Corrections

NEUROSCIENCE. For the article “Histone deacetylase inhibitors prevent oxidative neuronal death independent of expanded polyglutamine repeats via an Sp1-dependent pathway,” by Hoon Ryu, Junghee Lee, Beatrix A. Olofsson, Aziza Mwidau, Alpaslan Deodoglu, Maria Escudero, Erik Flemington, Jane Azizkhan-Clifford, Robert J. Ferrante, and Rajiv R. Ratan, which appeared in issue 7, April 1, 2003, of Proc. Natl. Acad. Sci. USA (100, 4281–4286; First Published March 14, 2003; 10.1073/pnas.0737363100), the author name Alpaslan Deodoglu should have appeared as Alpaslan Dedeoglu. The corrected author line appears below. The online version has been corrected.

Hoon Ryu, Junghee Lee, Beatrix A. Olofsson, Aziza Mwidau, Alpaslan Dedeoglu, Maria Escudero, Erik Flemington, Jane Azizkhan-Clifford, Robert J. Ferrante, and Rajiv R. Ratan

www.pnas.org/cgi/doi/10.1073/pnas.1231735100

COMMENTARY. For the article “What happens to genes in duplicated genomes,” by Elizabeth A. Kellogg, which appeared in issue 8, April 15, 2003, of Proc. Natl. Acad. Sci. USA (100, 4369–4371; First Published April 7, 2003; 10.1073/pnas.0831050100), the pull quote in the second column on page 4370 read “Subfunctionalization occurs in some genes and is not an immediate product of polyploidization.” It should have read “Subfunctionalization occurs in some genes and is an immediate product of polyploidization.” This error occurred during the editorial process and is not the fault of the author. PNAS regrets this error.

Lian Fan, Brian W. Busser, Traci Q. Lifsted, Mohamed Oukka, David Lo, and Terri M. Laufer

www.pnas.org/cgi/doi/10.1073/pnas.1232295100

CELL BIOLOGY. For the article “Prospective identification of tumorigenic breast cancer cells,” by Muhammad Al-Hajj, Max S. Wicha, Adalberto Benito-Hernandez, Sean J. Morrison, and Michael F. Clarke, which appeared in issue 7, April 1, 2003, of Proc. Natl. Acad. Sci. USA (100, 3983–3988; First Published March 10, 2003; 10.1073/pnas.0530291100), the authors note that the following statement was inadvertently omitted from the acknowledgements: “The results of this study support a patent-pending technology that is exclusively licensed to Cancer Stem Cell Genomics (CSCG) in which the authors and the University of Michigan have a financial interest.”

www.pnas.org/cgi/doi/10.1073/pnas.1131491100

IMMUNOLOGY. For the article “Antigen presentation by keratinocytes directs autoimmune skin disease,” by Lian Fan, Brian W. Busser, Traci Q. Lifsted, David Lo, and Terri M. Laufer, which appeared in issue 6, March 18, 2003, of Proc. Natl. Acad. Sci. USA (100, 3386–3391; First Published March 10, 2003; 10.1073/pnas.0437899100), the authors request that Mohamed Oukka, Department of Immunology and Infectious Diseases, Harvard School of Public Health, Boston, MA 02115, be added to the list of authors between Traci Q. Lifsted and David Lo. The revised author line appears below.

Lian Fan, Brian W. Busser, Traci Q. Lifsted, Mohamed Oukka, David Lo, and Terri M. Laufer

www.pnas.org/cgi/doi/10.1073/pnas.1232295100
For the article “3β-Acetoxyandrost-1,5-diene-17-ethylene ketal functions as a potent antiandrogen with marginal agonist activity,” by Hiroshi Miyamoto, Padma Marwah, Ashok Marwah, Henry Lardy, and Chawnshang Chang, which appeared in issue 8, April 15, 2003, of Proc. Natl. Acad. Sci. USA (100, 4440–4444; First Published April 2, 2003, 10.1073/pnas.0831001100), in Fig. 1A the hydroxyl group in position 17 for Δ⁴-androstenediol (Adiol), testosterone, and dihydrotestosterone (DHT) should be connected with a single, rather than a double, bond. The conclusions are unchanged by this typographical error. The corrected figure and its legend appear below.

Fig. 1. The structures of DHEA derivatives and effects on AR transcriptional activity. (A) The structures of compounds nos. 5, 10, 14, 15, 16, and 17, DHEA, Adiol, testosterone, and DHT. (B) PC-3 cells were transfected with the WT AR expression plasmid pSG5-AR and MMTV-Luc. After transfection, cells were cultured for 24 h with 1 nM DHT or 1,000 nM of various DHEA derivatives. The Luc activity is presented relative to that of EtOH treatment (white bar; set as 1-fold). Values represent the mean ± SD of at least three determinations. (C) PC-3 cells were transfected with the pSG5-AR and MMTV-Luc. After transfection, cells were cultured for 24 h with various concentrations of compounds nos. 5, 10 (ADEK), 14, or 16 in the presence of 1 nM DHT. The Luc activity is presented relative to that in the presence of DHT (black bar; set as 100%). Values represent the mean ± SD of at least three determinations.

www.pnas.org/cgi/doi/10.1073/pnas.1232068100
Prospective identification of tumorigenic breast cancer cells

Muhammad Al-Hajj*, Max S. Wicha*, Adalberto Benito-Hernandez†, Sean J. Morrison*†‡, and Michael F. Clarke*†§

Departments of *Internal Medicine and †Pathology, Comprehensive Cancer Center, ‡Department of Developmental Biology, and §Howard Hughes Medical Institute, University of Michigan Medical School, Ann Arbor, MI 48109

Communicated by Jack E. Dixon, University of Michigan Medical School, Ann Arbor, MI, January 16, 2003 (received for review December 18, 2002)

Breast cancer is the most common malignancy in United States women, accounting for >40,000 deaths each year. These breast tumors are comprised of phenotypically diverse populations of breast cancer cells. Using a model in which human breast cancer cells were grown in immunocompromised mice, we found that only a minority of breast cancer cells had the ability to form new tumors. We were able to distinguish the tumorigenic (tumor initiating) from the non-tumorigenic cancer cells based on cell surface marker expression. We prospectively identified and isolated the tumorigenic cells as CD44+/CD24−/lowLinage− in eight of nine patients. As few as 100 cells with this phenotype were able to form tumors in mice, whereas tens of thousands of cells with alternate phenotypes failed to form tumors. The tumorigenic subpopulation could be serially passaged: each time cells within this population generated new tumors containing additional CD44+/CD24−/lowLinage− tumorigenic cells as well as the phenotypically diverse mixed populations of nontumorigenic cells present in the initial tumor. The ability to prospectively identify tumorigenic cancer cells will facilitate the elucidation of pathways that regulate their growth and survival. Furthermore, because these cells drive tumor development, strategies designed to target this population may lead to more effective therapies.

Despite advances in detection and treatment of metastatic breast cancer, mortality from this disease remains high because current therapies are limited by the emergence of therapy-resistant cancer cells (1, 2). As a result, metastatic breast cancer remains an incurable disease by current treatment strategies. Cancers are believed to arise from a series of sequential mutations that occur as a result of genetic instability and/or environmental factors (3, 4). A better understanding of the consequences of these mutations on the underlying biology of the neoplastic cells may lead to new therapeutic strategies.

In solid tumors, it has been demonstrated that only a small proportion of the tumor cells are able to form colonies in an in vitro clonogenic assay (5–11). Furthermore, large numbers of cells must typically be transplanted to form tumors in xenograft models. One possible explanation for these observations is that every cell within a tumor has the ability to proliferate and form new tumors but that the probability of an individual cell completing the necessary steps in these assays is small. An alternative explanation is that only a rare, phenotypically distinct subset of cells has the capacity to significantly proliferate and form new tumors, but that cells within this subset do so very efficiently (12). To distinguish between these possibilities, it is necessary to identify the clonogenic cells in these tumors with markers that distinguish these cells from other non-tumorigenic cells. This identification has been accomplished in acute myelogenous leukemia, where it was demonstrated that a specific subpopulation of leukemia cells (that expressed markers similar to normal hematopoietic stem cells) was consistently enriched for clonogenic activity in nonobese diabetic/severe combined immunodeficient (NOD/SCID) immunocompromised mice, whereas other cancer cells were depleted of clonogenic activity (13–15). Such experiments have not been reported in solid cancers. If this model were also true for solid tumors, and only a small subset of cells within a tumor possess the capacity to proliferate and form new tumors, this finding would have significant implications for understanding the biology of and developing therapeutic strategies for these neoplasms.

To investigate the mechanisms of solid tumor heterogeneity, we developed a modification of the NOD/SCID mouse model in which human breast cancers were efficiently propagated in the mouse mammary fat pad (16). In the present study, we show that solid tumors contain a distinct population of cells with the exclusive ability to form tumors in mice. We refer to these cells as tumorigenic cells, or cancer-initiating cells, because they consistently formed tumors, whereas other cancer cell populations were depleted of cells capable of tumor formation. We identified cell surface markers that can distinguish between these cell populations. Our findings provide a previously uncharacterized model of breast tumor biology in which a defined subset of cells drives tumorigenesis, as well as generating tumor cell heterogeneity. The prospective identification of this tumorigenic population of cancer cells should allow for the identification of molecules expressed in these cells that could serve as targets to eliminate this critical population of cancer cells.

Materials and Methods

Mouse Preparation. Eight-week-old female NOD/SCID mice were anesthetized by an i.p. injection of 0.2 ml of ketamine/xylazine (300 mg ketamine combined with 20 mg of xylazine in a 4-ml volume; 0.02 ml of the solution was used per 20-g mouse). Dilution to 200 μl was done by using Hanks’ balanced salt solution (HBSS). Mice were then treated with VP-16 (etoposide) via an i.p. injection (30-mg etoposide dose per 1-kg mouse, diluted in serum-free HBSS for a final injection volume of 200 μl). At the same time, estrogen pellets were placed s.c. on the back of the mouse’s neck by using a trocar. All tumor injections/implants were done 5 days after this procedure. In the following procedures, mice were always anesthetized as described above.

Primary Tumor Specimen Implantations. For the implantation of fresh specimens, samples of human breast tumors were received within an hour after surgery. The tumors were cut up with scissors into small pieces, and the pieces were then minced with a blade to yield 2 × 2-mm pieces. Mincing was done in sterile RPMI medium 1640 under sterile conditions on ice. The tumor pieces were washed with serum-free HBSS before implantation. A 2-mm incision was then made in the mid abdomen area, and by using a trocar, one to two small tumor pieces were implanted in the region of the upper right and upper left mammary fat pads (right below the second nipple on both sides). A 6-0 suture was wrapped twice around the mammary fat pad nipple, allowing it to hold the implanted pieces in place. Sutures were removed after 5 days. Nexaban was used to seal the incision, and mice were monitored weekly for tumor growth.

Plural Effusion Injections. For the injection of the pleural effusions, cells were received shortly after thoracentesis and washed

Abbreviations: NOD/SCID, nonobese diabetic/severe combined immunodeficient; HICS, heat-inactivated calf serum; ESA, epithelial-specific antigen; Tn, tumor n.

See commentary on page 3547.

*To whom correspondence should be addressed. E-mail: mclarke@med.umich.edu.
with serum-free HBSS. Cells were then suspended in serum
free-RPMI/Matrigel mixture (1:1 volume) and then injected
into the upper right and left mammary pads by using a 22-gauge
needle. An amount equal to 0.2 ml, containing 1–2 million cells,
was typically injected. The site of the needle injection was sealed
with Nexaban to prevent any cell leakage.

Preparation of Single Cell Suspensions of Tumor Cells. Before diges-
tion with collagenase, xenograft tumors or primary human tumors
were cut up into small pieces and then minced completely by using
sterile blades. To obtain single cell suspensions, either pleural
effusion cells or the resulting tumor pieces were then mixed with
ultra-pure collagenase III in medium 199 (200–250 units of colla-
genase per ml) and allowed to incubate at 37 °C for 3–4 h. Pipetting
with a 10-ml pipette was done every 15–20 min. At the end of the
incubation, cells were filtered through a 45-μm nylon mesh and
washed with RPMI/20% FBS, then washed twice with HBSS.
Cells to be injected were then suspended in HBSS/Matrigel mix (1:1
volume) and injected into the area of the mammary fat pad as
described above. Nexaban was used to seal the injection site.

Table 1. Engraftment of human breast cancers into NOD/SCID mice

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Origin</th>
<th>Formation in mice</th>
<th>Passage in mice</th>
<th>Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>Metastasis</td>
<td>Yes</td>
<td>Yes</td>
<td>Infiltrating ductal carcinoma</td>
</tr>
<tr>
<td>T2</td>
<td>Breast primary</td>
<td>Yes</td>
<td>Yes</td>
<td>Adenocarcinoma</td>
</tr>
<tr>
<td>T3</td>
<td>Metastasis</td>
<td>Yes</td>
<td>Yes</td>
<td>Invasive lobular carcinoma</td>
</tr>
<tr>
<td>T4</td>
<td>Metastasis</td>
<td>Yes</td>
<td>No</td>
<td>Invasive lobular carcinoma</td>
</tr>
<tr>
<td>T5</td>
<td>Metastasis</td>
<td>Yes</td>
<td>Yes</td>
<td>Inflammatory breast carcinoma</td>
</tr>
<tr>
<td>T6</td>
<td>Metastasis</td>
<td>Yes</td>
<td>Yes</td>
<td>Inflammatory breast carcinoma</td>
</tr>
<tr>
<td>T7</td>
<td>Metastasis</td>
<td>Yes</td>
<td>Yes</td>
<td>Invasive lobular carcinoma</td>
</tr>
<tr>
<td>T8</td>
<td>Metastasis</td>
<td>Yes</td>
<td>Yes</td>
<td>Inflammatory breast carcinoma</td>
</tr>
<tr>
<td>T9</td>
<td>Metastasis</td>
<td>Yes</td>
<td>Yes</td>
<td>Adenocarcinoma</td>
</tr>
</tbody>
</table>

Mice were injected with unsorted T1 and T3 cells and a 2-mm piece of T2. Cells from T4–T9 were isolated by
flow cytometry as described in Fig. 1. All nine tumors tested engrafted in our NOD/SCID mouse model. Except for
T2, which was a primary breast tumor, all other tumors were metastases. All of the tumors were passaged serially
in mice except for T4.

Table 2. Tumor formation ability of sorted cells

<table>
<thead>
<tr>
<th>Tumors/injections</th>
<th>8 × 10⁵</th>
<th>5 × 10⁵</th>
<th>2 × 10⁵</th>
</tr>
</thead>
<tbody>
<tr>
<td>Passed T1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD44⁻</td>
<td>0/2</td>
<td>0/2</td>
<td></td>
</tr>
<tr>
<td>CD44⁺</td>
<td>2/2</td>
<td>2/2</td>
<td></td>
</tr>
<tr>
<td>B38.1⁻</td>
<td>0/2</td>
<td>0/2</td>
<td></td>
</tr>
<tr>
<td>B38.1⁺</td>
<td>2/2</td>
<td>2/2</td>
<td></td>
</tr>
<tr>
<td>CD24⁺</td>
<td>—</td>
<td>—</td>
<td>1/6</td>
</tr>
<tr>
<td>CD24⁻</td>
<td>—</td>
<td>—</td>
<td>6/6</td>
</tr>
<tr>
<td>Passed T2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD44⁻</td>
<td>0/2</td>
<td>0/2</td>
<td></td>
</tr>
<tr>
<td>CD44⁺</td>
<td>2/2</td>
<td>2/2</td>
<td></td>
</tr>
<tr>
<td>B38.1⁻</td>
<td>0/2</td>
<td>0/2</td>
<td></td>
</tr>
<tr>
<td>B38.1⁺</td>
<td>2/2</td>
<td>2/2</td>
<td></td>
</tr>
<tr>
<td>CD24⁺</td>
<td>—</td>
<td>—</td>
<td>1/6</td>
</tr>
<tr>
<td>CD24⁻</td>
<td>—</td>
<td>—</td>
<td>6/6</td>
</tr>
</tbody>
</table>

Fig. 1. Isolation of tumorigenic cells. Flow cytometry was used to isolate
subpopulations of T1 (a and b), T3 (c), T5 (d), T6 (e), and T7 (f) cells that were
tested for tumorigenicity in NOD/SCID mice. T1 (b) and T3 (c) had been
passaged (P) once in NOD/SCID mice, whereas the rest of the cells were frozen
or unfrozen samples obtained directly after removal from a patient (UP). Cells
were stained with antibodies against CD44, CD24, Lineage markers,
and mouse-H2K for passaged tumors obtained from mice, and 7AAD. Dead cells
(7AAD⁻), mouse cells (H2K⁺), and Lineage⁻ normal cells were eliminated from
all analyses. Each plot depicts the CD24 and CD44 staining patterns of live
human Lineage⁻ cancer cells, and the frequency of the boxed tumorigenic
cancer population as a percentage of cancer cells in each specimen is shown.

that contained 7-aminoactinomycin D (7AAD, 1 resuspended in 0.5 ml (per million cells) of HBSS/H2O862 twice with HBSS antibody) were then added and incubated for 20 min on ice, and then washed twice with HBSS concentration).

FITC (Biomeda, Foster City, CA), and anti-H2Kd, (PharMingen). cyanin (APC), phycoerythrin (PE), or biotin, anti-CD24 (PE or CD44, CD24 Lineage populations and CD44 CD24 lowLineage cells were isolated by flow cytometry as described in Fig. 1. The indicated number of cells of each phenotype was injected into the breast of NOD/SCID mice. The frequency of tumorigenic cells calculated by the modified maximum likelihood analysis method is $5 \times 10^3$ if single tumorigenic cells were capable of forming tumors, and every transplanted tumorigenic cell gave rise to a tumor (33). Therefore, this calculation may underestimate the frequency of the tumorigenic cells because it does not take into account cell-cell interactions and local environmental factors that may influence engraftment. In addition to the markers that are shown, all sorted cells in all experiments were Lineage−, and the tumorigenic cells from T1, T2, and T3 were further selected as B38.1. The mice were observed weekly for 4–6.5 mo, or until the mice became sick from the tumors.

**Table 3. Tumorigenic breast cancer cells were highly enriched in the ESA**

<table>
<thead>
<tr>
<th>Tumors/injections</th>
<th>T0</th>
<th>T5</th>
<th>T10</th>
<th>T50</th>
<th>T200</th>
<th>T100</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 $\times 10^5$</td>
<td>8/8</td>
<td>8/8</td>
<td>10/10</td>
<td>0/10</td>
<td>3/12</td>
<td>0/12</td>
</tr>
<tr>
<td>5 $\times 10^6$</td>
<td>8/8</td>
<td>8/8</td>
<td>10/10</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td>2 $\times 10^6$</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
<td>14/14</td>
<td>10/10</td>
</tr>
<tr>
<td>5 $\times 10^7$</td>
<td>10/10</td>
<td>10/10</td>
<td>10/10</td>
<td>10/10</td>
<td>0/10*</td>
<td>4/4</td>
</tr>
<tr>
<td>5 $\times 10^8$</td>
<td>0/10*</td>
<td>4/4</td>
<td>4/4</td>
<td>1/6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Flow Cytometry.** The antibodies used were anti-CD44 [allphocoecyanin (APC), phycoerythrin (PE), or biotin], anti-CD24 (PE or FITC), anti-B38.1 (APC), anti-epithelial-specific antigen (ESA)–FITC (Biomed, Foster City, CA), and anti-H2Kd (PharMingen). Lineage marker antibodies were anti-CD2, -CD3 –CD10, -CD16, -CD18, -CD31, -CD64, and -CD140b. Unless noted, antibodies were purchased from PharMingen. Antibodies were directly conjugated to various fluorochromes, depending on the experiment. In all experiments, mouse cells and mouse cells were removed by eliminating H2Kd cells (mouse histocompatibility class I).

**Identification of Tumorigenicity Markers.** Breast cancer cells were heterogeneous with respect to expression of a variety of cell surface markers (including CD44, CD24, and B38.1). CD24 and CD44 are adhesion molecules whereas B38.1 has been described as a breast/ovarian cancer-specific marker (17–19). To determine whether these markers could distinguish tumorigenic from nontumorigenic cells, flow cytometry was used to isolate cells that were positive or negative for each marker from passage 1 T1 or T2 cells. When $2 \times 10^5$ to $8 \times 10^5$ cells of each population were injected, all injections of CD44+ cells (8/8), B38.1+ cells (8/8), or CD24+ cells (12/12) gave rise to visible tumors within 12 wk of injection, but none of the CD44− (0/8), or B38.1− cell (0/8) injections formed detectable tumors (Table 2). Although no tumors could be detected by palpation in the locations injected with CD24+ cells, 2 of 12 mice injected with CD24+ cells did contain small growths at the injection site that were detected on necropsy. These growths most likely arose from the 1–3% of CD24− cells that invariably contaminate the sorted CD24+ cells or alternatively from CD24− cells with reduced proliferative capacity (Table 2). Because the CD44+ cells were exclusively B38.1+, we focused on the CD44 and CD24 markers in subsequent experiments.

**Results and Discussion**

**Tumor Specimens and Engraftment Rate.** Human breast cancer specimens obtained from primary or metastatic sites in nine different patients [designated tumors 1–9 (T1–T9)] all engrafted in the NOD/SCID mice (Table 1). In one case, the cancer cells were obtained from a primary breast tumor (T2) whereas in other cases the cells were obtained from metastatic pleural effusions (T1 and T3–T9). Some experiments were conducted on cells after they had been passaged once or twice in mice (designated passage 1 and 2) whereas other experiments were conducted on unpassed or fresh tumor samples obtained directly from patients. During use of human cancer cells from tumors passaged in mice, contaminating mouse cells were removed by eliminating H2Kd cells (mouse histocompatibility class I).

**Patients’ tumor cells**

<table>
<thead>
<tr>
<th>CD44</th>
<th>CD24</th>
<th>CD44 lowESA</th>
</tr>
</thead>
<tbody>
<tr>
<td>5/3</td>
<td>0/4</td>
<td>5/3</td>
</tr>
<tr>
<td>3/3</td>
<td>0/4</td>
<td>3/3</td>
</tr>
<tr>
<td>2/2</td>
<td>2/2</td>
<td>2/2</td>
</tr>
</tbody>
</table>

**Table 2**
Whether the CD44 and the remaining Lineage \textsuperscript{low} populations of phenotypically diverse cancer cell types, and in both cases only the CD44\textsuperscript{+}CD24\textsuperscript{low}Lineage\textsuperscript{−} cells had the capacity to proliferate to form new tumors (P < 0.001).

In three of the tumors, further enrichment of tumorigenic activity was possible by isolating the ESA\textsuperscript{+} subset of the CD44\textsuperscript{+}CD24\textsuperscript{low} population. ESA has been used in the past to distinguish epithelial cancer cells from benign reactive mesothelial cells (20). When ESA\textsuperscript{−}CD44\textsuperscript{−}CD24\textsuperscript{−}Lineage\textsuperscript{−} cells were isolated from passaged T1, as few as 200 cells consistently formed tumors of ~1 cm between 5 and 6 mo after injection, whereas 2,000 ESA\textsuperscript{−}CD44\textsuperscript{+}CD24\textsuperscript{low}Lineage\textsuperscript{−} cells or 20,000 CD44\textsuperscript{+}CD24\textsuperscript{+} cells always failed to form tumors (Table 3). Ten thousand unsorted cells formed tumors in only 3 of 12 mice. This result suggests that the ESA\textsuperscript{−}CD44\textsuperscript{+}CD24\textsuperscript{low}Lineage\textsuperscript{−} population was >50-fold enriched for the ability to form tumors relative to unfractonated tumor cells (Table 3). The ESA\textsuperscript{−}CD44\textsuperscript{+}CD24\textsuperscript{−}Lineage\textsuperscript{−} population accounted for 2–4% of passage 1 T1 cells (2.5–5% of cancer cells). The ESA\textsuperscript{−}CD44\textsuperscript{+}CD24\textsuperscript{−}Lineage\textsuperscript{−} population (0.6% of cancer cells) from unpassaged T5 cells was also enriched for tumorigenic activity, compared with ESA\textsuperscript{−}CD44\textsuperscript{−}CD24\textsuperscript{−}Lineage\textsuperscript{−} cells, but both the ESA\textsuperscript{−} and ESA\textsuperscript{+} fractions had some tumorigenic activity (Table 3). Among unpassaged T5 cells, as few as 1,000 ESA\textsuperscript{−}CD44\textsuperscript{+}CD24\textsuperscript{−}Lineage\textsuperscript{−} cells consistently formed tumors.

To determine whether the difference in tumorigenicity of the cell populations was due to differences in cell cycle, we analyzed these populations by flow cytometry. Comparison of the cell cycle status of tumorigenic and nontumorigenic cancer cells from T1 revealed that both exhibited a similar cell cycle distribution (Fig. 2). Therefore, neither population was enriched for...
cells at a particular stage of the cell cycle, and the nontumori-
genic cells were able to undergo at least a limited number of
divisions in the xenograft model.

Six months after injection, the injection sites of 20,000 tumori-
genic CD44+/CD24+/lowLineage− cells and 20,000 nontumorigenic
CD44+/CD24+/Lineage− cells were examined by histology. The
CD44+/CD24+/lowLineage− injection sites contained tumors ≈1 cm
in diameter, whereas the CD44+/CD24+/Lineage− injection sites
contained no detectable tumors (Fig. 3c). Only normal mouse
mammary tissue was seen by histology at the sites of the
CD44+/CD24+Lineage− injections (Fig. 3a), whereas the tumors
formed by CD44+/CD24+/lowLineage− cells contained malignant
cells as judged by hematoxylin and eosin-stained sections (Fig. 3b).
Even when CD44+/CD24+ Lineage− injection sites from 58 mice
each administered 1,000–50,000 cells) were examined after 16–29
wk, no tumors were detected. Furthermore, the tumorigenic and
nontumorigenic populations were indistinguishable morphologi-
cally. Both the tumorigenic and nontumorigenic subsets of Line-
age− cells from passed and unpassed tumors contained >95% cancer cells as judged by Wright staining or Papanicolaou
staining and microscopic analysis. By histology, the CD44+/CD24+/low
Lineage− cells and the rest of the Lineage− cells had the appearances of epithelial cancer cells (Fig. 3d and e).

The Tumorigenic Population Is Capable of Generating the Phenotypic Heterogeneity Found in the Initial Tumor. The ability of small
numbers of CD44+/CD24+/lowLineage− tumorigenic cells to give
rise to new tumors was reminiscent of the organogenic capacity
of normal stem cells. Normal stem cells self-renew and give rise to
phenotypically diverse cells with reduced proliferative potential. To
test whether tumorigenic breast cancer cells also exhibit these
properties, tumors arising from 200 ESA+/CD44+/CD24+/low
Lineage− T1 or 1,000 CD44+/CD24+/lowLineage− T2 cells were
dissociated and analyzed by flow cytometry. The heterogeneous
expression patterns of ESA, CD44, or CD24 in the secondary
tumors resembled the phenotypic complexity of the tumors from
which they were derived (Fig. 4a and b vs. e and f). Within these
secondary tumors, the CD44+/CD24+/lowLineage− cells remained
tumorigenic, whereas other populations of Lineage− cancer cells
remained nontumorigenic (Table 3). Thus, tumorigenic cells gave
rise to both additional CD44+/CD24+/lowLineage− tumorigenic
cells as well as to phenotypically diverse nontumorigenic cells that
recapitulated the complexity of the primary tumors from which the
tumorigenic cells had been derived. These CD44+/CD24+/low
Lineage− tumorigenic cells from T1, T2, and T3 have now been
serially passaged through four rounds of tumor formation in mice,
yielding similar results in each passage with no evidence of de-
creased tumorigenicity (data not shown). These observations sug-
gest that CD44+/CD24+/lowLineage− tumorigenic cancer cells un-
dergo processes analogous to the self-renewal and differentation
of normal stem cells.

Our results demonstrate that heterogeneous populations of cells
in breast cancers consist of a phenotypically distinct tumorigenic
population, as well as a much larger population that lacks this
tumorigenic potential. It is known that breast cancer cells are
genetically unstable, and thus individual breast cancer cells from
the tumorigenic population may sometimes be unable to proliferate
as a consequence of chromosomal aberrations acquired during mitosis (21–23). Nevertheless, the observation that in eight of nine tumor
specimens the tumorigenic subpopulation displayed a common
phenotype that allowed for their identification suggests that com-
mon pathways may drive this tumorigenic population.

The tumorigenic CD44+/CD24+/lowLineage− population shares
with normal stem cells the ability to proliferate extensively, and to

Fig. 4. Phenotypic diversity in tumors arising from CD44+/CD24+/lowLineage− cells. The plots depict the CD24 and CD44 or ESA staining patterns of live human
Lineage− cancer cells from T1 (a, c, and e) or T2 (b, d, and f). T1 CD44+/Lineage− cells (a) or T2 Lineage− cells (b) were obtained from tumors that had been passaged
once in NOD/SCID mice. ESA+/CD44−/CD24+/lowLineage− tumorigenic cells from T1 (c) or CD44+/CD24+/lowLineage− tumorigenic cells from T2 (d) were isolated and
injected into the breasts of NOD/SCID mice; e and f depict analyses of the tumors that arose from these cells. In both cases, the tumorigenic cells formed tumors
that contained phenotypically diverse cells similar to those observed in the original tumor.

give rise to diverse cell types with reduced developmental or proliferative potential (24). The extensive proliferative potential of the tumorigenic population was demonstrated by the ability of as few as 200 passaged or 1,000 unpassaged ESA⁺CD44⁺CD24⁻ low Lineage⁻ cells to give rise to tumors (>1 cm in diameter) that could be serially transplanted in NOD/SCID mice. The tumorigenic population from T1, T2, and T3 has now been purified and serially passaged four times through NOD/SCID mice. This extensive proliferative potential contrasts with the bulk of CD44⁺/H11002 VS tumors. Not only was the CD44 instance, it has been demonstrated that up to 30% of breast cancer tumors have this result may also be true for tumorigenic breast cancer cells, as the majority of tumor cells that can be derived from this population have only limited proliferative potential in vivo. It has previously been shown that the phenotype of acute myelogenous leukemia leukemogenic cells is similar to that of early hematopoietic progenitor stem cells (14). Our results suggest that this result may also be true for tumorigenic breast cancer cells, because early multipotent epithelial progenitor cells also have been reported to express ESA and CD44 (26–28).

The ability to separate tumorigenic and nontumorigenic populations of tumor cells should allow the molecular characterization of these cells and elucidation of the pathways that account for their tumorigenic potential. Furthermore, the existence of a subset of tumorigenic cells within a tumor would provide an explanation for a number of clinical observations in breast cancer patients. For instance, it has been demonstrated that up to 30% of breast cancer patients may show micrometastatic disease in their bone marrow at the time of presentation. However, after 5 yr, only ~50% of these patients will demonstrate clinically evident metastases. This finding has been speculated to be due to tumor dormancy. However, an alternative explanation consistent with a cancer stem cell model is that cancer cells in the bone marrow of patients may arise from the spread of either tumorigenic or nontumorigenic cancer cells, and only when tumorigenic cells metastasize will frank tumors that are clinically significant develop. This second explanation suggests that the development of diagnostic reagents that allow for the prospective identification of tumorigenic cells may have prognostic significance for patients with breast cancer.

The identification of tumorigenic and nontumorigenic breast cancer cells also has important therapeutic implications. Traditionally, drug therapies have been developed based on the ability of these agents to cause tumor regression in animal models. Because we have shown that the majority of cancer cells within a tumor are nontumorigenic, therapies directed at these cells would cause tumor regression. However, if therapies fail to target the tumorigenic cells, then these cells would persist after therapy and be able to regenerate the tumor, resulting in tumor relapse. It is known that normal stem cells have mechanisms that make them relatively resistant to chemotherapy, such as increased expression of BCL-2 family proteins, increased expression of membrane transporters like breast cancer drug resistance protein, and multiple drug resistance (29–32). The expression of such proteins in tumorigenic breast cancer cells may make them inherently more resistant to current therapies. The prospective identification of the tumorigenic population of cancer cells should allow the identification of molecules expressed in these cells that could be targeted to eliminate this crucial population of cancer cells, leading to more effective cancer therapies.

We thank Mark Kukaruga and Ann Marie Deslaurier for flow cytometry, Steve Ethier for tumor specimens, and Brian Clarke for the mathematical calculation of the frequency of tumorigenic cancer cells. This work was supported by National Cancer Institute Grant CA-075136. Flow cytometry was supported by National Cancer Institute Grant CA-46592.