiation toward a certain lineage or up to a certain point in the differentiation program, thus further contributing to the heterogeneity of breast cancers (Lim et al., 2009; Shipitsin et al., 2007).

Finally, we have shown, in a model of murine cancerogenesis, that reduced tumor growth can be achieved by pharmacological interference with the self-renewing properties of CSCs (Cicalese et al., 2009). Here, we show that the number of CSCs in human breast cancers can vary greatly, with discernible impact on several clinical and pathological features. Together, these data provide strong support for the concept of “cancer stem cell-targeted therapy” to eradicate cancer.

EXPERIMENTAL PROCEDURES

Clinical Samples

Fresh, frozen, or archival formalin-fixed paraffin-embedded (FFPE) mammary tissue specimens were collected at the European Institute of Oncology (Milan, Italy). All tissues were collected via standardized operative procedures approved by the Institutional Ethical Board, and informed consent was obtained for all tissue specimens linked with clinical data.

Cultivation of Mammospheres and FACS Procedures

Epithelial cells, from reductive mammoplasties (Pece et al., 2004), were allowed to adhere for 24 hr in complete SC medium (Dontu et al., 2003). Cells were trypsinized, filtered through a 100 μm cell strainer, resuspended in PBS (~500,000 cells/ml), and labeled with PKH26 (Sigma, 10⁻³ M, 5 min). Labeled cells were plated (30,000 cells/ml) in suspension (Dontu et al., 2003). After 7–10 days, mammospheres were harvested, dissociated.
enzymatically (0.05% trypsin/0.5 mM EDTA for 10 min, plus filtering through a 40 μm cell strainer), and subjected to FACS analysis with a FACS Vantage SE ﬂow cytometer (Becton & Dickinson) to yield PKHPOS and PKHNEG cells.

IF of PKHPOS and PKHNEG cells was performed on cytospin preparations fixed with 4% formaldehyde (5 min)/cold methanol (5 min). IF on mammary glands (from OCT-embedded, snap-frozen samples) was performed on 3 μm thick sections, ﬁxed for 10 min with cold methanol/acetic acid (1:1, v/v). IHC was performed as previously described (Pece et al., 2004).

The preparation of FACS-sorted samples, directly from bulk mammary gland (Figure S4), is described in the Extended Experimental Procedures.

**Diﬀerentiation Assays**

For 2D diﬀerentiation assays, PKHPOS and PKHNEG cells were plated at clonogenic density (500 viable cells/well) onto glass slides coated with Matrigel (BD Biosciences) and grown for 10 days in primary mammary epithelial cell medium (Pece et al., 2004), followed by IF with the appropriate antibodies. 3D Matrigel cultures were performed as described (Dontu et al., 2003); where indicated, treatment with prolactin (Sigma) was for 10 days at 5 μg/ml.

**Immunofluorescence, Immunohistochemistry, and Image Quantitative Analysis**

For the immunophenotypical characterization of PKHPOS cells and PKHNEG cells shown in Figure 2 and in Figure 3C, we employed the following primary antibodies: for IF analysis, anti-CD49F and anti-TP63 (BD Bioscience PharMingen), anti-SOX4 and ﬂuorescein isothiocyanate (FITC) anti-ASMA (Sigma), anti-CD24 and biotinylated anti-EpCAM (Neomarkers), antikeratin 5 (DAKO), and biotinylated anti-DNER (R&D Systems). Samples were analyzed under an AX-70 Provis (Olympus) ﬂuoroscence microscope equipped with a black and white cooled CCD camera (Hamamatsu c5985), or with a Leica TCS SP2 AOBs confocal microscope equipped with 405, 488, 543, and 633 nm laser lines. Digital images were computer processed with Adobe Photoshop CS2. For the quantitative analysis of IF experiments performed on PKHPOS and PKHNEG cells, the ImageJ image-analysis software (W. Rasband, National Institutes of Health) was used to measure the speciﬁc mean intensity on an average of ten cells for each antibody staining. Background intensity was initially subtracted by placing “regions of interest” over areas devoid of specific signal.

For immunohistochemical analysis of FFPE sections obtained from normal and tumor breast biopsies or from mammospheres, the primary antibodies used were as follows: anti-CK5, anti-Estrogen receptor, anti-EpCAM (Dako, clone Ber EP4), and anti-TP63 (Dako), anti-HER2 (Neomarkers), anti-HER1 (Abcam), anti-E-cadherin (BD Biosciences PharMingen), anti-JAG1 and anti-Delta/DLL1 (Santa Cruz Biotechnology), anti-ADRM1 (Abnova), and biotinylated anti-DNER (R&D Systems). Samples were analyzed under an AX-70 Provis (Olympus) ﬂuorescence microscope equipped with a black and white cooled CCD camera (Hamamatsu c5985), or with a Leica TCS SP2 AOBS confocal microscope equipped with 405, 488, 543, and 633 nm laser lines. Digital images were computer processed with Adobe Photoshop CS2. For the quantitative analysis of IF experiments performed on PKHPOS and PKHNEG cells, the ImageJ image-analysis software (W. Rasband, National Institutes of Health) was used to measure the specific mean intensity on an average of ten cells for each antibody staining. Background intensity was initially subtracted by placing “regions of interest” over areas devoid of specific signal.

**RNA Extraction and GeneChip Hybridization**

Total RNA was extracted with the Arcturus PicoPure RNA Isolation Kit (Arcturus Engineering, CA) according to the manufacturer’s instructions and analyzed with the Agilent 2100 Bioanalyzer (Agilent Technologies). Total RNA (50–100 ng) was amplified with the Affymetrix T7 Polymerase-based double linear amplification protocol. Complementary RNA probes (10 μg) were hybridized onto the Affymetrix HG-U133_Plus_2 Chip Set (Santa Clara, CA), according to Affymetrix technical protocols. The average intensity of each array was scaled to a predefined value (target intensity) of 500, in order to make arrays comparable.

**Expression Proﬁles and Meta-Analysis**

Hybridization onto the Affymetrix (Santa Clara, CA) HG-U133_Plus_2 Chip Set was as previously described (Vecchi et al., 2007). The statistical analysis was performed with the Affymetrix MAS5 “Comparison Analysis” algorithm (Affymetrix Statistical Algorithm Reference Guide, version 5 edition) by direct comparison of each PKHPOS fraction (baseline array) against its corresponding PKHNEG fraction (experimental array), within individual matched pairs. Among all the psets diﬀerentially expressed in at least one comparison (n = 10575), we selected only those showing a concordant trend of regulation (either increased or decreased) in at least two comparisons and assigned a “not changed” call in the third comparison. Given the diﬀerences in RNA yield and hybridization eﬃciency among the three pools of samples, for data presented in Figures 3A and 3B, expression data were independently mean normalized within each comparison, and normalized values were then imported in GeneSpring GX software version 7.3.1 (Agilent Technologies, Santa Clara, CA) without any further adjustment. Additional details (also with reference to validation experiments performed by quantitative RT-PCR) are in the Extended Experimental Procedures. Data sets have been deposited in NCBI’s Gene Expression Omnibus, and are accessible through GEO Series accession number GSE18931.

**Procedures for meta-analysis of published breast cancer data sets** (Ivshina et al., 2006; Pawitan et al., 2005) are in the Extended Experimental Procedures.

**Transplantation Assays into the Cleared Fat Pads of NOD-SCID Mice**

Three-week-old female NOD/SCID mice were anesthetized by i.p. injection of Tribroromeoanethol (Avertin) (150 mg/kg). For the xenotransplant of human normal mammary cells (either PKHPOS or PKHNEG cells, or bulk mammary cells, or normal mammospheres), the number 4 inguinal mammary glands were cleared and humanized with 2.5 x 10^6 nonirradiated telomerized human mammary fibroblasts (a generous gift from G. Dontu, University of Michigan, and R. B. Clarke, University of Manchester) and 2.5 x 10^6 irradiated (4 Gy) fibroblasts, as previously described (Kuperwasser et al., 2004; Proia and Kuperwasser, 2006). Two weeks after humanization, donor normal mammary cells, combined with a new batch of irradiated and nonirradiated fibroblasts to enhance the engraftment, were resuspended in an equal volume of a Phenol-red free Matrigel (Becton Dickinson) mixture, supplemented with 0.04% trypan blue (Sigma), to a final volume of 35 μl, and injected into the humanized site.

For the xenotransplant of human breast cancer cells, tumor biopsies were dissociated mechanically and enzymatically and cells, resuspended as above, were injected directly into cleared mammary fat pads of NOD/SCID mice. At the same time, a 60 day release Estradiol (17β estradiol) pellet (0.72 mg/pellet; Innovative Research of America) was placed subcutaneously on the back of the mouse’s neck with a trocar, when indicated.

Animals injected with normal breast cells were euthanized after 10 weeks. Animals injected with cancer cells were euthanized when the tumors were approximately 0.5–1.2 cm in the largest diameter, to avoid tumor necrosis and in compliance with regulations for use of vertebrate animal in research. For whole-tumor analysis, the fat pads were removed, ﬁxed in Carnoy’s solution (ethanol, glacial acetic acid, chloroform), and stained with 0.2% carmine alum (carmine, AKSO4). For IHC analysis, samples of normal mammary whole mounts and of tumor xenotransplants were FFPE and assayed with the appropriate antibodies. Measurements of total ductal outgrowth area on whole glands were taken essentially as previously described (McCaffrey and Macara, 2009). All measurements were made with ImageJ imaging software (W. Rasband, National Institutes of Health). In brief, fat pad ﬁlling was measured by drawing a shape around the fat pad, with the Freehand tool. Ductal areas were measured by drawing a shape that connected the tips of ducts. The percentage of fat pad ﬁlled was measured by the formula (ductal area/fat pad area) x 100, and is shown in the corresponding whole-mount images with a graphic indication (pie) of the extent to which each transplant ﬁlled the fat pad.

The transplantation frequency was calculated by Poisson statistics, using the “statmod” software package for the R computing environment (http://www.R-project.org/) as previously described (Shackleton et al., 2006) and a complementary log-log generalized linear model (two-sided 95% Wald conﬁdence intervals or, in case of zero outgrowths, one-sided 95% Clopper-Pearson intervals), respectively. The single-hit assumption was tested as recommended and was not rejected for any dilution series (p > 0.05).

**ACCESSION NUMBERS**

Data sets reported in this paper have been deposited in NCBI’s Gene Expression Omnibus (GEO Series) with the accession number GSE18931.
SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, seven figures, and two tables and can be found with this article online at doi:10.1016/j.cell.2009.12.007.

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REFERENCES


Supplemental Information

EXTENDED EXPERIMENTAL PROCEDURES

General Strategy for Mammosphere Experiments

The general strategy for all mammosphere experiments described in this paper is illustrated in Figure S1A. Cells were dissociated from the mammary gland to yield the “bulk mammary” cellular population. This population was allowed to adhere for 24 hr (see Experimental Procedures) to remove the majority of non-epithelial cellular contaminants, mostly lymphocytes, that do not adhere under our culture conditions. A similar strategy has been recently described to improve the preparation of epithelial-enriched populations from freshly dissociated mammary tissue, without affecting key properties associated with the stem cell state, such as multi-lineage differentiation (Raouf et al., 2008). The adherent population, which we operationally define as “bulk epithelial” population, was then trypsinized and labeled with the PKH26 dye (PKH-labeled bulk epithelial, in Figure S1A). In preliminary experiments, by immunostaining analysis of disaggregated bulk mammosphere cells for CD45 (Leukocyte-Common Antigen, LCA), we determined that the short-term adherence step, followed by detachment and subsequent mammosphere culture in suspension, removed residual contaminants of leukocyte origin, that were able to adhere and that were therefore still present at the first adhesion step. Of note, other possible contaminants present in the mammary parenchyma, mostly endothelial cells and fibroblasts, are removed during the well-standardized procedure for mammary epithelial cell preparation (Eirew et al., 2008; Ginestier et al., 2007). PKH26-labeled cells were then plated for mammosphere assays (mammosphere generations are indicated as F1 through F6 in Figure S1A). F1 mammospheres were disaggregated and subjected to FACS-sorting to purify PKHPOS and PKHNEG cells (FACS PKHPOS, in Figure S1A = . PHKPOS (and PHKNEG cells, if needed) were then used for various experiments, as indicated in Figure S1A. In other instances, we performed mammosphere experiments directly starting from the bulk mammary population, or from the bulk epithelial population (see Figure S1A).

Determination of SFE in Various Experimental Conditions

In Figure S1B, we report the frequency of mammosphere-initiating cells (Sphere Forming Efficiency, SFE) in various cell preparations. In the bulk mammary and bulk epithelial populations (lines 1-2 in Figure S1B), the SFE at F1 was 0.003 and 0.011%, respectively. Thus, the 24 hr adherence procedure, used to remove the non-adherent cellular contaminants, yielded a ~3.5-fold enrichment. The SFE at F1 of PKH-labeled bulk epithelial cells (line 3 in Figure S1B) was indistinguishable from that of unlabeled bulk epithelial cells, demonstrating that the labeling procedure did not affect the viability or the number of mammosphere-initiating cells. When F1 mammospheres (obtained from either the bulk mammary or the bulk epithelial populations, lines 1-2 in Figure S1B) where disaggregated and passed to obtain F2 mammospheres, the SFE was ~0.1%.

The SFE of PKHPOS cells was established before and after the FACS procedure (lines 4-5 in Figure S1B). For the pre-FACS experiments (line 4 in Figure S1B), we plated single cells, obtained from dissociated F1 mammospheres, and monitored them, by visual inspection, for their ability to form F2 mammospheres, as a function of their PKH26 positivity. PKHPOS cells were able to form mammospheres, with an SFE of 23%. hNMSCs loose mammosphere-forming ability in an exponential manner: at every generation, only 22.7% of hNMSCs are able to form mammospheres (reported in Figure S1C). Thus the SFE of 23% detected in pre-FACS PKHPOS cells is in excellent agreement with the maximum expected SFE (22.7%) calculated on the bases of cellular kinetics (reported in Figure S1C). For the post-FACS experiments (line 5 in Figure S1B), we plated PKHPOS cells gated such as to isolate the most epifluorescent cells (top 0.2%–0.4%) from F1 mammospheres. In this case, the SFE was around 11%, which is ~50% of the theoretical maximum and of the value detected in the pre-FACS experiment (line 4 in Figure S1B). This indicates that, at worst, our FACS-sorted PKHPOS population is ~50% pure.

The discrepancy between the values obtained for the SFE of PKHPOS cells pre- and post-FACS (as seen on lines 4 and 5 in Figure S1B) can be due a number of reasons. The most obvious possibility is that the gating procedure allowed the inclusion of cells not endowed with mammosphere-forming ability (operationally: non-hNMSCs). In this case, the fraction [SFE of PKHPOS post-FACS/ SFE of PKHPOS pre-FACS = ~50%] would represent the true purity of the FACS-sorted PKHPOS cells. However, other explanations are possible. For example, there may be detrimental effects of the FACS procedure per se on cell viability (Seidl et al., 1999), something that recently also has been described in the prospective purification of other cell types, such as human embryonic-derived neuronal cells (Pruszak et al., 2007). We decided to test experimentally whether this bias was present in our experiments. To do so, we determined the SFE of cells derived from F1 mammospheres, before FACS and after mock-FACS in which the cells were simply passed through the machine and recovered en bloc, without any fractionation. The SFE of the pre-FACS cells was 0.11 ± 0.02%, as expected. The mock-sorted cells displayed an SFE of 0.06 ± 0.01%. Thus the FACS procedure per se reduced the clonogenic ability of hNMSCs to around 55% of its theoretical maximum value. We used this correction factor, to normalize the SFE of our FACS-sorted PKHPOS cells (line 6 in Figure S1B, PKHPOS post-FACS corrected for FACS effect). By this normalization, the SFE became ~21%, which corresponds to ~90% of the maximum theoretical value obtained in the PKHPOS pre-FACS population (23%, see line 4 in Figure S1B). By this adjustment, we estimated that our PKHPOS population is ~90% pure.

Finally, PKHNEG cells were also tested in pre-FACS and post-FACS conditions (lines 7-8, in Figure S1B) and found repeatedly to be unable to support mammosphere growth.

Kinetic Analysis of Mammosphere Formation

In Figure S1C, we show the mammosphere-forming ability, as a function of mammosphere generation, in bulk epithelial cells from two different patients (lines 1-2 in Figure S1C, actual curves are in Figure 1B) and in PKHPOS cells obtained by FACS from two different
pools of normal bulk epithelial cells (one representative experiment is shown in Figure 1C). The cumulative sphere number decreased exponentially, over various generations, with excellent fit (see R2 values in Figures 1B and 1C). From the fitted curves, we calculated the exponential function, which was remarkably similar in the four analyzed cases: The mean was 0.227 ± 0.02 (line 5, Figure S1C), indicating that at every generation, hNMSCs or PKH\textsuperscript{POS} cells retain ~23% of their mammosphere-forming ability. There was no significant difference between the exponential functions of cells from bulk epithelial cells ("pt." in line 7 of Figure S1C) and PKH\textsuperscript{POS} cells. In turn, this shows that PKH\textsuperscript{POS} cells behave kinetically in manner indistinguishable from authentic hNMSCs present in the bulk epithelial population.

### Frequency of hNMSCs in Various Types of Cellular Populations

In Figure S1D, we estimated the number of mammosphere-initiating cells (assumed to be hNMSCs) in various types of cell populations, based on the calculations shown in Figure S1B and S1C. In each case, the observed SFE (from Figure S1B) was corrected for the exponential decrease of 0.227 (as from Figure S1C), according to the formula: [observed SFE/22.7]x100 = estimated hNMSCs (%)

For bulk mammary preparations: [0.003 (from Figure S1B, line 1)/22.7]x100 = 0.013%, i.e., 1 hNMSC/7567 cells.

For bulk epithelial preparations: [0.011 (from Figure S1B, line 2)/22.7]x100 = 0.048%, i.e., 1 hNMSC/2064 cells.

For F1 mammospheres: [0.10 (from Figure S1B, line 2-column 2)/22.7]x100 = 0.441%, i.e., 1 hNMSC/227 cells. Since normal mammospheres contained ~300 cells (306 ± 37, see Figure 6A), we calculated that ~1.3 hNMSC is present per mammosphere. In addition, since virtually all PKH\textsuperscript{POS} cells (in the pre-FACS exp, see Figure S1B) gave rise to mammospheres, we concluded that PKH\textsuperscript{POS} cells most likely represent hNMSCs in mammospheres.

### Reconstitution of the Mammary Gland in Humanized Epithelium-Cleared Fat Pads of Immunocompromised NOD/SCID Mice

In Figure S1E, we report a comparison between the frequency of true hNMSCs, established by transplantation experiments, with that obtained by theoretical estimates based on the frequency of mammosphere-initiating cells in various populations (shown in Figures S1B, S1C, and S1D). Experimentally we determined the frequency of hNMSCs by reconstitution of the mammary gland in humanized epithelium-cleared fat pads of immunocompromised NOD/SCID mice. These experiments were performed for the bulk mammary population (Figure 6C, Figure S7B), F1 mammospheres (Figure S7C), and FACS-purified PKH\textsuperscript{POS} cells (Figure 2F). The observed ranges of estimated frequencies are reported in Figure S1E, and were calculated as described in Experimental Procedures.

The expected frequencies were calculated as follows:

- Bulk mammary: estimated hNMSCs in the initial population 1 in 7567 (from Figure S1D); exponential factor 0.227 (decrease of clonogenic ability at every passage, from Figure S1C) -> expected frequency 1 in 7567/0.227 = 1:33,335.
- F1 mammospheres: estimated hNMSCs in the initial population 1 in 227 (from Figure S1D); exponential factor 0.227 (decrease of clonogenic ability at every passage, from Figure S1C) -> expected frequency 1 in 227/0.227 = 1:1,000.
- For PKH\textsuperscript{POS} cells, we started from an estimated value of 1 PKH\textsuperscript{POS} = 1 hNMSC (as from Figure S1D) and corrected this value for both the exponential factor (0.227, from Figure S1C), and for the reduction in clonogenic ability brought about by the FACS procedure (0.55 as calculated in Figure S1B). This yielded an expected frequency of reconstitution of 1 per 8 PKH\textsuperscript{POS} cells.

### Comparison between the Immunophenotypes of hNMSCs in This and Other Studies

The immunophenotype of hNMSC herein reported shows some apparent discrepancies with respect to other studies, in particular with regard to the expression (or the levels of expression) of EpCAM and CD24. In this section, we analyze and comment on these differences.

Our immunophenotypical analysis shows that hNMSC express, in addition to basal markers [a finding consistent with other studies (Eirew et al., 2008; Lim et al., 2009; Raouf et al., 2008)], also markers of the luminal cell lineage, such as CD24 and EpCAM. The expression of EpCAM on hNMSC is in agreement with previous findings in which human mammary epithelial cells able to generate mammary organotypic structures in Matrigel (Villadsen et al., 2007), or functionally characterized as bipotent colony-forming cells (Raouf et al., 2008), displayed an EpCAM\textsuperscript{+} phenotype. However, these findings are discordant with recent reports in which human mammary cells able to form mammary structures in xenotransplantation models display low or negative EpCAM (and also CD24, see below) expression (Eirew et al., 2008; Lim et al., 2009).

One possible general explanation for the variable expression levels of some cell surface markers, in different studies, may be found in the heterogeneity of the procedures used for the prospective isolation of purified cell fractions, which predominantly rely on the use of antibodies directed against surface epitopes combined with immuno-magnetic or FACS sorting. A caveat intrinsic to these procedures is that even subtle differences in the complex manipulations required to dissociate mammary tissues into single cells (mechanically and enzymatically), or in the antibody labeling/sorting procedures (type of fluorochrome-conjugation or antibody source, gating strategies) may have an impact on the cell surface immunophenotype. For instance, enzymatic treatments of solid tissues may significantly affect a number of surface epitopes, including among others, EpCAM (Gray et al., 2002). Furthermore, even when comparable procedures are used to prospectively isolate mammary cell fractions, it is possible that different expression levels for a given marker
are attributed to a given cell fraction, based on interpretation of FACS profiles and different terminology. A relevant example is represented by the somewhat different EpCam status attributed to hNMSCs in two recent studies (Eirew et al., 2008; Lim et al., 2009), in which similar FACS strategies were employed yielding similar FACS profile for CD49F and EpCam expression (compare Figure 3B in the study of Eirew et al. with Figure 1B in that of Lim et al.).

While a higher level of standardization of procedures seems, therefore, desirable to allow direct comparison of results across studies, it is possible that the present degree of (partial) heterogeneity might account for some of the discrepancies among different studies, including ours.

With reference to the EpCam status of hNMSCs, we would also like to point out that the mentioned discrepancies might actually be more apparent than real. In our study, we report that PKHPOS (and PKHNEG) cells are EpCam-positive (Figure 2A), and that in vivo cells displaying markers of hNMSCs are also EpCam-positive (Figure 3E and Figure S3B). These results do not bear quantitative implications (since they were obtained with a qualitative method, i.e., by IF), and we did not use EpCam to prospectively isolate hNMSCs. It is therefore possible that our “EpCam positivity” corresponds to a “low level” of expression of EpCam, in line with the levels of expression reported by Eirew et al., and Lim et al. (Eirew et al., 2008; Lim et al., 2009) in their prospective isolation of hNMSCs.

In the case of CD24, the situation is more complex. A recent study indicates that hNMSCs are CD24-negative (Lim et al., 2009), in line with the observation that CD24 is predominantly associated with a terminally differentiated luminal cell phenotype in the human mammary gland (Shipitsin et al., 2007). Conversely, we show here that anti-CD24 Ab can be used for the prospective isolation of hNMSCs, and that actually the CD24HIGH fraction (and not the CD24MEDIUM or CD24LOW fractions) possesses mammosphere-forming ability (Table 1, Figure S4E). In addition, CD24HIGH/CD49FHIGH/DNERHIGH cells could reconstitute the mammary gland in transplantation experiments (Figures S4A and S4H; it should be noted, however, that we did not perform reconstitution experiments in vivo with CD24MEDIUM or CD24LOW fractions). Finally, PKH1POS (but not PKHNEG) cells are CD24-positive (Figure 2A). Thus, the sum of our results is in favor of the possibility that hNMSCs are CD24-positive. Of note, this conclusion is consistent with similar conclusions reached for the murine mammary gland (Liao et al., 2007; Shackleton et al., 2006; Stingl et al., 2006).

We can only speculate concerning the differences between our results and those of Lim et al. (Lim et al., 2009) on the CD24 status of hNMSCs. Some of the mentioned considerations, about heterogeneity of procedures might account for the differences. In particular, we note that different Abs were used in the two studies. In a hypothetical scenario, isoform-specific recognition of CD24 by the two Abs might explain the differences, a scenario worth of additional experimental attention.

Finally, some issues concerning the CD24 status of human breast cancer, in light of our results and previous literature (Al-Hajj et al., 2003) should be discussed. The results depicted in Figure S6C confirm data from the literature, showing that while CD24 expression is mainly restricted to the plasma membrane in normal mammary tissue, it is characterized by a more predominant cytoplasmic distribution in neoplastic tissues (Bircan et al., 2006; Fogel et al., 1999; Kristiansen et al., 2003). This is also in line with data reporting that the cell surface immunophenotype of breast cancer SCs is CD24NEGLOW (Al-Hajj et al., 2003). For this reason, we did not use anti-CD24 Ab in our prospective isolation of breast cancer SCs (Figures 5D–5F and Figure S6D), and relied instead on tri-parametric CD49F/DLL1/DNER FACS.

We would like to note, however, that the fact that CD24 shows a predominant cytosolic expression in breast cancers has only implications for its use as a cell surface marker and not for its utility as a cancer SC marker altogether. Indeed, we show in this study that aggressive, poorly-differentiated G3 breast tumors display a higher content in cells expressing CD24 (together with several other SC markers), compared to less aggressive, well-differentiated G1 tumors (Figure 5A, Figure S6A). This is in line with previous reports showing a strong correlation between a high proportion of CD24POS cells (detected by IHC) and clinical/pathological parameters of aggressive breast neoplastic disease, including histological grading and poorer prognosis (Athanassiadou et al., 2009; Bircan et al., 2006; Fogel et al., 1999; Kristiansen et al., 2003).

Quantitative RT-PCR
A major limiting step for the validation of a sizable number of transcripts by quantitative RT-PCR was represented by the very low amount of total RNA obtained from the purified PKH26 fractions, especially from PKHPOS cells. To circumvent this technical limitation, cDNA derived from reverse transcription was enriched in the content of each gene (pre-amplification) through a short PCR (10-12 cycles) performed with the TaqMan® PreAmp Master Mix (Applied Biosystems) in the presence of specific primers for the selected genes. To optimize conditions for the pre-amplification reaction and ensure that this procedure did not alter the relative concentrations of the different genes, we performed a series of preliminary tests for a selected number of genes with variable degree of expression, using different PCR cycles and different starting amounts of cDNA (not shown). For both the pre-amplification reaction and the subsequent RT-PCR analysis, the following TaqMan Gene Expression Assay Identifications were used: ACTB (Hs00607939_m1), 18S (Hs03929290_m1), HSPA1B (Hs00355772_m1), IL4R (Hs00166237_m1), ICAM1 (Hs00164932_m1), ADRM1 (Hs00199645_m1), APOE (Hs00171168_m1), TOP2A (Hs00172214_m1), CIRBP (Hs00154457_m1), GAP43 (Hs00176645_m1), SIAH1 (Hs00361758_m1), SOX4...
PKHPOS versus PKHNEG cells. In particular, upregulation of Wnt/b-catenin and Notch pathways imparts instructive cues at the boundary between progenitors (PKHNEG cells), together with various Notch transcriptional targets. Thus, one might speculate that, because of the type, whereas upregulation of the Notch pathway was detected in PKHNEG/progenitor cells. Intriguingly, the Wnt/b-catenin target, JAG1 (Estrach et al., 2006), upregulated in hNMSCs, is a ligand for Notch receptors. These receptors are, in turn, upregulated in progenitors (PKHNEG cells), together with various Notch transcriptional targets. Thus, one might speculate that, because of the JAG1 liaison, the crosstalk between the Wnt/b-catenin and Notch pathways imparts instructive cues at the boundary between PKHPOS and PKHNEG cells. In particular, upregulation of Wnt/b-catenin and Notch pathway [reviewed in (Pardal et al., 2003)], are differentially regulated in PKHPOS versus PKHNEG cells. In particular, upregulation of Wnt/b-catenin signaling was associated with the hNMSC/PKHPOS phenotype, whereas upregulation of the Notch pathway was detected in PKHNEG/progenitor cells. Intriguingly, the Wnt/b-catenin target, JAG1 (Estrach et al., 2006), upregulated in hNMSCs, is a ligand for Notch receptors. These receptors are, in turn, upregulated in progenitors (PKHNEG cells), together with various Notch transcriptional targets. Thus, one might speculate that, because of the JAG1 liaison, the crosstalk between the Wnt/b-catenin and Notch pathways imparts instructive cues at the boundary between PKHPOS and PKHNEG cells.

For the experiments reported in Table S1, sheet “Taqman validated list,” sixty-nine genes from the hNMSC signature were selected for validation by quantitative-RT-PCR (Q-PCR). We selected genes for validation that would represent the entire range of up- or downregulation (~1.3 to ~2.0 fold-change, and ~0.7 to ~1.2 fold-change, for upregulated and downregulated genes, respectively). Expression was tested on 3 independent mRNA preparations, different from those used for the Affymetrix screening, extracted from pools each containing an equal number of mammospheres from 5-6 individuals (pools 4, 5, and 6).

Identification of the hNMSC Signature

Only a small number of PKHPOS cells can be obtained using our procedure, so the quantity of RNA that can be extracted from these cells is limited. To overcome this limitation, we extracted RNA from FACS-sorted cells derived from three independent pools of mammospheres, each of which originated from 5-6 individuals. We compared the gene expression profiles of PKHPOS and PKHNEG cells for each pool and obtained three separate gene lists of differentially expressed genes. The analysis was performed using the Affymetrix MAS5 “Comparison Analysis” algorithm (Affymetrix Statistical Algorithm Reference Guide, Affymetrix, Santa Clara, CA, USA; version 5 edition) by directly comparing each PKHPOS fraction (baseline array) against its corresponding PKHNEG fraction (experimental array), in order to detect and quantify changes in gene expression within individual matched pairs (Hubbell et al., 2002; Liu et al., 2002). Comparison of the global gene expression profiles associated with the two PKH26 fractions, in each sample, resulted in 4023 probesets differentially expressed in Pool #1, 4659 probesets in Pool #2, and 5278 probesets in Pool #3 (Figure 3A).

To define the “hNMSC signature,” we considered the overlap between the three pools of probesets. There were 2889 probesets that were regulated in the same direction (either increased or decreased) in the majority of the pools (at least two out of three). Of these, however, 583 showed discordant regulation in one of the pools. These 583 probesets were therefore discarded. The remaining 2306 probesets (which constitute what we call the hNMSC signature) therefore contained either probesets showing the same trend of regulation in all 3 pools (which once enucleated constitute the 3/3 signature, see below), or probesets showing a concordant trend of regulation (increased or decreased) in at least two pools and assigned a “not changed” call in the third pool (Figure 3A). The P-value for having 2306 or more probesets classified consistently under the null hypothesis was then computed, using a Monte Carlo simulation that randomized the positions of the Increased/Decreased signals with a fixed scheme for the Not Changed signals; the probability of having 2306 or more concordant signals by chance was found to be < 1x10^-14 (Figure 3A).

The “3/3 signature” is defined by the probesets (377) showing the same trend of regulation in all 3 pools, and is therefore a subset of the hNMSC signature (Figure 3A). The significance of the overlap was verified by means of a binomial estimation. The total probability that a single probeset is classified in a consistent manner across all the three pools (all Increased or all Decreased) is, under the assumption of the null hypothesis, ~1.53x10^-6; the expected number of probesets coherently classified on a population of 54675 would be 8.35, vis-à-vis 377 actually classified (over-representation factor 45.11). The probability that 377 or more probesets can be “classified coherently” can be estimated as the cumulative binomial probability distribution of 377 successes in 54675 trials with a probability of success of 1.53x10^-6. This number, which represents an estimate of the P-value for the null hypothesis, is below 1x10^-60 (Figure 3A).

Functional Classification of the hNMSC Signature

In Table S1, sheet “Functional classification,” we report the functional classification of genes upregulated or downregulated in hNMSCs. In addition to the biological and molecular classification based on Gene-Ontology (GO), we performed a manual annotation of the genes of the hNMSC signature, to identify groups of genes involved in specific biochemical pathways or cellular functions useful in considering distinguishing features of hNMSCs relative to their progeny.

While a comprehensive analysis of the molecular features of the hNMSC signature will be impossible here, some issues are worthy of a more in-depth discussion. For instance: does the complexity of the hNMSC signature represent the end point of a limited number of “critical circuitries” governing stem cell function? In this regard, we note that several components of pathways implicated in self-renewal, such as the canonical Wnt/b-catenin and Notch pathway [reviewed in (Pardal et al., 2003)], are differentially regulated in PKHPOS versus PKHNEG cells. In particular, upregulation of Wnt/b-catenin signaling was associated with the hNMSC/PKHPOS phenotype, whereas upregulation of the Notch pathway was detected in PKHNEG/progenitor cells. Intriguingly, the Wnt/b-catenin target, JAG1 (Estrach et al., 2006), upregulated in hNMSCs, is a ligand for Notch receptors. These receptors are, in turn, upregulated in progenitors (PKHNEG cells), together with various Notch transcriptional targets. Thus, one might speculate that, because of the JAG1 liaison, the crosstalk between the Wnt/b-catenin and Notch pathways imparts instructive cues at the boundary between
the stem cell and the TA compartment. This projects a complex regulatory role for Wnt/β-catenin and Notch pathways in mammary morphogenesis, a notion further reinforced by the frequent deregulation of these pathways in breast cancer (Dontu et al., 2004; Leong et al., 2007; Li et al., 2003; Liu et al., 2004; Pece et al., 2004).

In this context, it should also be remarked that a similar regulation of key components of the Notch and β-catenin pathways was identified in a recent report by Raouf et al., in which the transcriptomic profile of human mammary pluripotent cells was compared to that of their luminal-restricted progenitors or to their terminally differentiated progeny (Raouf et al., 2008). However, a comparison between our transcriptomic analysis of PKH26POS versus PKH26NEG cells and the one generated by the analysis of the distinct subsets of cells in the study of Raouf et al. revealed only a limited overlap. In particular 101 probesets were concordantly regulated between the hNMSC signature and the bipotent versus luminal-restricted cells signature, and 79 probesets were concordantly regulated between the hNMSC signature and the bipotent versus differentiated myoepithelial cells signature.

Such a limited overlap is not surprising and might actually be of help in dissecting the complex molecular programs leading from a mammary stem cell to a differentiated cell. As already discussed in the main text, signatures do not portray the “absolute” molecular picture of a given condition, but only the comparative picture with respect to another condition. Thus, a “stem cell signature” can be different according to whether it was derived by comparing the stem cell to a pluripotent progenitor, a committed progenitor, or a differentiated cell. Even assuming that our PKHPOS cells are the same as the bipotent CFC of Raouf et al. (as in all likelihood they are), the comparison was clearly made with different progenies of the mammary stem cell, as our PKHNEG cells represent immediate progeny, while the biological features of the luminal-restricted cells and of the differentiated myoepithelial cells indicate that they represent later differentiated progeny. Indeed, it is tempting to speculate that the integrated analysis of our data and of that of Raouf et al. might unveil different molecular steps in the complex mammary cell hierarchy.

Notwithstanding the above problems, commonly regulated pathways could be identified in the two studies. This is relevant, since it points to regulatory mechanisms that might represent master regulators of the normal mammary stem cell identity. A case in point is represented by the Notch pathway, which has been demonstrated to be functionally involved in cell fate specification and mammary morphogenesis (Bouras et al., 2008; Raouf et al., 2008).

We would like to discuss one final issue, concerning genes of the hNMSC signature, i.e., that of quiescence. This is important since the PKH26 functional labeling strategy rests on the assumption that hNMSCs are quiescent in nature, as also shown by the findings that PKHPOS cells, but not PKHNEG cells, selectively retain BrdU and do not express the proliferative marker Ki-67. This is also reflected by genes present in the hNMSC signature. First, as also discussed in the main text, a number of putative transcriptional targets of the tumor suppressor protein p53, which has recently been described as a key regulator of quiescence in other tissues, such as the hematopoietic system (Liu et al., 2009), are enriched in our hNMSC signature (see “p53 putative targets” sheet in Table S1 for details). Second, a variety of regulated genes in PKHPOS cells are involved in balancing cellular proliferation and apoptosis (this Table). These genes include the two cyclin-dependent kinase inhibitors CDKN1A (p21) and CDKN1C (p57), which may maintain hNMSCs in a quiescent state through the induction of cell cycle arrest.

Identification of Putative p53 Targets in the hNMSC Signature

By using chromatin immunoprecipitation (ChIP) combined to the paired-end ditag (PET) sequencing strategy, and to searches for p53 binding motifs, Wei and colleagues (Wei et al., 2006) identified 474 bona fide p53 binding loci in the human genome, matching to 426 unique RefSeq annotated genes. This number corresponds to 1.69% of the entire RefSeq collection of annotated human transcripts (25,126 non-redundant genes). Among these 426 genes, 42 are present in our hNMSC signature (thus constituting 2.66% of the 1576 unique RefSeq annotated transcripts present in the hNMSC signature), a number which is significantly higher than what would be expected by chance (chi square = 10.011; p = 0.0016). These results are shown in Table S1, sheet “p53 putative targets.”

Meta-Analysis of Published Breast Cancer Data Sets

G1/G3 data sets derived from the studies of Ivshina et al., and Pawitan et al. (Ivshina et al., 2006; Pawitan et al., 2005). Original RAW data (CEL files) were downloaded from the GEO database (Gene Expression Omnibus http://www.ncbi.nlm.nih.gov/geo/) accession code GSE4922 and GSE1456, respectively (there is no patient overlap between the two data sets). The G1/G3 data sets were built by extracting, from the data sets, information for patients for which both tumor grade (G1 or G3) and clinical follow-up was available (Ivshina: 68 G1s, 55 G3s; Pawitan: 28 G1s, 61 G3s).

The molecular type data set derived from the Pawitan et al. study, as annotated in the original publication, contained 25 basal-type, 15 ErbB2-type, 39 luminal A-type, and 23 luminal B-type tumors. CEL files were reprocessed by our Affymetrix Microarray Unit (http://services.ifom-ieu-campus.it/), with the Affymetrix’s proprietary MAS5 pre-processing algorithm, in order to make all samples comparable with those used in the present study. Processed files were then imported into GeneSpring GX software version 7.3.1 (Agilent Technologies, Santa Clara, CA). According to the GeneSpring normalization procedure, in each analysis the 50th percentile of all measurements was used as a positive control, within each hybridization array, and each measurement for each gene was divided by this control. The bottom 10th percentile was used for background subtraction. Among different hybridization arrays, each gene was divided by the median of its measurements in all samples. Data were then log transformed for subsequent analysis. Of the 2306 probesets found in the HG-U133_Plus_2 chipset only 2207 are present in the HG-U133 chipset and were used in the subsequent analyses, while of 377 (3 out of 3) only 366 are present. All clustering
analyses were performed with GeneSpring, using the Pearson Correlation as a similarity measure and Average Linkage as a clustering algorithm for both genes and samples.

**GSEA**

Gene set enrichment analysis was performed as reported previously (Subramanian et al., 2005), using GSEA (version 2.0) provided by the Broad Institute of the Massachusetts Institute of Technology (Cambridge, MA, USA). The enrichment score is a measure of the degree to which a gene set is over-represented at the extremes of the entire ranked gene list (ratio G3/G1 expression values). Significance was assessed by permuting (1000 times) class labels (that is, G3 versus G1) and calculating enrichment scores for the permuted data sets that yielded a null distribution.

As a control 20 additional lists of randomly selected probeset, of comparable size, were used (see Table S2, “specificity controls” sheet). This was done to address a possible caveat related to the enrichment of genes of the hNMSC signature in G3 versus G1 tumors (see Figure 4A). One could postulate that G3 and G1 tumors are so different that any list of random genes could be found significantly associated with them. Thus, we built 20 lists of randomly selected probesets of similar size (identical to the size of the lists resulting from the GSEA analysis of Figure 4A). None of the random lists was as significantly enriched in genes associated with a specific class of tumors (either G1 or G3) as the lists of PKHPOS or PKHNEG genes, in terms of FDR (False Discovery Rate) corrected P-values or number of enriched genes (see Table S2, “specificity controls” sheet).

**Genomic Fluorescence In Situ Hybridization (FISH)**

Tissue sections were assayed for the presence of mouse and human cells by performing FISH with species-specific genomic probes as described (Andersen et al., 2002). Briefly, 5-μm sections were de-paraffinized in xylene, dehydrated in ethanol, treated with NaSCN, RNase, pepsin, and finally HCl, and subsequently de-hydrated in 70%, 85%, and 100% ethanol series. Sections were denatured and hybridized with probes against CY3-d-UTP-labeled mouse Cot-1 DNA (InVitrogen), or FITC-12-d-UTP-labeled human Cot-1 DNA (InVitrogen), as previously described (Minucci et al., 2002).

**Flow-Cytometry Procedures**

Primary antibodies were: CD24-FITC, CD49F-PE (dilution 1/100 for 10^6 cells /100 μl, BD Biosciences PharMingen), biotinylated DNER (dilution 1:55 for 10^6 cells /100 μl, RD System) and DLL1 (dilution 1 μg for 10^6 cells /100 μl, Santa Cruz). Incubation was for 30 min, on ice, in Leibovitz’s L15 medium (GIBCO) with 3% bovine serum albumine (BSA), followed by washing in PBS/3% BSA. For detection of DLL1 and DNER, anti-goat IgG-Cy5 or IgG-Alexa 488 (Jackson Labs) or Streptavidin-Phycoerythrin-Cy7 (SAv-PE-Cy7, BD Biosciences) were used (1:100 dilution, for 20 min on ice). After incubation, cells were washed once with PBS and re-suspended in L15 medium. To assess viability, cells were stained with 1 μg/ml propidium iodide (Sigma) for 5 min. Analysis was performed using a FACS Vantage SE flow cytometer (Becton&Dickinson).

**BrdU Retention Assay**

Dissociated cells, from F1 mammospheres, were harvested, dissociated, and subjected to FACS analysis to yield PKHPOS and PKHNEG cells, which were then assayed for BrdU retention and Ki67 expression. For IF, cells were fixed using 4% paraformaldehyde, permeabilized with 0.25% Triton-X and 0.1% 30 min, on ice, in Leibovitz’s L15 medium (GIBCO) with 3% bovine serum albumine (BSA), followed by washing in PBS/3% BSA. For detection of DLL1 and DNER, anti-goat IgG-Cy5 or IgG-Alexa 488 (Jackson Labs) or Streptavidin-Phycoerythrin-Cy7 (SAv-PE-Cy7, BD Biosciences) were used (1:100 dilution, for 20 min on ice). After incubation, cells were washed once with PBS and re-suspended in L15 medium. To assess viability, cells were stained with 1 μg/ml propidium iodide (Sigma) for 5 min. Analysis was performed using a FACS Vantage SE flow cytometer (Becton&Dickinson).

**Detection of Numb Asymmetric Partitioning at Mitosis**

The pLentiLox3.7Numb-GFP vector was generated by sub-cloning of the Numb-GFP fusion protein (a generous gift of J. M. Verdi, University of Maine, USA) into the Nhel/EcoRI restriction sites of the pLentiLox3.7 lentiviral vector (Rubinson et al., 2003). Details are available upon request. The construct was sequence-verified. Lentivirus production was performed as previously described (Rubinson et al., 2003). Briefly, after transfection of HEK293 cells, the viral supernatant was collected and used to infect bulk mammary epithelial cells. Cells were then stained with PKH26 and grown in suspension for the formation of F1 mammospheres. Six days after infection, mammospheres were harvested, dissociated, and subjected to FACS analysis to yield PKHPOS and PKHNEG cells. Sorted cells were re-suspended in methylcellulose in complete medium, plated at clonal density and analyzed by epifluorescence 30-36 hr later.

To detect endogenous Numb, FACS-sorted PKHPOS cells were cultured in suspension for 30-36 hr ± 25 μM Blebbistatin (Sigma) (Straight et al., 2003) (see below for rationale of blebbistatin use). Numb distribution was visualized by IF on fixed cells, permeabilized with 0.05% Triton-X and 3% BSA in PBS and incubated with a mouse monoclonal anti-Numb (Colaluca et al., 2008) and an anti-mouse Alexa 488 Ab (Jackson lab).

To better visualize the asymmetric partitioning of endogenous Numb as a cortical crescent in dividing PKHPOS cell, we used blebbistatin, a small molecule inhibitor that is specific for non-muscle myosin II (Straight et al., 2003). Cells treated with blebbistatin enter
mitosis and proceed through anaphase. However, because they lack actin/myosin contractility, the cleavage furrow does not contract during cytokinesis, thus originating two interconnected daughter cells (Straight et al., 2003). In Figure 1G (bottom), the cleavage plane, between the two interconnected daughter cells, is indicated. Four consecutive, adjacent 0.5 μm optical sections of a mitotic PKH26-stained cell were collected. The shown cell is a representative example in which, for each optical section, the sister nuclei of the prospective daughter cells appear similar in size and shape, indicating that the planes of scanning of the individual optical sections were perpendicular to the cleavage plane, which in such a case can be deduced accurately as illustrated (white line in 1G) (Kosodo et al., 2004).

SUPPLEMENTAL REFERENCES


Figure S1. Related to Figure 1
Synopsis of various experiments performed with mammospheres and PKH^{POS} cells. A. General strategy for mammosphere experiments. A schematic representation of all the experiments performed with mammospheres is shown. Inset: left, a typical FACS profile of a PKH26-labeled mammosphere population with gated populations; right, suspension cultures of FACS-sorted PKH^{POS} or PKH^{NEG} cells. Bar, 100 μm. B. SFE under various conditions. The frequency of mammosphere-initiating cells (Sphere Forming Efficiency, SFE), in various cell preparations, is shown. ND, not done; NA, not applicable. C. Kinetic analysis of mammosphere formation. The mammosphere-forming ability - as a function of mammosphere generation in bulk epithelial cells from two different patients, and in PKH^{POS} cells obtained by FACS from two different pools of normal bulk epithelial cells – is shown. D. Frequency of hNMSCs in various types of cells. The number of mammosphere-initiating cells (assumed to be hNMSCs) in various types of cell populations is shown. E. Reconstitution of the mammary gland in humanized epithelium-cleared fat pads of immunocompromised NOD/SCID mice. Comparison of the frequency of true hNMSCs, established by transplantation experiments, and the frequency expected from the theoretical estimates of panels B-D. As shown, the expected frequencies and the experimentally observed frequencies were in good agreement. Please refer to the Supplemental Experimental Procedures section for experimental detail (A-E).
Figure S2. Related to Figure 2

Additional characterizations of hNMSCs. A. A quantification of the experiment in Figure 2A is shown (cumulative results for three experiments, in each of which 10 cells/sample were counted). The number and type of colonies were counted in three experiments performed in duplicate (mean ± s.e.m; 500 cells plated per condition). Top, number and type of colonies that developed per 100 plated cells. Results are comparable to those obtained by Raouf et al. in their analysis of bipotent CFC from the human mammary gland (Raouf et al., 2008). Bottom, number and type of colonies normalized to 100 colonies. As a comparison, the results obtained with cells from disaggregated F1 mammospheres (5,000 cells plated in triplicate) are also reported. (C). Additional data to Figure 2F of the main text. Left, IHC of reconstituted mammary glands (from PKHPOS cells as in Figure 2F), showing myoepithelial (TP63-positive, top) and epithelial (EpCAM-positive, bottom) cells properly organized in the mammary structures. Please note that the anti-EpCAM Ab (Dako, clone Ber-EP4) is human-specific (right panels). Bar, 50 μm. D. Mammary gland reconstitution by PKHPOS cells. Analysis of typical outgrowths obtained after injection of 10 or 100 PKHPOS cells. From left to right: Carmine-stained whole-mounts of outgrowths; graphic indications (pies) of the percentage of fat pad filled by the outgrowths are shown (see Experimental Procedures); bright field images of paraffin-embedded sections; serial paraffin-embedded sections used for genomic FISH with specific human (hCOT, red) or mouse (mCOT, green) Cot-1 probes, and for IHC (on a consecutive, not serial section) with a human-specific anti-EpCAM Ab (see panel A). Bar, 500 μm. Original magnifications are also indicated.
Figure S3. Related to Figure 3
Additional validation of hNMSC markers. A. IHC analysis of mammospheres with markers of hNMSCs. Mammospheres were embedded in paraffin and analyzed in IHC with the indicated Abs. Bar, 100 μm. B. Identification of hNMSCs in vivo using markers from the hNMSC signature (supplemental data to Figure 3E). Fresh or paraffin-embedded (last panel) sections of normal mammary glands were analyzed in IF or IHC (last panel) with the indicated Abs. For the IF experiments, magnifications of the boxed regions are shown on the top of each panel; bar, 10 μm. In the last panel, arrows point to rare stained cells; original magnification x40. As discussed in the main text, in IF experiments, we used double-labeling with EpCAM, to facilitate the visualization of the epithelial layer.
The experiments of this figure and of Figure 3E confirm that some markers (CD49F, CK5, JAG1) were expressed both by myoepithelial cells and by putative hNMSCs, while DLL1 appeared expressed exclusively by putative hNMSCs.
Figure S4. Related to Table 1

Flow cytometric analysis of normal breast epithelial cells, and additional biological characterization of sorted cells. A-D, FACS with anti-CD24, anti-CD49F, anti-DNER, and anti-DLL1, in various combinations. Representative flow cytometric analyses of normal breast epithelial cells (bulk mammary cells) using the indicated markers. The shown fractions of FACS-sorted cells were used to produce data shown in Table 1 of the main text. A. Mono-parametric FACS. B-D. Bi- and tri-parametric FACS with the indicated Abs. Boxes in the various profiles indicate the gating strategies to obtain the different cell fractions used in the assays reported in Table 1. The different fractions are also indicated on the right of each profile, with their cellular content. E-H. Additional biological characterization of sorted cells. E. Cells from the indicated FACS experiments were tested for their mammosphere-forming ability.

An analysis of the differences between the cell surface immunophenotypes of hNMSCs in our study and in other reports is in Supplemental Experimental Procedures.
Figure S5. Related to Figure 4
The hNMSC signature can partially distinguish among the molecular subtypes of breast cancer. Top, unsupervised two-way hierarchical clustering of the Pawitan cohort by the hNMSC signature. The molecular subtypes and the tumor grade, as annotated in the original publication are indicated at the bottom (labels are shown in the inset). Bottom, gene set enrichment results for genes upregulated in PKH$^{POS}$ cells, considering basal-like tumors versus all other subtype.
Figure S6. Related to Figure 5
A-B. Cells expressing markers of the hNMSC signature are enriched in G3 versus G1 tumors. A. Additional images for the experiments shown in Figure 5A. Paraffin-embedded (top) or frozen OCT-embedded sections (bottom) were analyzed in IHC or IF with the indicated Abs (see also Figure 5A of the main text). Original magnification x20 (top). Bar (bottom), 50 μm. B. Serial 3 μm-thick sections were analyzed in IHC with the indicated Abs. The boxed areas point to a nest of cells stained by the three Abs. Results were confirmed on multiple sections from 3 different G3 tumors. Original magnification x20. C. Immunohistochemical analysis of CD24 expression in normal and tumor breast tissues. Shown are two examples of normal breast tissue (Normal) displaying predominantly apical membranous CD24 immunoreactivity in the ductal luminal epithelium and three different instances of high grade breast cancers (Tumor) displaying diffuse and primarily cytoplasmic CD24 reactivity. Please see additional discussion in Supplemental Experimental Procedure (section “Comparison between the immunophenotypes of hNMSCs in this and other studies”). D. Representative bi-tri-parametric FACS showing the expression of CD49F, DLL1 and DNER in cells from G1 and G3 tumor tissues. Bulk epithelial cells from different tumor tissues were immunostained with the indicated Abs (details are in Experimental Procedures). Boxes in the various profiles indicate the gating strategies to obtain the different cell fractions used in the assays reported in Figure 5D–F of the main text. The different fractions are also indicated on the right of each profile, with their cellular content.
Figure S7. Related to Figure 6

A. Analysis of PKH26 distribution in mammospheres derived from G1 and G3 tumors. Left, Primary epithelial cells obtained from G1 and G3 tumors (examples from 2 G1s and 3 G3s are shown, out of more than 10 tumors analyzed per each subtype) were stained with PKH26 and cultured in methylcellulose to obtain tumor mammospheres. Shown are representative images of G1- and G3-mammospheres with their typical pattern of PKH26 distribution. Bar, 100 μm. Right, We addressed a possible caveat in the formation of G3 mammospheres, that – in principle – might be bigger than G1 mammosphere and display higher numbers of PKHPOS cells as the results of the coalescence of mammospheres. Starting from bulk epithelial cells from 2 G1s and 4 G3s, we divided each sample in two and infected the two subpopulation separately with lentiviruses encoding GFP (green) or RFP (red), respectively. The two subpopulations, derived from individual tumors, were then combined and plated in a mammosphere assay for 1 week. As shown, each spheroid (either in G1 or G3 tumors) is the result of the clonal expansion of either a red or a green cell, and no mixed color spheroids were detected. This excludes that tumors spheroids (especially G3 mammospheres) are the result of coalescence events.

The above data support the notion, that while G1 tumors (at least from what can be inferred from a mammosphere assay) retain by-and-large the asymmetric mode of division, typical of hNMSC, G3 tumors tend to skip rounds of asymmetric division, in favor of symmetric ones, thus leading to an expansion of the PKHPOS/SC pool. This is also supported by kinetic studies performed, in our laboratories, in murine systems of NMSCs and breast cancer SCs (Cicalese et al., 2009), see also Discussion in the main text).

B-C. Limiting dilution transplantation assay into the cleared fat pad of NOD/SCID mice using normal and tumor mammary epithelial cells derived from freshly dissociated tissues or from mammospheres. The indicated amounts of bulk mammary normal cells (Nor) or tumor cells (G1, G3) from G1 or G3 tumors (B) or of cells derived from normal and tumor mammospheres (C) were xenografted into either the humanized epithelium-cleared mammary fat pads (Nor) or into the cleared mammary fat pads (G1, G3) of immunocompromised NOD/SCID mice. Results are shown as number of outgrowths per number of cell injections. MRU (mammary repopulating unit, for Normal) or CIC (cancer-initiating cell, for G1 and G3) frequency (estimates with upper/lower limits) was calculated by limiting dilution analysis, as described in Experimental Procedures.

Data in panel B represent the complete set of data from which the condensed information shown in Figure 6C of the main text was extracted. In panel B, the asterisks indicate that a pool of three tumors (either G1s or G3s) was used.