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Transformation of Different Human Breast Epithelial Cell Types Leads to Distinct Tumor Phenotypes

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SUMMARY

We investigated the influence of normal cell phenotype on the neoplastic phenotype by comparing tumors derived from two different normal human mammary epithelial cell populations, one of which was isolated using a new culture medium. Transformation of these two cell populations with the same set of genetic elements yielded cells that formed tumor xenografts exhibiting major differences in histopathology, tumorigenicity, and metastatic behavior. While one cell type (HMECs) yielded squamous cell carcinomas, the other cell type (BPECs) yielded tumors closely resembling human breast adenocarcinomas. Transformed BPECs gave rise to lung metastases and were up to 10^4-fold more tumorigenic than transformed HMECs, which are nonmetastatic. Hence, the pre-existing differences between BPECs and HMECs strongly influence the phenotypes of their transformed derivatives.

INTRODUCTION

The histopathological and clinical behavior differences among epithelial cancer subtypes arising within a single organ can be as large as those arising in different organs. For instance, more than a dozen distinct histopathological subclasses of breast cancer are encountered in the clinic (Rosen, 2001), and subtypes with differing patient outcomes have also been defined through gene expression profiling (Gusterson et al., 2005; Sorlie et al., 2001). The phenotypic diversity of tumors has been generally ascribed to subtype-specific genetic and epigenetic alterations. However, some have suggested that the heterogeneity among human breast cancers is also due to their derivation from a variety of distinct normal epithelial cell types (Bocker et al., 2002; Dontu et al., 2003; Welm et al., 2003), this notion being supported both by mouse tumor models (Dimri et al., 2005; Li et al., 2003) and by expression profiling of human breast tumors (Sorlie et al., 2003). While it seems evident from clinical observations that cells from different organs give rise to distinct tumors, it has been less clear whether transformation of neighboring epithelial cells residing within a single organ can lead to different tumor phenotypes.

It has been difficult to retrospectively identify the precise cell type that gives rise to a particular tumor in clinical samples or rodent tumor models, since the normal cell from which the tumor arose is already transformed and no longer available in its original state. This suggests that prospective transformation of different cell subtypes

SIGNIFICANCE

Tumor phenotype is influenced by multiple factors, including genetic and epigenetic alterations, tumor stroma, and systemic environment. Because of this complexity, it has been difficult to investigate the influence of normal cell phenotype on the behavior of its tumorigenic derivatives. We developed a cell culture method that allows direct comparison of two genetically matched tumors derived from two different breast epithelial cell types. This revealed that tumor cell phenotype, including metastatic tendency and gene expression profile, can be strongly influenced by the normal mammary epithelial cell type that serves as the precursor of the tumorigenic cells. Hence, analyses of normal cell populations that give rise to various tumor types will be essential for a complete understanding of tumor phenotypes.

from the same normal epithelium is essential to uncover the influence of the normal cell phenotype on the phenotype of a tumor derived from a particular normal cell population.

In the case of human breast tissue, a specific culture medium (termed MEGM or MCDB-170) has been widely used to propagate a subpopulation of human mammary epithelial cells (HMECs) in vitro since its development more than two decades ago (Hammond et al., 1984; Stampfer and Yaswen, 2000). We have previously reported that experimental transformation of HMECs grown in MEGM medium resulted in tumorigenic breast epithelial cells that gave rise, after implantation into immunocompromised host mice, to poorly differentiated carcinomas with areas of squamous differentiation (Elenbaas et al., 2001). This particular tumor phenotype is rare among naturally occurring human breast cancers, representing less than 1% of human breast tumors. Interestingly, it has been reported previously that the normal HMEC population from which these tumors were derived is equally rare in vivo (Brenner et al., 1998; Holst et al., 2003; Tlsty et al., 2004; Yaswen and Stampfer, 2001). Accordingly, we suspected that the outgrowth of other normal epithelial cell types might well be favored in alternative culture media, and that experimental transformation of these other cell types might yield tumor phenotypes that differed from those observed previously. This motivated us to develop alternative means of propagating normal human breast epithelial cells in vitro.

RESULTS

Isolation of Two Normal Human Mammary Epithelial Cell Types

In an attempt to culture normal human mammary epithelial cell types other than the MEGM-derived HMECs, we recently developed a serum-free, chemically defined medium termed WIT. Normal breast tissue from disease-free reduction mammoplasties was digested with collagenase, and the resulting multicellular structures (mammary organoids) were plated either directly in WIT medium on a modified plastic surface (Primaria, Becton Dickinson) or in MEGM medium on standard tissue culture plastic; dissociation of organoids into single-cell suspensions at this stage precluded establishment of successful cultures.

As reported previously, the majority of cells that grew out of organoids underwent growth arrest within 3 weeks of in vitro propagation in MEGM; in contrast there was no significant growth arrest in WIT cultures (Figure 1A). It has been previously shown that, during the first several passages in MEGM, expression of the $p16^{INK4A}$ tumor suppressor protein is increased 10- to 15-fold in HMECs, causing the replicative arrest referred to as M0 (Romanov et al., 2001; Sandhu et al., 2000; Yaswen and Stampfer, 2001), which was not seen in the WIT medium (Figures 1A and 1B). This result is reminiscent of the behavior of HMECs grown on feeder layers, whose presence also allowed propagation in the absence of p16 induction (Herbert et al., 2002).

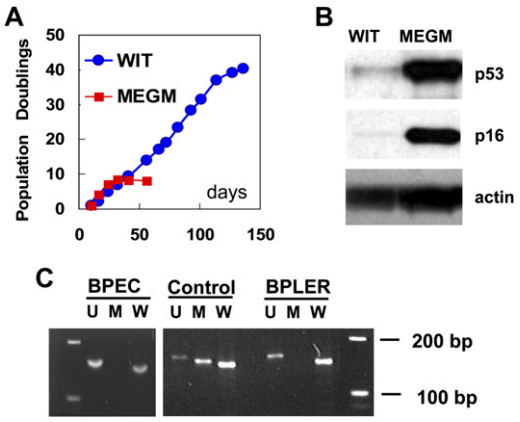


Figure 1. Primary Culture of Normal Human Mammary Epithelial Cells

(A) Comparison of population doublings of mammary epithelial cells simultaneously cultured from organoids isolated from the same donor in WIT (blue circles) medium on Primaria plates or in MEGM (red squares) medium on regular culture plates. Cells that were cultured in MEGM growth arrested after five to six population doublings; in contrast, cells that were cultured in WIT proliferated past 40 population doublings.

(B) Comparison of p53 and p16 protein expression levels in mammary epithelial cells cultured in WIT medium on Primaria plates versus MEGM medium on regular plates on day 21, immediately prior to growth arrest of cells in MEGM medium. Western blot, β-actin loading control.

(C) Comparison of $p16^{INK4A}$ gene promoter methylation analysis by using DNA methylation-specific PCR primers (U, unmethylated; M, methylated; W, wild-type. $p16^{INK4A}$ promoter DNA-specific primers produce a single PCR product of different sizes with a complete chemical modification reaction; U primers amplify only unmethylated DNA (154 bp), M primers amplify only methylated DNA (145 bp), and W primers amplify only DNA that is not chemically modified, or "wild-type" (142 bp).

Others have shown that M0 arrest imposes a severe in vitro selection step on HMECs propagation and permits only a rare subset ($<1 \times 10^{-5}$ cells) with an already in vivo methylated $p16^{INK4A}$ promoter to proliferate past M0 arrest (Holst et al., 2003; Tlsty et al., 2004). In contrast, the p16 protein is not significantly induced in cells propagated in WIT medium on Primaria plates (Figure 1B), allowing long-term propagation of a population of mammary cells that do not exhibit $p16^{INK4A}$ promoter methylation (Figure 1C). Hereafter, we refer to human mammary epithelial cells growing in the WIT medium as BPECs (breast primary epithelial cells) in order to distinguish them from the HMECs selected for growth in MEGM medium.

The two mammary epithelial cell populations that proliferated in the WIT and MEGM media have distinct growth requirements. When primary BPECs that had been cultured in WIT medium on Primaria plates during the initial 3 weeks in vitro were subsequently transferred into MEGM medium and on a regular plastic surface, all of these cells entered into permanent growth arrest within 7–10 days (Figure S1A in the Supplemental Data available with this article online). Moreover, these BPECs could not be successfully propagated on regular plastic surfaces,

even in WIT medium (Figure S1B). Conversely, it was not possible to transfer early-passage HMECs that had been propagated in MEGM medium on regular plates for 3 weeks into WIT medium. None of the HMEC cells survived in WIT medium beyond a few days due to widespread cell death; this was observed on either Primaria or regular plastic tissue culture surfaces (Figure S1C).

We note that, in addition to the differing attachment surfaces, these two epithelial cell populations are propagated in substantially different media formulations: 37 of the 78 components that are present in basic WIT medium formulation are either completely absent or present at >5-fold different concentration in MEGM medium (Supplemental Experimental Procedures; Freshney, 2000; Freshney and Freshney, 2002). In addition, the standard MEGM medium is supplemented with bovine pituitary extract, which contains numerous undefined components, unlike the WIT medium, which is chemically defined. Hence, the combination of these two sets of distinct media and physical substrates appeared to encourage the outgrowth of cell populations that were unable to readily interconvert into the other type simply by switching from one set of growth conditions to the other.

Differentiation State of BPE and HME Cells

We examined the mRNA expression profiles of BPECs and HMECs in order to understand the differences between these two cell populations and found that there were nearly 2000 mRNA transcripts in each cell population that were differentially expressed ≥2-fold compared to the other cell population (Table S1). The mammary epithelium consists of an inner, luminal layer of milk-producing cells and an outer myoepithelial cell layer. The two cell types forming these two epithelial layers have distinct functions and gene expression profiles. Recently, a set of transcripts that are differentially expressed between these cell types was identified following immunomagnetic separation of these two cell populations isolated directly from normal human breast tissue (Grigoriadis et al., 2006; Jones et al., 2004). We compared these reported luminal- and myoepithelial-specific expression signatures with mRNA transcripts that were 2-fold or more differentially expressed between HMEC and BPEC populations in the analysis described above.

While neither cell population showed a gene expression program characteristic of either fully differentiated luminal or myoepithelial cells, there was a significant difference in the relative number of myoepithelial-specific genes expressed in these two populations. In particular, HMECs overexpressed more than twice as many myoepithelial-specific genes relative to BPECs (Figure 2A; Table S2). The differential expression of several of these genes was also confirmed at the protein level. For example, Claudin-4, a protein that is exclusively expressed in the inner luminal layer of normal breast epithelium, is highly expressed in BPECs and is absent in HMECs (Figures 2B and 2C). Conversely, CD-10, which is exclusively expressed in the outer myoepithelial layer of the normal mammary epithelium, is highly expressed in HMECs but is absent in BPECs (Figures 2B and 2C). These results indicated that BPECs and HMECs differ in their differentiation state, revealing that HMECs are considerably more myoepithelial-like than BPECs.

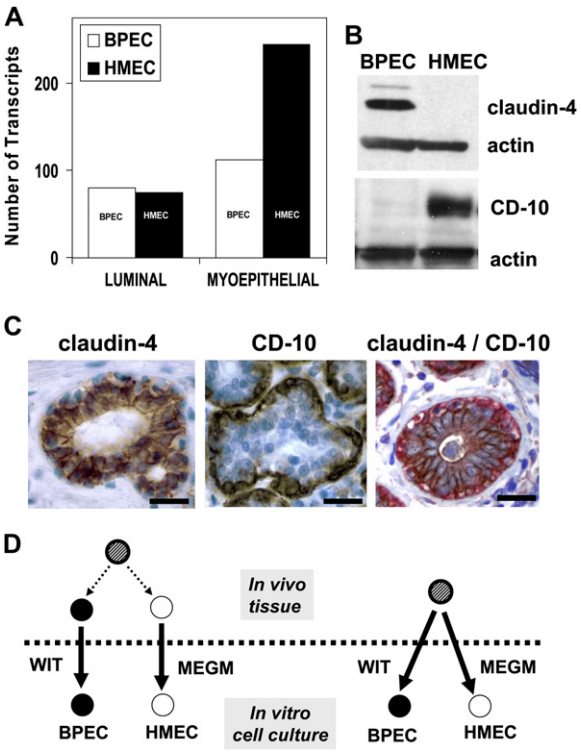


Figure 2. Differentiation State of BPE and HME Cells

(A) The comparison of luminal- and myoepithelial-specific expression signatures with genes that are differentially expressed ≥2-fold between HMEC and BPEC populations. Each bar represents the number of luminal- or myoepithelial-specific transcripts expressed at a higher level (≥2-fold) in one cell type relative to the other; open bars (BPEC), filled bars (HMEC), luminal-specific genes (columns at left), myoepithelial-specific genes (columns at right). The mRNA from three independently derived BPECs and HMECs was analyzed and compared to the luminal- or myoepithelial-cell-specific transcripts previously identified (Grigoriadis et al., 2006). A full list of genes that are differentially expressed between BPECs and HMECs, and the list of genes that correspond to each specific bar in this figure is available in Tables S1 and S2.

(B) Comparison of luminal-specific Claudin-4 and myoepithelial-specific CD-10 protein expression in HME and BPE cells. Western blot, β-actin loading control.

(C) Immunoperoxidase staining of formalin-fixed paraffin embedded normal human breast tissue with luminal-specific Claudin-4 (left panel) and myoepithelial-specific CD-10 antibodies (middle panel). The double immunostain (right panel) was performed by sequential Claudin-4-HRP staining (brown) followed by CD-10-alkaline phosphatase (red) staining (scale bar = 50 μm).

(D) Schematic representation of alternative mechanisms for the derivation of the two normal in vitro breast epithelial cell populations: selection of pre-existing cell types (left panel) versus in vitro differentiation from a single in vivo cell type (right panel).

We postulate that these two cell phenotypes—BPECs and HMECs—arose either because of selection of two preexisting cell types within the normal breast tissue

(Figure 2D, left panel), or through in vitro differentiation from a common oligopotential in vivo precursor (Figure 2D, right panel). In either case, based on their mutually exclusive growth requirements and differences in their differentiation state, BPECs and HMECs isolated from the same donor provided us with an experimental platform with which we could examine prospectively whether differences in the phenotype of normal cells from the same epithelium exert lasting influences on the behavior of their transformed, tumorigenic derivatives.

Immortalization and Transformation of BPE and HME Cell Types

In order to examine the influence of normal cell phenotype on that of derived transformants, we determined whether transformation of the BPECs growing in WIT medium on Primaria plates would give rise to tumors that were biologically different from those arising following transformation of the HMECs grown in MEGM on regular culture plates. BPECs and HMECs were transformed in three consecutive steps using retroviral vectors expressing *hTERT*, *SV40 early region*, and H-*ras*, respectively, as previously described (Elenbaas et al., 2001; Hahn et al., 1999). Prior to complete transformation with *SV40 early region* and H-*ras*, we determined the differentiation state of hTERT-expressing cells by comparing their gene expression pattern with the set of previously reported luminal- and myoepithelial-specific human breast genes (Grigoriadis et al., 2006; Jones et al., 2004).

The gene expression differences indicated that, in parallel with the results described above, BPE-hTERT and HME-hTERT cells remained partially differentiated along luminal and myoepithelial pathways, respectively. The BPE-hTERT cells expressed many more luminal-specific genes at a $\geq$2-fold higher level relative to the corresponding HME-hTERT cells; conversely, the HME-hTERT cells expressed more myoepithelial-specific genes relative to BPE-hTERT cells (Figure S2A). Furthermore, the ratio of luminal-to-myoepithelial-specific gene expression within each cell type was very different in this comparison; while BPE-hTERT cells expressed predominantly luminal-specific genes, the corresponding HME-hTERT cells predominantly expressed myoepithelial-specific genes (Figure S2A; Table S3). Hence, the hTERT-expressing populations retained the distinct gene expression patterns of their primary BPEC and HMEC precursors.

The hTERT-expressing BPECs and HMECs were subsequently transformed in parallel with retroviral vectors expressing the *SV40 early region* and the H-*ras* oncogene, as described before (Elenbaas et al., 2001; Hahn et al., 1999) (Figure 3A and Figure S2B). The tumorigenic cells arising from HMECs following introduction of vectors expressing *hTERT* (L), the *SV40 early region* (E), and H-*ras* (R) are termed hereafter HMLER cells, while those arising from BPECs are termed BPLER cells (Figure 3A). The resulting transformed progeny remained polyclonal throughout multiple steps of transformation (Figures S2C and S2D; Hahn et al., 1999). Moreover, expression levels of the products of the introduced genes in the two cell lines were comparable, i.e., less than 2-fold different between the HMLER and BPLER cells, as determined by immunoblots, immunofluorescence, and RT-PCR analyses (Figures 3B–3D; Figure S3). Importantly, the continued presence of polyclonal populations of these two cell types in vitro made it unlikely that rare variant subtypes were selected during the generation of these two transformed cell populations (Figure S2D).

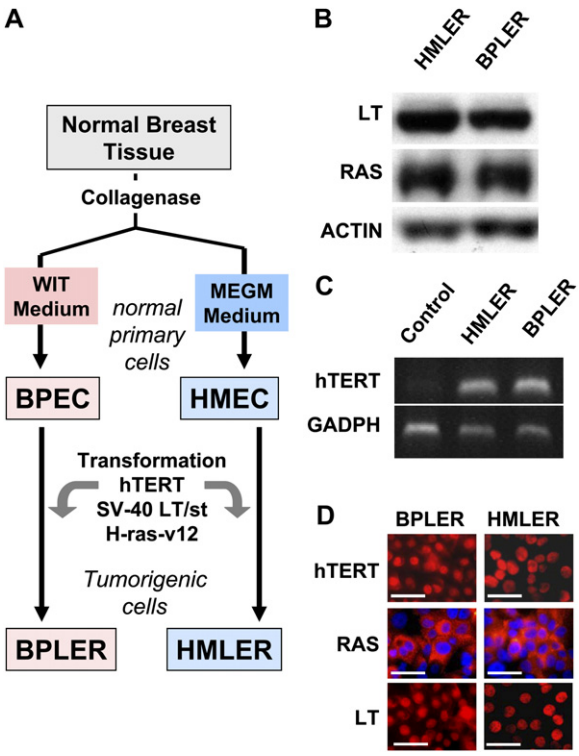


Figure 3. Tumorigenic Transformation of Normal Breast Epithelial Cells

(A) Schematic steps for the creation of two breast cancer cell types (BPLER and HMLER) with defined genetic elements.

(B) Comparison of SV40-Large T Ag (LT) and H-Ras (RAS) protein expression levels in BPLER and HMLER cells, in vitro culture (Western blot, β-actin as loading control). The difference between the two cell populations was less than 2-fold, based on serial dilutions (see Figure S3A).

(C) Comparison of ectopic hTERT mRNA expression levels in BPLER and HMLER cells with RT-PCR shows similar expression levels in both cells. Primers for GADPH were used as internal control; first lane was a control RT-PCR reaction with mRNA from HMECs without ectopic hTERT.

(D) SV40-Large T Ag (LT), H-Ras (RAS), and hTERT protein expression levels in BPLER and HMLER cells, in vitro culture (immunofluorescence). SV40-LT and hTERT were detected in the nucleus, and Ras was detected in the cytoplasm (red, signal; blue, nuclear counterstain; scale bar = 15 μm; see Figure S3B for corresponding DAPI nuclear stains).

Histology of HMLER and BPLER Tumors

Most human breast carcinomas (>90%) retain some form of normal glandular architecture, which explains their classification as ductal adenocarcinomas (Figures S4E and

S4F). Moreover, human breast tumors are associated with a desmoplastic stromal response, which is composed of a newly formed extracellular matrix and multiple nonneoplastic cell types, in particular, abundant α-Smooth muscle actin (α-SMA)-positive myofibroblasts. Both the ductal architecture and the stromal response seen in human tumors are absent in most commonly used breast tumor xenograft models.

As reported previously, HMLER cells form poorly differentiated tumors with areas of squamous cell differentiation when injected into the mammary fat pad of immunocompromised mice (Elenbaas et al., 2001). We observed the same results with a second independently isolated and transformed HMLER cell population (Figure 4, left column panels). Microscopic examination of representative tumor sections showed that these HMLER tumors grew as a solid mass of neoplastic cells with little desmoplastic stroma and that they formed keratin pearls—a typical feature of squamous differentiation (Figure 4, left H&E panel). No ductal or glandular structures that are characteristic of breast adenocarcinomas were apparent. Furthermore, HMLER tumor cells lacked Cytokeratins 8 and 18 (CK 8/18), which are expressed in >85% breast adenocarcinomas but are absent in squamous cell carcinomas (Figure 4, left) (Chu and Weiss, 2002).

Significantly, the BPLER tumors that formed in the mammary fat pads of immunocompromised mice focally displayed well-formed epithelial ductal structures that expressed Cytokeratins 8 and 18 (Figure 4, right column panels), which were surrounded by a strong desmoplastic response composed of numerous α-SMA-positive mouse myofibroblasts; α-SMA was not expressed by the tumor cells themselves (Figures S5C–S5E). In addition to areas of ductal differentiation, there were areas of papillary differentiation and scattered poorly differentiated regions in BPLER tumors (see Figures S4 and S5 for additional images). Thus, the histopathological appearance of BPLER tumors was closer to actual human tumors compared to most breast tumor xenograft models.

As described here, the histomorphology of BPLER and HMLER xenografts is reminiscent of adenocarcinomas and squamous cell carcinomas of the breast. Since both cell populations were transformed with the same set of introduced genetic alterations, the observed difference in tumor histomorphology appeared to be influenced by the phenotype of the starting normal cell populations.

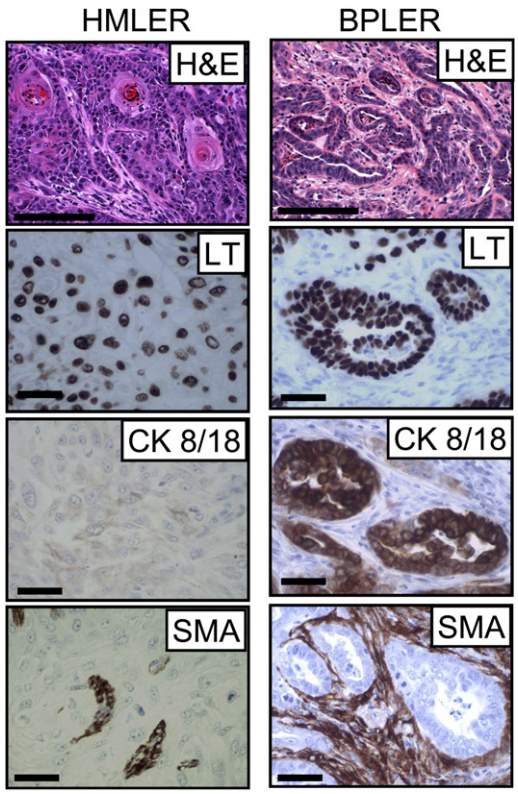


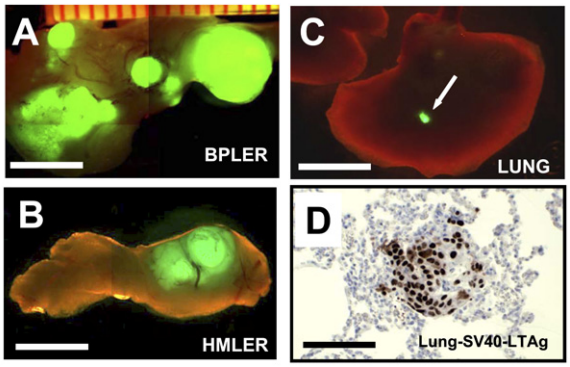
Figure 4. Microscopic Examination of Mouse Mammary Fat Pad Tumor Xenografts

HMLER cells (left column) and BPLER cells (right column). H&E, hematoxylin-eosin staining; scale bar = 200 μm. Immunoperoxidase stains of representative tumor sections: LT, SV40-TL Ag; SMA, α-Smooth muscle actin; and CK8/18, Cytokeratin 8/18. Brown, specific staining; blue, counterstain; scale bar = 50 μm. All histological sections were prepared from tumor tissue explanted 4 to 6 weeks after implantation of tumorigenic cells from tissue culture into the mammary fat pad of NOD/SCID mice.

Cell Type and Metastatic Ability

BPLER tumor xenografts exhibited a multifocal growth pattern in the mammary fat pad (Figure 5A); in human breast tumors, such behavior has been ascribed to intramammary gland metastasis (Andea et al., 2002, 2004; Norton and Massague, 2006). This prompted us to search for distant metastases. To do so, BPLER and HMLER cells were transduced with a Green fluorescent protein (GFP) gene and implanted in the mammary fat pads of NOD/SCID mice (Figures 5A and 5B). Ten weeks after injection, more than 70% of mice bearing BPLER tumors had lung micrometastases that generally ranged from single cells up to nodules of 20 cells, with occasional 1 mm diameter nodules (Figure 5C), as confirmed by immunohistochemical staining of the nodules with an antibody against LT-Ag (Figure 5D). Lung micrometastases were observed in multiple experiments using two independently derived BPLER cell lines originating from the BPECs isolated from two different patients (BPLER-1 and -2; Figure 5E). Of note, BPLER tumors were equally metastatic following subcutaneous injection in nude mice (Figure 5E).

Despite primary tumor burdens equivalent to BPLER-injected mice, none of the HMLER-injected animals developed lung micrometastases, as ascertained by dissection microscopy as well as histological and immunohistochemical examination of the lungs, confirming previous reports that also failed to detect distant metastases in mice bearing HMLER xenograft tumors (Elenbaas et al., 2001; Kuperwasser et al., 2005). Hence, transformation of HMECs and BPECs yielded tumors with differing metastatic disposition.



E Frequency of Metastasis	BPLER-1	BPLER-2	HMLER
Orthotopic	10 / 11	6 / 8 *	0 / 8 *
Subcutaneous	9 / 10	8/12	0 / 6

Figure 5. Differences in Primary Tumor Growth Pattern and Metastasis of BPLER and HMLER Cells

Fluorescence dissecting microscopic images of nodules composed of tumor cells expressing green fluorescent protein (GFP).

(A) BPLER cells; multifocal growth of five tumor nodules ranging 0.1–0.6 cm in diameter, in mammary fat pad of NOD/SCID mice, 4 weeks postinjection (1×, scale bar = 0.5 cm, composite image).

(B) HMLER cells; single 0.6 cm diameter primary tumor nodule in mammary fat pad of NOD/SCID mice, 4 weeks postinjection (1×, scale bar = 0.5 cm, composite image).

(C) BPLER metastasis to lungs from a mammary fat pad primary tumor; single 0.05 cm diameter green metastatic tumor nodule (white arrow), 10 weeks postinjection (1×, scale bar = 0.5 cm).

(D) Detection of metastatic BPLER cells (from [C]) with SV40-LT immunohistochemical staining of formalin-fixed, paraffin-embedded lung sections (scale bar = 250 μm).

(E) Frequency of BPLER and HMLER lung metastasis from orthotopic (mammary fat pad) and subcutaneous injection sites 10 weeks postinjection. BPLER-1 and -2 were derived from two different individuals. *There was no statistically significant difference in tumor burden between these groups (BPLER-2: 0.74 g ± 0.14; HMLER: 0.75 g ± 0.06).

Cell Type and Tumor-Initiating Cell Frequency

In most tumor xenograft experiments using established tumor cell lines, injection of at least 10^6 tumor cells is required in order to observe tumor growth. The rapid growth of the BPLER primary tumors forced us to inject fewer cells in order to allow for the long-term observations required to detect distant metastases. During the course of such experiments, we discovered a significant difference in the number of cells required for the seeding of tumors by the HMLER and BPLER cells: three independent BPLER cell lines, derived from three different patients, formed tumors when as few as 100 cells were injected subcutaneously into nude mice (BPLER-1, -2, and -3; Table 1). Furthermore, even BPLER tumors that arose from subcutaneous injection of 100 cells formed lung metastases (Table 2). In contrast, a minimum of 2–3 × 10^6 cells was needed per inoculum in order to observe subsequent outgrowth of HMLER tumors (Table 1, MEGM). Importantly, there was no significant difference in the in vitro growth rates of BPLER and HMLER cells. Thus, in addition to histomorphology and metastatic behavior, the differences in the phenotype of normal cells also influenced tumor-initiating cell frequency observed among their transformed derivatives.

Table 1. Number of BPLER and HMLER Cells Required for Tumor Initiation

BPLER				HMLER		
Cells Injected	Tumors/Injection 1	2	3	Cells Injected	Tumors/Injection MEGM	WIT
10^6	9/9	9/9	9/9	10^6	4/12	5/9
10^5	9/9	9/9	12/12	10^5	0/12	1/12
10^4	9/9	9/9	12/12	10^4	0/12	0/12
10^3	8/15	9/9	9/12	10^3	0/12	0/12
10^2	—	10/12	4/9	10^2	—	—

Tumor formation in nude mice was measured 10 weeks after injection of 10^2 to 10^6 BPLER cells subcutaneously. Three independent BPLER cell lines (1, 2, and 3) derived from normal mammary epithelial cells isolated from three different donors were tested. In addition, injection of 10^2 cells from two independent *single-cell clones* of BPLER formed tumors in 8/12 and 11/12 mice (data not shown). Tumor formation in nude mice was measured up to 24 weeks after injection of 10^2 to 10^6 HMLER cells that were grown in parallel in either MEGM or WIT medium for 3 weeks.

Influence of In Vitro Growth Conditions on Tumor Initiation, Metastasis, and Gene Expression Profile of Tumor Cells

We next determined whether the observed phenotypic differences in tumorigenicity and metastasis between the two transformed mammary epithelial cell types were due to adaptation to certain conditions of in vitro culture. While HMECs that had been adapted to MEGM medium could not survive in WIT medium (see above), their fully transformed derivatives proliferated equally well in both media.

Table 2. Number of BPLER Cells Required for Tumor Initiation and Metastasis

BPLER				
Cells Injected	Primary Tumor/ Injection	Lung Metastasis	Primary Tumor Burden (g)	Time (Weeks)
10^5	12/12	3/4	1.26 ± 0.11	10
10^4	12/12	3/4	1.12 ± 0.11	10
10^3	9/12	1/3	0.76 ± 0.24	10
10^2	8/12	2/4	2.04 ± 0.35	18

Tumor formation and lung metastases were examined after injection of 10^2 to 10^5 BPLER cells subcutaneously into nude mice (three injections per mouse; n = 4). Lung metastasis was assessed at 10 weeks (10^3 to 10^5 cells injected) and at 18 weeks (10^2 cells injected).

Accordingly, we tested the tumorigenicity and metastatic ability of HMLER cells that were transferred to WIT medium for 3 weeks prior to orthotopic or subcutaneous implantation.

The WIT-adapted HMLER cells were only slightly more tumorigenic than HMLER cells propagated exclusively in MEGM medium (Table 1, HMLER MEGM versus WIT) and, like those propagated exclusively in MEGM medium, lacked metastatic ability (data not shown). This slight increase in tumorigenicity following altered conditions of culture could not account for up to four orders-of-magnitude difference in the frequency of tumor-initiating cells between the HMLER and BPLER cell populations. These differences in behavior were apparently stably imprinted on the HMLER cells and could not be altered by propagating the HMLER cells in WIT medium.

We also compared the gene expression profiles of HMLER and BPLER cell populations that had been propagated in either MEGM or WIT media. This showed that their expression profiles did not change substantially when these cells were transferred from one medium to the other (Figure S6). This result indicated that the gene expression differences between the BPLER and HMLER cells were not susceptible to change when their growth medium was switched, consistent with the above-described biological observations.

Influence of Normal Precursor Cell Types HME and BPE on Tumor Expression Profile

In order to define the contribution of normal cell phenotype to tumorigenic cell phenotype, we compared the gene expression profiles of three independently derived in vitro cultured primary BPECs and HMECs and their hTERT-expressing, nontumorigenic, untransformed derivatives (BPEs and HMEs), with the profiles of their fully transformed, tumorigenic derivatives (BPLER and HMLER).

Hierarchical clustering analyses revealed that the tumorigenic cells were more similar to their untransformed parental cells than to one another. The BPE cells and their tumorigenic BPLER derivatives formed one common root cluster that was distinct from the cluster formed by HME cells and their HMLER tumorigenic derivatives (Figure 6A). These significant differences in gene expression patterns were in consonance with the biological differences between these various cell types that we described above.

Comparison of the gene expression profile of each tumorigenic in vitro cultured cell type (BPLER and HMLER) with its corresponding untransformed hTERT-expressing precursor population (BPE and HME) identified those genes that were significantly altered upon transformation. Out of a total of 15,399 expressed genes monitored in both lineages in these arrays, 1336 genes in BPLER (versus BPE) and 3022 genes in HMLER (versus HME) were either increased or decreased by more than a factor of two upon transformation (Figure 6B; see Table S4 for a full list of genes).

Among all of the genes altered upon transformation, only a small fraction of these genes (~15%) were altered in the same direction in both cell populations (HME versus BPE) following transformation (Figure 6B, group c). The remaining 85% of the genes were either altered in one lineage but not in the other, or were altered in opposite directions [increased in one lineage and decreased in the other; Figure 6B, group (a + b)]. The comparison of the gene expression profiles of the two transformed cell populations with those of their corresponding primary BPEC and HMEC populations that had not yet been immortalized with hTERT yielded very similar results (Figure S7A). Hence, the same set of introduced transforming genes elicited quite different cellular-context-dependent changes in gene expression profiles following transformation.

Contributions of Normal Precursor Cell Type to Tumor-Specific Gene Expression

We also examined the gene expression profiles of the transformed cell populations from another perspective. Based on the initial analyses above, it became clear that the gene expression profiles of tumor cells are partly inherited in a pattern that is unchanged from their normal precursor cells and partly acquired due to genetic and epigenetic alterations acquired during the course of transformation. In order to reveal and quantify the relative contributions of these two influences on gene expression, we compared the gene expression profiles of the two in vitro cultured tumorigenic cell types (BPLER and HMLER) directly with one another. This revealed 3213 genes that were expressed significantly differently between the two tumorigenic cell populations, being increased or decreased $\geq$2-fold [Figure 6C, group (a + b), BPLER/HMLER]. We then compared the expression levels of this set of genes in the untransformed hTERT-expressing parental cells (BPE and HME) in order to measure the scale of the contribution of the precursor cell gene expression profile to the tumor-cell-specific gene expression patterns of derived tumor cells. Interestingly, approximately 40% (1265/3213) of the mRNA expression differences between the BPLER and HMLER tumor cells were already apparent when the expression patterns of their respective normal BPE and HME precursors cells were compared, being increased or decreased in the same direction (1265 genes; Figure 6C, group a; see Table S4 for a full list of genes). The comparison of early-passage primary BPEC and HMEC populations that had not yet been hTERT-immortalized yielded similar results (Figure S7B). These results further support the notion that, in this model system, a significant portion of the gene expression profile that distinguished one tumor cell type from another derived from pre-existing differences that these tumor cells inherited from their normal cell precursors.

DISCUSSION

The multistep model of tumor progression emphasizes the accumulation of genetic alterations as the central mechanism driving tumorigenesis (Karakosta et al., 2005; Nowell, 1976; Vogelstein and Kinzler, 1993). According to this view, the normal cell is an almost passive recipient of

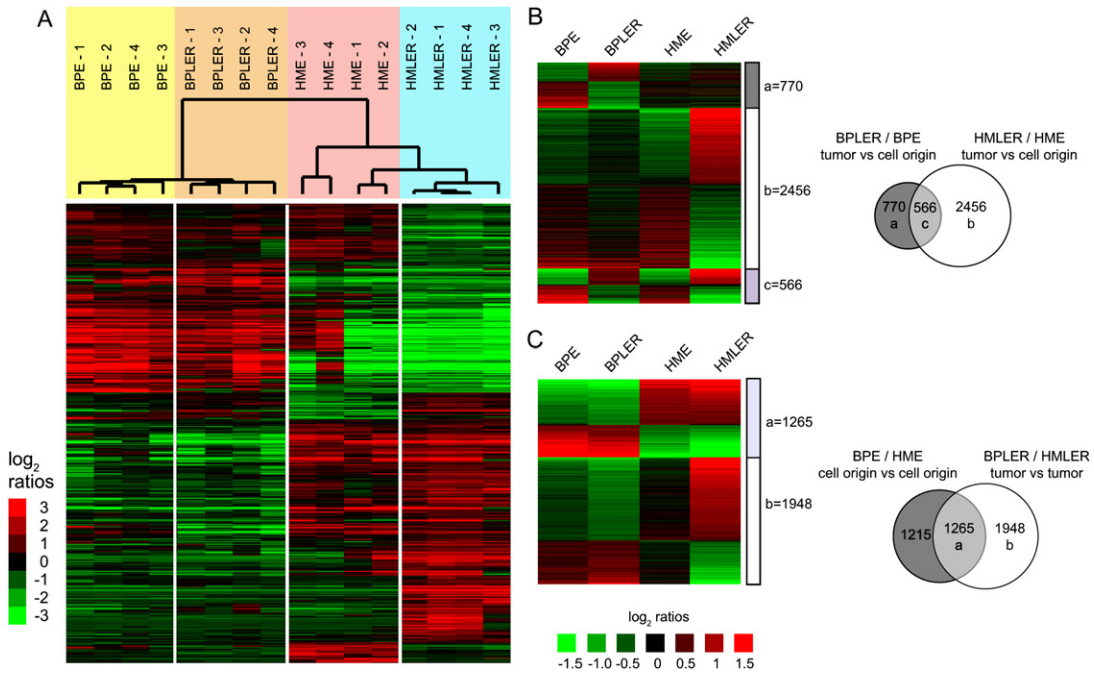


Figure 6. Influence of Precursor Cell Type on Gene Expression Signature of Tumorigenic Cells

The gene expression comparisons described in this figure were performed on cell populations isolated from three different individuals. The mRNA was prepared from in vitro cultured cells. The untransformed hTERT-expressing cell populations (BPE and HME) were compared with each other and with their fully transformed tumorigenic derivatives (BPLER and HMLER).

(A) Hierarchical clustering. Each column represents a cell line sample, and each row demonstrates the results of a different gene. Clustering orders the samples according to greatest similarity of gene expression, shown by the dendrogram at the top, and orders genes by similarity of expression level among the sample set. Mean levels of expression are depicted in black, overexpression is depicted in red, and underexpression is depicted in green for each probe set that was present and exhibited differential expression. Expression values were compared to the mean expression value across all replicates and log2 transformed.

(B) Transformation-specific gene expression differences—tumorigenic cells versus precursor cells. To the left is a heatmap in which each column represents a cell line sample and each row demonstrates the results of a different gene. Mean levels of expression are depicted in black, overexpression is depicted in red, and underexpression is depicted in green. To the right is a Venn diagram demonstrating the overlap of gene expression differences between in vitro cultured cell lines: BPLER versus BPE compared to HMLER versus HME. The full list of corresponding genes is available in Table S4. In vitro transformation induced changes that are cell type dependent: BPLER/BPE, tumorigenic cells versus hTERT-expressing cell origin (a), the mRNA expression level of these genes changed $\geq$2-fold upon transformation in BPE versus BPLER but not in HME versus HMLER (n = 770); and HMLER/HME, tumorigenic cells versus hTERT-expressing cell origin (b), the mRNA expression level of these genes changed $\geq$2-fold upon transformation in HME versus HMLER but not in BPE versus BPLER (n = 2456). In vitro transformation induced changes that are cell type independent: the genes with concordant $\geq$2-fold mRNA expression level change with transformation in both BPE and HME cell types (c) (n = 566). The number of probe sets that were statistically different in each group were 43 (a), 7 (b), and 1952 (c); $p < 0.05$.

(C) Tumor-specific gene expression differences between two tumorigenic cell populations (BPLER versus HMLER). To the left is a heatmap in which each column represents a sample from an in vitro cultured cell line and each row demonstrates the results of a different gene. Mean levels of expression are depicted in black, overexpression is depicted in red, and underexpression is depicted in green. To the right is a Venn diagram demonstrating the overlap of gene expression differences between in vitro cultured cell lines: BPLER versus HMLER compared to BPE versus HME. The full list of corresponding genes is available in Table S4. Shaded bar (a): the genes with concordant changes greater than or equal to 2-fold difference in their mRNA expression level between tumorigenic versus tumorigenic cells (BPLER/HMLER) and between the untransformed hTERT-immortalized cell origin (BPE/HME) (n = 1265). Among this group of genes, 287 probe sets were different statistically ($p < 0.05$). Open bar (b): the genes with greater than or equal to 2-fold difference in their mRNA expression level between tumorigenic versus tumorigenic cells (BPLER/HMLER) but *not* between the untransformed hTERT-expressing cell origin (BPE/HME) (n = 1948 genes). Among this group of genes, 308 probe sets were different statistically ($p < 0.05$).

these mutations, and its cancer-associated phenotypes are governed largely by the somatic mutations that its descendants happen to acquire during the course of tumor progression (Cahill et al., 1999; Fearon and Vogelstein, 1990). Indeed, the role of accumulated somatic mutations in determining tumor phenotype has been extensively documented and explains many of the observed differences among different tumors.

We provide evidence here supporting an additional, but far less studied, mechanism that governs tumor phenotype. HMLER and BPLER tumors that were created through introduction of the identical set of gene expression vectors differed significantly in their morphology, tumorigenicity, and metastatic behavior. Consequently, we conclude that, in this experimental model, the observed differences between the two tumor cell types can be

traced to differences inherent in their respective in vitro normal precursors, HMECs and BPECs. This observation raises the question of whether some of the clinical differences observed between subtypes of human breast cancers can be traced to their respective normal in vivo cells of origin (Olsson, 2000).

In the presently described work, the accumulation of genetic alterations other than those introduced experimentally might, in principle, explain the observed phenotypic differences between BPLER and HMLER cells. However, we have previously shown in multiple human cell types, including human mammary epithelial cells, that tumors that are generated by introduction of a defined set of genetic elements do not require accumulation of additional stochastically occurring mutations in order to become tumorigenic (Hahn et al., 2002; Lundberg et al., 2000; Zimonjic et al., 2001). Furthermore, accumulation of random mutations in BPLER cells during the course of their in vitro culture is unlikely to explain the high frequency of tumor-initiating cells in BPLER cultures ($\sim$1 in 10^2), which are present in concentrations up to four orders of magnitude higher than in HMLER cell populations ($\sim$1 in 10^6 cells). Such a high frequency of tumor-initiating cells might well result from the positive selection of such cells during propagation in vitro. However, Southern blot analyses of the chromosomal integration sites of retroviral vector DNAs have shown that the BPLER cells remain highly polyclonal throughout the multiple steps of experimental transformation, with no evidence of in vitro selection of rare variant subclones (Figures S2C and S2D). Moreover, it is difficult to envision a single mutational event that could account for all of the multiple observed differences between BPLER and HMLER cells, including their differentiation state (adenocarcinoma versus squamous carcinoma), tumorigenicity, stromal recruitment, and metastatic behavior.

We note, as well, that BPLER tumors derived by transforming normal mammary epithelial cells (BPECs) prepared from three different donors were very similar phenotypically, excluding the influences of specific donors and their respective genetic backgrounds on the observed behavior of BPLER cells.

During the course of tumor pathogenesis, human tumor cells acquire numerous mutations that perturb multiple, centrally acting cellular regulatory pathways. This might suggest that the acquired, mutation-specific gene expression pattern would obscure or dominate the pre-existing gene expression profile of the normal precursor cells. In the present case, the expression vectors that were used to transform HMEs and BPEs deregulate many pathways known to be altered in human tumors, doing so by inhibiting p53, pRB, p130, p107, and Protein phosphatase 2A (PP2A), as well as causing overexpression of oncogenic H-Ras (Hahn and Weinberg, 2002). If the actions of the introduced transforming genes were to dominate the neoplastic cells' gene expression patterns, then the BPE and HME cells should have become more similar to one another following transformation. This was not the case, however, since the great majority (>90%) of the genes whose expression was altered following transformation were changed in a cell-type-specific manner, being altered in either BPLERs or HMLERs but not in both. Furthermore, almost half of the mRNA expression differences between the BPLER and HMLER tumor cells closely reflected pre-existing differences between their corresponding untransformed precursors—the parental BPE and HME populations. At present, clues about the pathogenesis of human tumors are inferred from gene expression differences between tumor tissue and bulk normal tissue of origin. We suggest that, in the future, further insight into this question will require comparisons of tumor cells with their respective normal cells of origin.

The tumor phenotypes described here reflect one possible combination of genetic alterations that could be used to transform BPECs and HMECs to a tumorigenic state. It is therefore possible that other sets of introduced genetic alterations could lead to differing phenotypes in these two transformed cell populations. We note, however, that introduction of other combinations of transforming genes into HMECs has, to date, failed to yield tumors that are phenotypically different from the HMLER tumors described here (Rangarajan et al., 2004; Watnick et al., 2003; Zhao et al., 2003).

Lastly, we note that the adenocarcinoma phenotype has been difficult to recapitulate in tumor xenograft models (Cardiff et al., 2000; Liu et al., 2004; Lundberg et al., 2002), even though this tumor type constitutes the great majority of the tumors arising in a variety of visceral tissues, including breast, lung, ovary, colon, and prostate. We point out that, in contrast, the presently described tissue culture and xenograft model system has indeed been able to phenocopy many aspects of naturally occurring human adenocarcinomas, including their metastatic behavior.

EXPERIMENTAL PROCEDURES

Mammary Tissue

The normal disease-free breast tissues were collected from reduction mammoplasty procedures performed at the Brigham and Women's Hospital (BWH) with standard procedure consent. The donor patients were disease-free and between 26 and 48 years old. The collected tissues were confirmed to be disease and malignancy free by histopathological examination of tissue sections. The tissue collection protocol was reviewed by the Institutional Review Board (IRB) for Human Research at BWH that determined this study as "not involving human subjects" since (1) only discarded human tissue was used, (2) all the patient identifiers were removed from the samples before collection, and (3) there would be no identifiable private data/information obtained for this research in a form associable with the individual from whom the human material was obtained.

Isolation and Culture of BPECs

The normal tissue samples from reduction mammoplasty specimens of disease-free patients were minced and dissociated with collagenase (1 mg/ml, Roche) in Hank's buffered salt solution at 37°C, for 6 hr. The organoids liberated from the stroma were separated from single cells by centrifugation (10 × g, 5 min) and plated on Primaria plates (Becton Dickinson) in WIT medium (approximately 10–20 organoids/cm^2) at 37°C with 5% CO_2. Nearly every organoid that attached to the plate gave rise to BPEC colonies; it was not possible to establish

similar cultures following full dissociation of organoids into single cells. After 10–15 days, during which the medium was changed every 2 days, cells were lifted by 0.15% trypsin treatment at 37°C and subcultures were seeded at 1–2 × 10^4 cells/cm^2 density; lower plating densities diminished cell survival significantly. Twenty percent serum-containing medium (1:10) was used to inactivate trypsin, followed by centrifugation of cells in polypropylene tubes (500 × g, 5 min) to remove residual trypsin and serum. The medium was replaced 24 hr after replating cells and every 48 hr thereafter. HMECs were cultured from the same organoid preparations in MEGM medium on regular tissue culture plastic ware according to established protocols (Stampfer and Yaswen, 2000).

Cell Culture Medium

A working formulation of basic WIT medium for culturing transformed cells (expressing SV40 LT and Ras) can be prepared by mixing equal volumes of F12 (Sigma) and M199 media (JHR Biosciences), supplemented with the following: 10 mM HEPES (pH 7.4), glutamine (2 mM), insulin (10 μg/ml), EGF (0.5 ng/ml), hydrocortisone (0.5 ng/ml), transferrin (10 μg/ml), triiodothyronine (0.2 pg/ml), 0-phosphoryl ethanolamine (5 μg/ml), selenious acid (8 ng/ml), 17β estradiol (0.5 ng/ml), linoleic acid (5 μg/ml), all-trans retinoic acid (0.025 μg/ml), hypoxanthine Na (1.75 μg/ml), lipoic acid (0.05 μg/ml), cholesterol (0.05 μg/ml), glutathione (0.012 μg/ml), xanthine (0.085 μg/ml), ascorbic acid (0.012 μg/ml), α-tocopherol phosphate (0.003 μg/ml), calciferol (vitamin D, 0.025 μg/ml), choline chloride (3.5 μg/ml), folic acid (0.33 μg/ml), vitamin B12 (0.35 μg/ml), thiamine HCl (0.08 μg/ml), i-inositol (4.5 μg/ml), uracil (0.075 μg/ml), ribose (0.125 μg/ml), para-aminobenzoic acid (0.012 μg/ml), and bovine serum albumin (1.25 mg/ml). This formulation is supplemented with cholera toxin (25 ng/ml, Calbiochem) for culturing hTERT-immortalized BPECs. The basic WIT medium is supplemented with insulin (20 μg/ml), EGF (10 ng/ml), hydrocortisone (0.5 μg/ml), and cholera toxin (100 ng/ml, Calbiochem) for culturing primary BPECs. The primary cells were cultured in antibiotic-free conditions. All chemicals were purchased from Sigma unless otherwise indicated. Tissue culture ware with a modified surface chemistry was used (Primaria, Becton Dickinson) for BPEC and BPLER cultures. HMECs and HMLER cells were cultured in MEGM medium according to the manufacturer (Cambrex).

Analysis of Tumorigenicity and Metastasis

The protocol for tumorigenesis experiments in immunocompromised mice was approved by the Committee on Animal Care (CAC) at the Massachusetts Institute of Technology and Whitehead Institute. All such experiments were performed in accordance with relevant institutional and national guidelines and regulations. Single-cell suspensions were prepared in a WIT:Matrigel (1:1) mixture and injected in 25 μl (orthotopic) or 100 μl (subcutaneous) volumes. Female athymic nude mice (Balb/c nu/nu, Taconic) were γ-irradiated (400 rad) 12 hr prior to subcutaneous injections. Injections of tumorigenic cells into mammary fat pads were performed in 8-week-old female Nod/Scid mice that were anesthetized with intraperitoneal Avertin and implanted with a subcutaneous 60 day release pellet containing 2 mg estrogen and 20 mg progesterone (Innovative Research of America, FL). Metastasis of GFP-expressing tumor cells to lungs and other tissues was analyzed initially under a fluorescence dissecting microscope (Leica) in fresh tissues, followed by microscopic examination of hematoxylin-eosin and immunostained sections of formalin-fixed and paraffin-embedded tissues. Immunohistochemical staining was carried out by use of the conventional ABC technique.

Array Analysis

The microarray raw data were deposited in a public database (NCBI Gene Expression Omnibus, accession number GSE6885).

Supplemental Data

The Supplemental Data include Supplemental Experimental Procedures, seven supplemental figures, and four supplemental tables and can be found with this article online at http://www.cancercell.org/cgi/content/full/12/2/160/DC1/.

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Accession Numbers

The microarray raw data were deposited in a public database (NCBI Gene Expression Omnibus, accession number GSE6885).