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let-7 Regulates Self Renewal and Tumorigenicity of Breast Cancer Cells

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SUMMARY

Cancers may arise from rare self-renewing tumor-initiating cells (T-IC). However, how T-IC self renewal, multipotent differentiation, and tumorigenicity are maintained remains obscure. Because miRNAs can regulate cell-fate decisions, we compared miRNA expression in self-renewing and differentiated cells from breast cancer lines and in breast T-IC (BT-IC) and non-BT-IC from 1° breast cancers. *let-7* miRNAs were markedly reduced in BT-IC and increased with differentiation. Infecting BT-IC with *let-7*-lentivirus reduced proliferation, mammosphere formation, and the proportion of undifferentiated cells in vitro and tumor formation and metastasis in NOD/SCID mice, while antagonizing *let-7* by antisense oligonucleotides enhanced in vitro self renewal of non-T-IC. Increased *let-7* paralleled reduced H-RAS and HMGA2, known *let-7* targets. Silencing *H-RAS* in a BT-IC-enriched cell line reduced self renewal but had no effect on differentiation, while silencing *HMGA2* enhanced differentiation but did not affect self renewal. Therefore *let-7* regulates multiple BT-IC stem cell-like properties by silencing more than one target.

INTRODUCTION

The cancer stem cell hypothesis suggests that many cancers are maintained in a hierarchical organization of rare, slowly dividing "cancer stem cells" (or tumor-initiating cells, T-IC), rapidly dividing amplifying cells (early precursor cells, EPC) and differentiated tumor cells (Dalerba et al., 2007). Tentatively defined T-IC have been identified in hematologic, brain, breast, prostate, liver, pancreas, and colon cancers. T-IC, which are self renewing and can differentiate into multiple lineages, are highly tumorigenic in immunodeficient mice. According to the hypothesis, T-IC are not only the source of the tumor but also may be responsible for tumor progression (Dalerba et al., 2007), metastasis (Wicha, 2006), resistance to therapy, and subsequent tumor recurrence (Al-Hajj, 2007). Breast T-IC (BT-IC) can be enriched by sorting for $CD44^{+}CD24^{-/low}$ cells (Al-Hajj et al., 2003), by selecting for side-population (SP) cells that efflux Hoechst dyes (Patrawala et al., 2005), or by isolating spherical clusters of self-replicating cells ("mammospheres") from suspension cultures (Ponti et al., 2005). However, these methods purify both T-IC and some EPC (Al-Hajj et al., 2003; Ponti et al., 2005).

Since miRNAs regulate differentiation and can function as either tumor suppressors or oncogenes to regulate tumor development and prognosis (Lu et al., 2005), we looked at whether differences in miRNA expression might distinguish BT-IC/EPC from their more differentiated progeny. miRNAs are known to contribute to preserving stemness of embryonic stem (ES) cells, because ES cells deficient in miRNA processing cannot be maintained (Shcherbata et al., 2006). Previous studies have shown an overall reduction in miRNA expression in embryonic or tissue stem cells (Croce and Calin, 2005), and changes in specific miRNAs have been associated with ES cell self renewal and differentiation (Shcherbata et al., 2006). Moreover, miRNA-expression profiling can help characterize the stage, subtype, and prognosis of some cancers (Lu et al., 2005).

Because BT-IC are relatively resistant to chemotherapy, we are able to generate large numbers of BT-IC-enriched cells by in vivo passage of breast cancer cells in NOD/SCID mice treated with chemotherapy. We find that miRNA expression is globally reduced in BT-IC compared with more differentiated cancer cells. In particular, the *let-7* family is not expressed by BT-IC generated from cell lines or 1° patient tumors and increases with differentiation. By expressing *let-7* but not other differentially expressed oncomirs, in BT-IC or antagonizing *let-7* in more differentiated cells, we find that *let-7* regulates the key features of breast cancer stem cells—self renewal in vitro, multipotent differentiation, and the ability to form

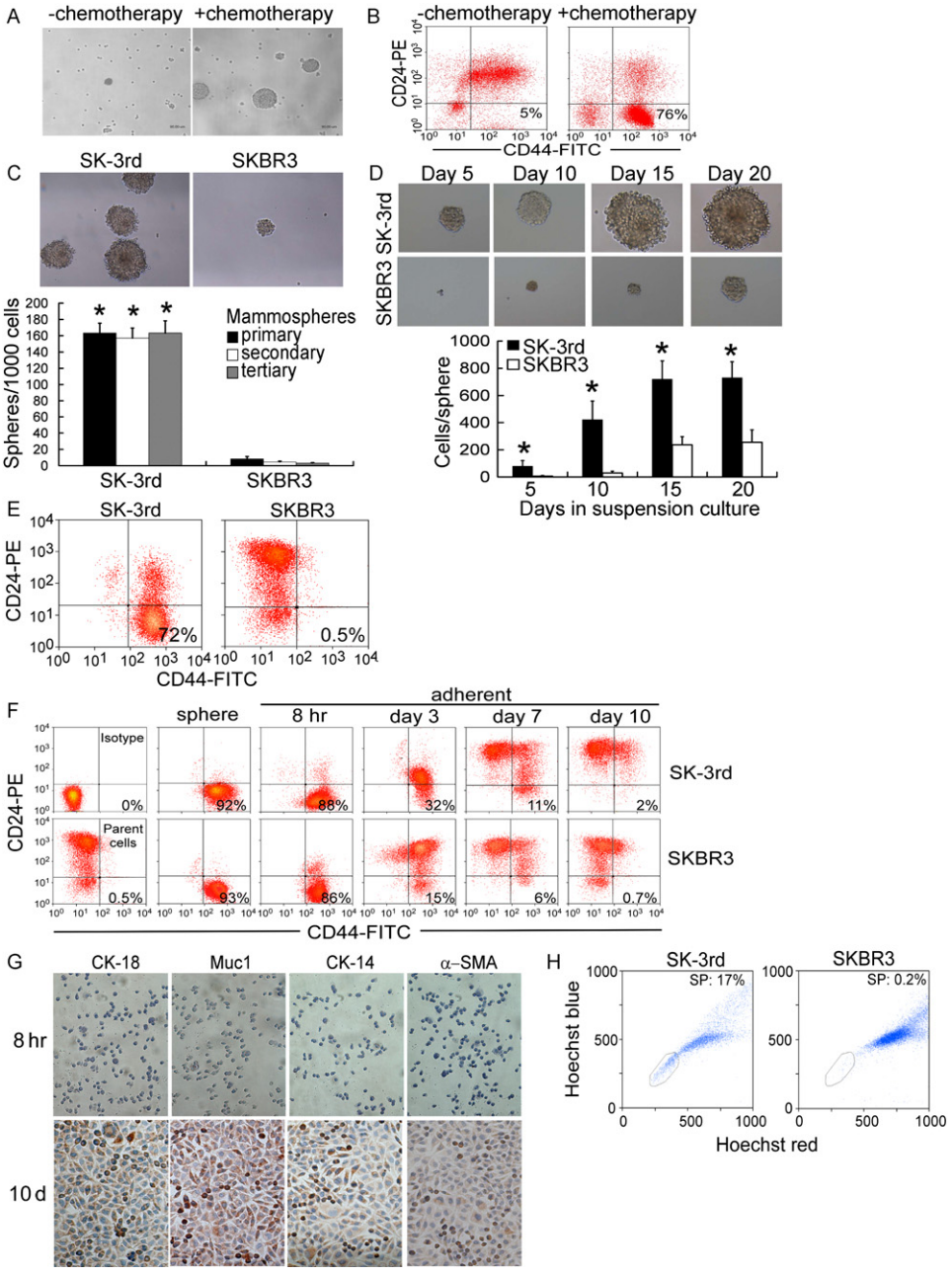


Figure 1. Breast Cancer Cells under Pressure of Chemotherapy Are Enriched for BT-IC

(A and B) 1° breast cancers from patients who received neoadjuvant chemotherapy are substantially enriched for self-renewing cells with the expected properties of BT-IC, compared to untreated patients. Representative images show increased numbers of mammospheres after 15 days of culture (A) and a higher percentage of $CD44^+CD24^-$ cells in freshly isolated tumors (B) from a patient who received chemotherapy. (Tumors resected from eight patients who did not receive chemotherapy and five patients who received neoadjuvant chemotherapy were analyzed [Table S1A]).

(C) Similarly, passaging the human breast cancer line SKBR3 in epirubicin-treated NOD/SCID mice enriches for cells with BT-IC properties. SK-3rd cells from the third passage xenograft form more mammospheres than the parent line, and the mammospheres can be repetitively passaged in vitro and are larger. Shown are numbers of 1°, 2° (generated from dissociated 1° spheres), and 3° (generated from dissociated 2° spheres) mammospheres on day 15 from 1000 cells. *, $p < 0.001$ compared with SKBR3. Error bars correspond to mean ± SD.

(D) Mammospheres generated from single-cell cultures of SK-3rd and SKBR3, imaged on indicated day of suspension culture. Shown are the mean ± SD number of cells/sphere for each time. *, $p < 0.001$ compared with SKBR3. Error bars correspond to mean ± SD.

(E) The majority of freshly isolated SK-3rd cells are $CD44^+CD24^-$, as expected for BT-IC, while cells with this phenotype are rare in SKBR3 (representative data of five experiments shown).

(F) SK-3rd and SKBR3 cells cultured as spheres are $CD44^+CD24^-$. When they differentiate in adherent cultures, they gradually assume the parental SBKR3 phenotype, but somewhat more rapidly for SKBR3 mammospheres.

tumors that can be serially transplanted and metastasize in NOD/SCID mice. Protein expression of the *let-7* targets *RAS* (Johnson et al., 2005) and *HMGA2* (Mayr et al., 2007) is high in BT-IC and silenced during differentiation. *RAS* silencing contributes to loss of self renewal but does not affect multipotent differentiation, while silencing *HMGA2* enhances multilineage differentiation but has no effect on self renewal.

RESULTS

Chemotherapy Selectively Enriches for Self-Renewing Breast Cancer Cells

Resistance to chemotherapy distinguishes T-IC from other cancer cells (Al-Hajj, 2007). To examine whether chemotherapy might enrich for BT-IC, we compared the proportion of in vitro self-renewing cancer cells in 1° breast cancers from patients who received neoadjuvant chemotherapy with tumors resected from chemotherapy-naive patients (Table S1A). Freshly isolated cells were cultured in suspension to generate mammospheres, a method for culturing both mammary gland progenitor cells (Dontu et al., 2003) and BT-IC (Ponti et al., 2005). Mammosphere generation is an in vitro assay of self-renewal potential (Ponti et al., 2005). After 15 days, 5.8% ± 2.6% of tumor cells from 5 neoadjuvant chemotherapy patients formed mammospheres as compared with 0.4% ± 0.3% from 8 chemotherapy-naive patients, a 14-fold increase ($p < 0.001$, Figure 1A). Furthermore, 1° mammospheres from chemotherapy patients could be passaged for at least eight to ten generations (endpoint of the study), while those from patients without chemotherapy vanished within two to three generations. In the 1° breast cancers; 74% ± 7% of tumor cells from chemotherapy-treated patients, but only 9% ± 4% of cells from untreated patients, were $CD44^+CD24^{-/low}$, the phenotype ascribed to BT-IC (Al-Hajj et al., 2003; Ponti et al., 2005) ($p < 0.001$, Figure 1B). Enrichment of BT-IC by chemotherapy was confirmed by studying paired specimens from seven patients obtained by biopsy prior to chemotherapy and at surgery following neoadjuvant chemotherapy. Only 0.5% ± 0.3% of tumor cells before chemotherapy, but 5.9% ± 1.7% of cells obtained after chemotherapy, formed mammospheres after 15 days of suspension culture ($p < 0.001$, Figure S1A). Similarly, the proportion of $CD44^+CD24^{-/low}$ cells was 9.5-fold higher in samples after chemotherapy ($p < 0.001$, Table 1B). In another patient group with metastatic pleural effusions who had received chemotherapy 2–6 years before, pleural cancer cells were highly enriched (31% ± 10%) for $CD44^+CD24^{-/low}$ cells (Table S1C). These data from three cohorts suggest that chemotherapy selectively enhances the proportionate survival of BT-IC.

We took advantage of this finding to see if we could enrich for BT-IC by consecutively passaging breast cancer cells in NOD/SCID mice treated with chemotherapy. Mice injected in the mammary fat pad with SKBR3 cells were treated with epirubicin weekly for 10–12 weeks until xenografts reached ~2 cm diameter. Cells from the third passage (SK-3rd) were cultured in suspension to generate mammospheres. The number of mammospheres reflects the quantity of cells capable of in vitro self renewal, while the number of cells/sphere measures the self-renewal capacity of each sphere-generating cell (Dontu et al., 2003; Dontu et al., 2004). SK-3rd formed ~20-fold more spheres than SKBR3 (16.3% versus 0.8%, $p < 0.001$; Figure 1C). Moreover, dissociated SK-3rd cells from 1° mammospheres generated an equivalent proportion of 2° and 3° spheres (Figure 1C), demonstrating their in vitro self-renewing potential. SK-3rd mammosphere cultures could be maintained for >50 passages, while within three to four passages, SKBR3 mammospheres failed to generate 2° spheres, became adherent, and differentiated. These findings were confirmed by single-cell cloning (Figure S2). SK-3rd mammospheres were observed beginning at day 5 and increased in size and cell number until day 15 (Figure 1D). Mammospheres could be passaged >40 times from single-cell SK-3rd clones. However, mammospheres did not appear until day 15 in parental SKBR3 cells and were about 18-fold fewer in number and much smaller (Figure 1D). In addition, freshly isolated SK-3rd formed 4-fold more colonies than SKBR3 in a colony-forming assay that correlates with self renewal (Patrawala et al., 2005) (Figure S3).

Seventy-two percent of freshly isolated SK-3rd cells, compared to 0.5% of SKBR3 cells, were $CD44^+CD24^-lin^-$ (Figure 1E). Moreover, 93% of SK-3rd sphere cells were $CD44^+CD24^-lin^-$. When cells were plated on collagen under differentiating conditions in serum, only 2% remained $CD44^+CD24^-$ by day 10 (Figure 1F). Similarly, 93% of freshly isolated mammospheric cells from SKBR3 were $CD44^+CD24^-lin^-$, while after 10 days of differentiating conditions, <1% maintained this phenotype (Figure 1F). Furthermore, sphere-derived SK-3rd and SKBR3 cells, but not parental SKBR3 cells, highly expressed stem cell-associated OCT4 (Ponti et al., 2005), which declined with in vitro differentiation (Figure S4). Therefore, SK-3rd and SKBR3 mammospheric cells not only have self-renewing and differentiating capability in vitro but also have the phenotype expected of BT-IC. SKBR3 mammosphere cells, however, may be subtly different from SK-3rd mammospheres; although they initially had similar proportions of $CD44^+CD24^-lin^-$ cells, the SKBR3-derived mammospheres lost this phenotype more rapidly—on day 3 of differentiation, 15% of SKBR3 mammospheric cells versus 32% of SK-3rd cells were $CD44^+CD24^-lin^-$,

(G) When SK-3rd spheres are dissociated, removed from growth factors, and plated on collagen for 8 hr (top), they do not express luminal (Muc1 and CK-18) or myoepithelial (CK-14 and α-SMA) differentiation markers, while after further differentiation (bottom), they develop into elongated cells with subpopulations staining for either differentiated subtype.
(H) Freshly isolated SK-3rd cells are enriched for $Hoechst^{low}$ SP cells compared with SKBR3 cells.

Table 1A. Incidence of Tumors and Metastasis by Mammospheric SK-3rd Cells and SKBR3 Cells in NOD/SCID Mice

		2×10^3			2×10^4			2×10^5		
Number of Cells Inoculated		Tumors	Lung Metastasis	Liver Metastasis	Tumors	Lung Metastasis	Liver Metastasis	Tumors	Lung Metastasis	Liver Metastasis
Mammospheric SK-3rd cells	untransduced	8/10	6/10	3/10	10/10	7/10	4/10	10/10	8/10	6/10
	lentivector	8/10	5/10	3/10	10/10	8/10	5/10	10/10	8/10	5/10
	lenti-*let-7*	2/10*	1/10	0/10	5/10*	3/10	1/10	7/10	4/10	3/10
	RAS-shRNA	3/10*	2/10	1/10	7/10	5/10	3/10	10/10	7/10	4/10
parent SKBR3		0/10$^{\$}$	0/10*	0/10	0/10$^{\#}$	0/10$^{\$}$	0/10	3/10$^{\$}$	0/10$^{\#}$	0/10*

*, $p \leq 0.05$; $^{\$}$, $p \leq 0.01$; $^{\#}$, $p \leq 0.001$ compared with untransduced mammospheric SK-3rd cells.

CD24 staining was brighter on differentiated SKBR3 cells, and a larger proportion of SKBR3 cells were $CD44^-$ (Figure 1F). Taken together with the slower growth and smaller size of SKBR3 mammospheres and our inability to passage them as spheres, these data suggest that SKBR3 mammospheres may have undergone a first step toward losing self-renewing capacity.

A key property of T-IC is multipotency. Mammospheric SK-3rd cells were round and did not stain for cytokeratins (CK) or other differentiation markers, such as mucin 1 (MUC1) and α-smooth-muscle actin (α-SMA), even after they adhered to collagen for 8 hr (Figure 1G). However, after further differentiation, they developed into elongated cells with subpopulations staining for either myoepithelial (CK14, α-SMA) or luminal epithelial (CK18, MUC1) markers (Figure 1G). As expected, the differentiated cells also lost self-renewal potential since only 1.5% ± 0.5% of cells maintained under differentiating conditions for 10 days formed spheres, an 11-fold reduction compared to cells grown as spheres.

T-IC are also believed to be resistant to chemotherapy, in part from overexpressing an ATP-binding cassette half-transporter ABCG2 (Patrawala et al., 2005). This property correlates with the ability to expel dyes, defined as a flow cytometry side population (SP). Freshly isolated SK-3rd cells contained 26-fold more SP cells than SKBR3 (15.7% ± 4.2% versus 0.6% ± 0.4%, n = 5, $p < 0.001$; Figure 1H). In line with this, mammospheric SK-3rd, but not differentiated SK-3rd or SKBR3, stained for ABCG2 by immunoblot (Figure S4). Reduced ABCG2 was not a direct consequence of chemotherapy, as a similar reduction was observed in mammospheric SK-3rd and chemotherapy-unexposed mammospheric SKBR3. *ABCG2* mRNA paralleled differences in protein (not shown). Moreover, mammospheric SK-3rd and SKBR3 cells were relatively resistant to epirubicin, compared to differentiated SK-3rd or SKBR3 (not shown).

An important T-IC feature is efficient xenograft formation (Clarke et al., 2006). When 2×10^3 mammospheric SK-3rd cells were inoculated into NOD/SCID mice, eight out of ten mice generated tumors (Table 1A). All animals injected with 10- or 100-fold more cells developed tumors. By contrast, no mice inoculated with 2×10^3 or 2×10^4 SKBR3 cells developed tumors, while tumors developed in only three out of ten animals inoculated with 2×10^5 SKBR3 cells. Therefore, mammospheric SK-3rd cells were at least 100-fold more tumorigenic than SKBR3. Importantly, mammospheric tumor cells from SK-3rd xenografts could be serially passaged into 2° and 3° recipients, while the unselected cells could not (Table S2). The potent tumorigenic capability of mammospheric SK-3rd cells upon serial xenotransplantation suggests in vivo self-renewing capacity (Clarke et al., 2006).

It has been hypothesized that only cancer cells with T-IC properties can initiate metastases (Dalerba et al., 2007). We therefore compared lung and liver metastases of

Table 1B. Incidence of Tumors from 1° Breast Cancer Cells Serially Transplanted in NOD/SCID Mice

		2×10^3 cells			5×10^3 cells		
		1° Tumor Cells	Passage 1	Passage 2	1° Tumor Cells	Passage 1	Passage 2
$lin^-CD44^+CD24^{-/low}$	untransduced	6/8	8/8	8/8	8/8	8/8	8/8
	lentivector	6/8	8/8	8/8	7/8	8/8	8/8
	lenti-*let-7*	2/8*	2/8$^{\$}$	2/8$^{\$}$	4/8$^{\$}$	4/8$^{\$}$	5/8*
lin^- Not$CD44^+CD24^{-/low}$		0/8$^{\#}$	0/8$^{\#}$	0/8$^{\#}$	0/8$^{\#}$	0/8$^{\#}$	0/8$^{\#}$

*, $p < 0.05$; $, $p < 0.01$; #, $p < 0.001$ compared with untransduced $lin^-CD44^+CD24^{-/low}$ cells.
For the initial inoculation, each mouse was inoculated with sorted cells, transduced or not, from a different chemotherapy-naïve patient. The clinical features of these eight patients are described in Table S1A. For subsequent passages, cells were isolated, sorted, and transduced from mice injected with tumor cells from the two patients whose lenti-*let-7* transduced cells established xenografts.

SK-3rd and SKBR3 xenografts. Five weeks after inoculation with 2 × 10^5 mammospheric SK-3rd cells, massive lung metastases were visualized by microscopy in eight out of ten mice, but no mice injected with the same number of SKBR3 developed microscopic lung metastases, analyzed when 1° SKBR3 tumors reached the same size (~2 cm diameter) as SK-3rd xenografts (Table 1A). Similarly, 2 × 10^5 mammospheric SK-3rd cells generated liver micrometastases in six out of ten mice, but SKBR3 cells produced none.

To determine whether chemotherapy is needed to maintain a stable percentage of self-renewing cells, we compared mammospheric SK-3rd-generated tumors further passaged in NOD/SCID mice treated or not with chemotherapy (SK-4th[+] and SK-4th[−], respectively). SK-4th(+) contained an equal percentage of sphere-forming cells as SK-3rd (Table S3 and Figure 1), suggesting that the proportion of BT-IC had already plateaued by the third passage. In contrast, SK-4th(−) cells generated 8-fold fewer spheres, implying that selective pressure from chemotherapy is required to maintain the proportion of self-renewing cells in vivo. When 2 × 10^3 or 2 × 10^4 mammosphere-selected SK-4th(+) or SK-4th(−) cells were injected into NOD/SCID mice, tumors developed in virtually all animals and the majority of these metastasized (Table S3). There was no significant difference in tumor formation or metastasis by mammospheric SK-3rd, SK-4th(+) or SK-4th(−) cells, while unselected SK-4th cells, whether from chemotherapy-exposed mice or not, generally did not generate tumors from this number of cells. Therefore, chemotherapy selects for self-renewing BT-IC and prevents their differentiation in xenografts but is not responsible for BT-IC tumor-forming capacity.

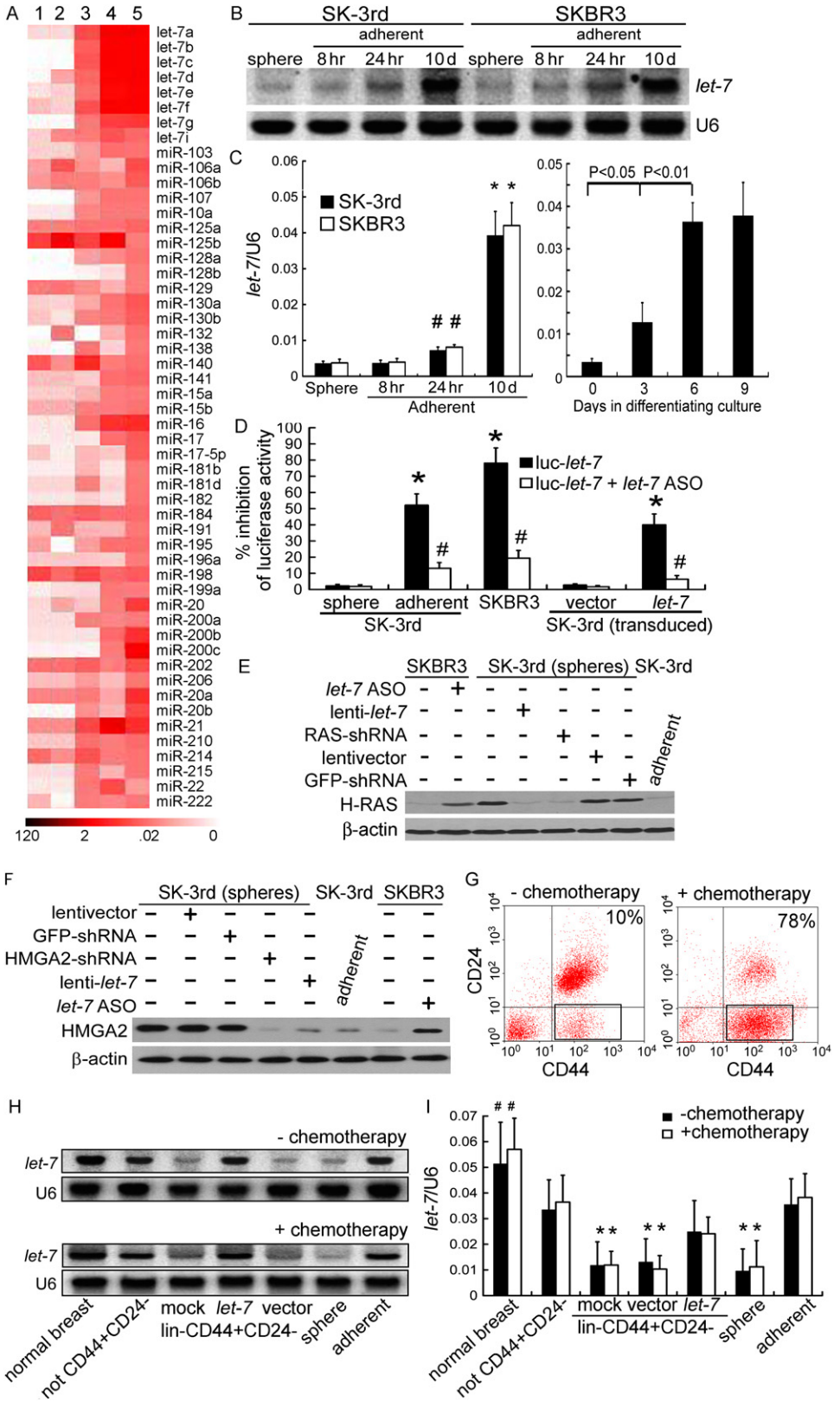
Collectively, these data show that in vivo passaging of breast cancer lines under chemotherapy pressure enriches for BT-IC. At least 16% of SK-3rd cells displayed all the expected properties of T-IC: in vitro stable mammosphere formation, growth under nonadherent conditions, multipotent differentiation, lin-$CD44^+CD24^-$ phenotype and drug-expelling SP, and a high rate of forming tumors capable of serial transplantation as xenografts (Clarke et al., 2006).

Mammospheric Cells Have Reduced *let-7*

Because miRNA help regulate cell differentiation, we took advantage of our ability to obtain large numbers of self-renewing cells to compare miRNA expression in mammospheric SK-3rd with their in vitro differentiated progeny and SKBR3. Most of the 52 miRNAs expressed above background in any of the lines had reduced expression in mammospheric SK-3rd cells either freshly dissociated (Figure 2A, lane 1) or briefly adhered (8 hr, lane 2) compared with cells differentiated under adherent conditions (1 day, lane 3; 10 days, lane 4) or SKBR3 (lane 5). During differentiation most reduced miRNAs gradually increased to the level in SKBR3. Cluster analysis of multiple samples showed a clear distinction between mammospheric versus differentiated cells and SKBR3 (not shown). Using ANOVA analysis on normalized chip data, we identified a number of miRNAs whose expression in mammospheres was significantly different from the differentiated and parent cells. Among them, the *let-7* family emerged as the most consistently and significantly reduced miRNAs. Some other miRNAs, including *miR-16*, *miR-107*, *miR-128a*, and *miR-20b*, showed the same expression pattern as *let-7*. *let-7* was initially identified as a miRNA that regulates *C. elegans* development (Pasquinelli et al., 2000), where it targets key genes including *lin-41*, *hbl*, *daf-12*, *pha-4*, and *let-60*, a *RAS* homolog (Grosshans et al., 2005). There are 11 human *let-7* family members, differentially expressed in different tissues, that are believed to have redundant targets and functions (Johnson et al., 2005). *let-7*, which is downregulated in some human cancers and associated with poor lung cancer prognosis (Takamizawa et al., 2004), targets *RAS* (Johnson et al., 2005) and *HMGA2* (Mayr et al., 2007), which encodes a DNA-binding protein implicated in mesenchymal cell differentiation and tumor formation. This paper focused on *let-7* because it is a known tumor suppressor.

To verify the reduction of *let-7* in mammospheric cells, we performed northern blot using a probe that recognizes all *let-7* homologs (Johnson et al., 2005) (Figure 2B) and qRT-PCR using *let-7a*-specific primers (Figure 2C). By both assays, *let-7* was barely detected in mammospheric SK-3rd, did not change after 8 hr of adherence, but began to increase within 1 day and increased further over 10 days of differentiation. *let-7* also was not expressed in SKBR3 mammospheres and was upregulated upon in vitro differentiation with similar kinetics. Therefore, although we cannot rule out the possibility that chemotherapy exposure alters properties of pre-existing self-renewing cells, reduced *let-7* is not a consequence of chemotherapy or anchorage-independent growth but rather a corollary of self-renewal capacity. After 10 days, *let-7* expression in the differentiated cells increased ~10-fold and was not significantly different from *let-7* expression in SKBR3. Reduction of some other tumor-related miRNAs (*miR-15a*, *miR-16*, *miR-21*) in SK-3rd spheres was also verified by qRT-PCR using specific miRNA primers (Figure S6). *miR-15a* was reduced ~3-fold ($p < 0.05$) and *miR-16* ~4-fold ($p < 0.01$) in mammospheric SK-3rd as compared with SKBR3 or differentiated SK-3rd. *miR-21* was reduced by ~50%, but not significantly ($p \sim 0.05$).

To investigate *let-7* function, we transfected a luciferase reporter containing a *let-7* target 3′UTR sequence into mammospheric or differentiated SK-3rd and SKBR3. Luciferase activity was suppressed by 52% in differentiated SK-3rd ($p < 0.001$) and by 78% in SKBR3 ($p < 0.001$), while there was no suppression in SK-3rd (Figure 2D). Infection of SK-3rd with a lentivirus expressing *let-7a* enhanced miRNA expression and function comparably to that of the differentiated cells (Figures S7 and 2D). Cotransfection of differentiated SK-3rd cells, SKBR3, or lenti-*let-7a*-infected SK-3rd with a *let-7a* antisense oligonucleotide (ASO) significantly reduced the suppression in luciferase activity by endogenous or exogenous *let-7* ($p < 0.01$, Figure 2D). Transfection of *let-7a* ASO in SKBR3 reduced not only *let-7a* but also *let-7b*, *let-7e*, and *let-7i*,


A
1 2 3 4 5
let-7a
let-7b
let-7c
let-7d
let-7e
let-7f
let-7g
let-7i
miR-103
miR-106a
miR-106b
miR-107
miR-10a
miR-125a
miR-125b
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miR-17-5p
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miR-202
miR-206
miR-20a
miR-20b
miR-21
miR-210
miR-214
miR-215
miR-22
miR-222
120 2 .02 0
B
SK-3rd
SKBR3
adherent
sphere 8 hr 24 hr 10 d sphere 8 hr 24 hr 10 d
let-7
U6
C
let-7/U6
SK-3rd
SKBR3
Sphere 8 hr 24 hr 10 d
Adherent
P<0.05 P<0.01
Days in differentiating culture
D
% inhibition of luciferase activity
luc-let-7
luc-let-7 + let-7 ASO
sphere adherent SKBR3 vector let-7
SK-3rd
SK-3rd (transduced)
E
SKBR3
SK-3rd (spheres)
SK-3rd
let-7 ASO
lenti-let-7
RAS-shRNA
lentivector
GFP-shRNA
adherent
H-RAS
β-actin
F
SK-3rd (spheres)
SK-3rd
SKBR3
lentivector
GFP-shRNA
HMGA2-shRNA
lenti-let-7
let-7 ASO
adherent
HMGA2
β-actin
G
- chemotherapy
+ chemotherapy
10%
78%
CD24
CD44
H
- chemotherapy
+ chemotherapy
let-7
U6
normal breast
not CD44+CD24-
mock let-7 vector
lin-CD44+CD24-
sphere
adherent
I
let-7/U6
-chemotherapy
+chemotherapy
normal breast
not CD44+CD24-
mock vector let-7
lin-CD44+CD24-
sphere
adherent

expression by qRT-PCR analyzed using specific primers ($p < 0.001$, Figure S8). Because of the close homology within the *let-7* family, a single ASO may be able to inhibit the entire family or many of its members.

Since *RAS* and *HMGA2* are known *let-7* targets, we compared their expression in the three cell lines. H-RAS and HMGA2 proteins were highly expressed in mammospheric SK-3rd cells but greatly reduced in differentiated SK-3rd cells and SKBR3 (Figures 2E and 2F). (Other RAS proteins were not detected in these cells [not shown].) Expressing lenti-*let-7a* or shRNA against either gene in mammospheric SK-3rd reduced H-RAS and/or HMGA2 to the level in differentiated cells, while *let-7* ASO in SKBR3 upregulated both proteins substantially. However, *H-RAS* and *HMGA2* mRNA, measured by qRT-PCR, did not differ significantly among the three cell lines (not shown). Therefore, *let-7* silences *RAS* and *HMGA2* expression by inhibiting translation as previously reported (Johnson et al., 2005; Mayr et al., 2007), rather than by cleaving mRNA (Lee and Dutta, 2007). Moreover, reduced *let-7* in mammospheric cells leads to RAS and HMGA2 overexpression.

let-7 Is Reduced in BT-IC from Clinical Cancer Specimens

Reduced *let-7* in SK-3rd or SKBR3 mammospheres suggested that *let-7* might be reduced in 1° BT-IC. We therefore examined *let-7* expression by northern blot and qRT-PCR in BT-IC selected from the three groups of patient specimens. BT-IC were enriched by culture as mammospheres or by sorting freshly isolated cells for $lin^-CD44^+CD24^-$ cells (Figure 2G). Both the mammospheres and sorted cells, composed mostly of BT-IC and EPC (Al-Hajj et al., 2003; Ponti et al., 2005), from patients in Table S1A, had reduced *let-7* as compared with either adherent cells or tumor cells that were not $CD44^+CD24^-$ ($p < 0.01$). Figure 2H shows northern blots from a representative patient who received chemotherapy and one who did not, while Figures 2I and S9A depict the mean ± standard deviation (SD) and individual patient data, respectively, of qRT-PCR analysis of *let-7a*/U6 expression. *let-7* expression in normal adjacent breast tissue was ~35% higher than in 1° cancer cells depleted of BT-IC, consistent with a prior report that breast cancers express less *let-7* than normal breast tissue (Iorio et al., 2005). Transduction with lenti-*let-7*, but not with lentivector, enhanced *let-7* expression comparably to that of the 1° cancers depleted of BT-IC ($p > 0.05$).

Although there were more BT-IC in the patients who received chemotherapy (Figures 1A and 1B and Table S1), *let-7* reduction in mammospheres or sorted $lin^-CD44^+CD24^-$ cells was comparable whether or not patients received preoperative chemotherapy. Reduced *let-7* in 1° cancer cells enriched for BT-IC, either by growth as mammospheres or by sorting for BT-IC phenotype, was also confirmed and shown to be independent of chemotherapy exposure or whether the tumors were 1° or metastatic in samples from the other two patient groups (Figures S9B and S9C). Therefore, reduced *let-7* is an intrinsic property of BT-IC/EPC.

Reduced *let-7* Is Required to Maintain Mammospheres

To test whether low *let-7* is important for self renewal, we studied the effect of enforced *let-7a* expression by mammosphere assay. SK-3rd cells infected with lenti-*let-7a* formed 5.3-fold fewer mammospheres than uninfected SK-3rd or SK-3rd cells infected with empty or eGFP-shRNA lentiviruses (Figure 3A). Mammosphere formation was also delayed, and the mammospheres that formed were 2- to 3-fold smaller in *let-7a*-expressing SK-3rd cells than control SK-3rd cells (Figure 3B). Importantly, *let-7a*-transduced mammospheres could only be passaged for eight to ten generations, whereas the untransduced spheres could by passaged for at least 50 generations (>1 year). Therefore *let-7a* weakened self-renewal capacity under nonadherent conditions. Conversely, transfecting *let-7* ASO into parental SKBR3 or differentiated

Figure 2. *let-7* miRNA Is Reduced in Mammospheric SK-3rd Cells and 1° Tumor BT-IC

(A–C) miRNA array analysis shows miRNAs differentially expressed in SK-3rd cells cultured in mammospheres (1) or adhered for 8 hr (2), 24 hr (3), or 10 days (4) and parent SKBR3 (5). Most miRNAs, including all *let-7* homologs, are reduced in SK-3rd cultured in mammospheres or just adhered for 8 hr, and increase during differentiation to similar levels as SKBR3. Northern blot probed for *let-7* (B) and qRT-PCR amplified for *let-7a* (C, mean ± SD relative to *U6*) verify the microarray results. Spheres derived from either SK-3rd or SKBR3 show similar low expression of *let-7* that increases gradually beginning 1 days following induction of differentiation and plateaus within 6 days. #, $p < 0.01$; *, $p < 0.001$ as compared with cells cultured in spheres. Error bars correspond to mean ± SD.

(D) *let-7* function, assayed by luciferase assay, is negligible in SK-3rd, but increases upon differentiation or infection with lenti-*let-7* (*, $p < 0.001$ compared with mammospheric SK-3rd). Transfection with *let-7* ASO reduces endogenous or exogenous *let-7* activity (#, $p < 0.01$ compared to untransfected cells). Error bars correspond to mean ± SD.

(E and F) H-RAS (E) and HMGA2 (F), targets of *let-7*, are highly expressed in mammospheric SK-3rd but not in differentiated adherent SK-3rd or SKBR3 (protein assayed by immunoblot relative to β-actin). Infection with lenti-*let-7* or lentivirus encoding RAS- or HMGA2-shRNA, but not GFP-shRNA or vector, suppresses H-RAS or HMGA2 expression, respectively, in mammospheric SK-3rd cells, while transfection of SKBR3 with *let-7* ASO augments H-RAS and HMGA2 protein.

(G–I) In addition, tumors from eight untreated patients and five patients treated with neoadjuvant chemotherapy were enriched for BT-IC by sorting for $lin^-CD44^+CD24^-$ cells or by growth as mammospheres. Tumors depleted of BT-IC by adherent growth or by excluding $CD44^+CD24^-$ cells also have reduced *let-7* compared to adjacent normal breast tissue. FACS analysis and northern blots probed for *let-7* and *U6* are shown in (G) and (H), respectively, for representative untreated (#7, Table S1) and neoadjuvant chemotherapy treated (#5) patients. Mean ± SD of relative *let-7* expression for all samples analyzed by qRT-PCR (I); data for each patient are in Figure S9A. Infection with lenti-*let-7* increases *let-7* in BT-IC-enriched 1° cells. #, $p < 0.05$; *, $p < 0.01$ compared with samples depleted of $CD44^+CD24^-$ cells.

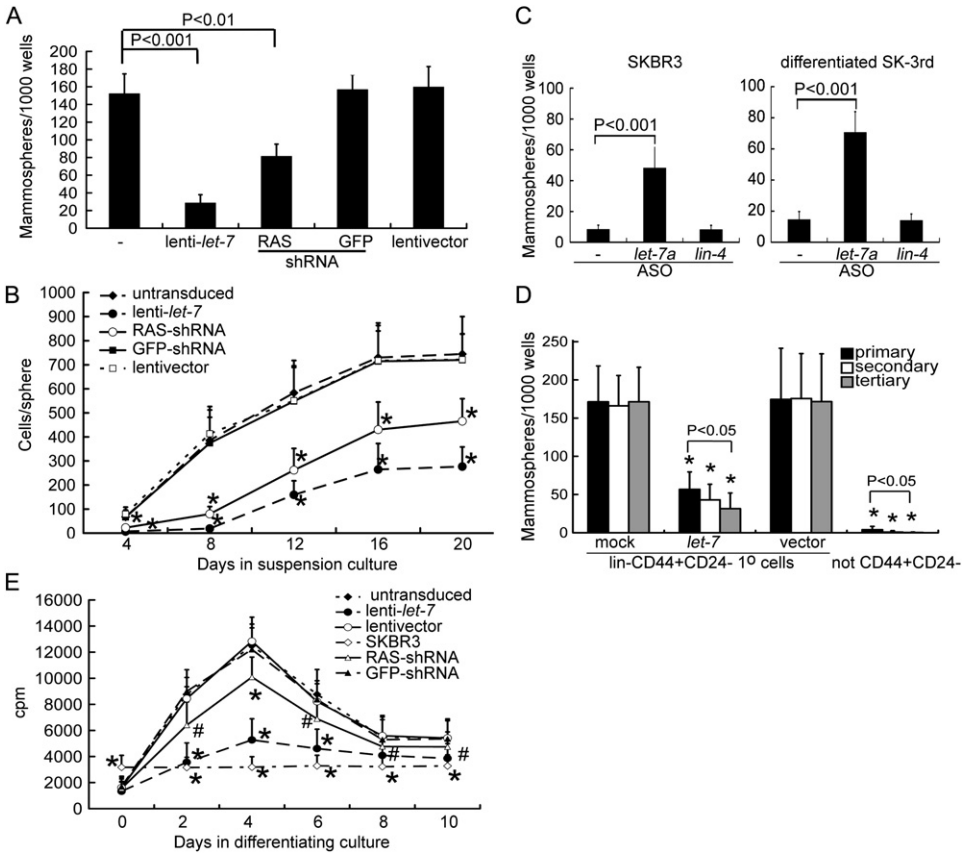

Figure 3. SK-3rd Cells Engineered to Express *let-7a* Lose Ability to Form Mammospheres and Proliferate Less under Differentiating Conditions

(A and B) Single-cell cultures of dissociated SK-3rd cells, infected with lenti-*let-7* or lentivirus-expressing RAS-shRNA but not GFP-shRNA or empty vector, form fewer mammospheres (A), and mammospheres that do form develop more slowly and are reduced in cell number (B) (*, $p < 0.0001$ compared to untransduced cells). Error bars correspond to mean ± SD.

(C) Conversely, SKBR3 and differentiated SK-3rd cells transfected with *let-7a*, but not control *lin-4,* ASO, generate 10-fold more mammospheres. Error bars correspond to mean ± SD.

(D) BT-IC-enriched cells, sorted for $lin^-CD44^+CD24^{-/low}$ phenotype from 1° chemotherapy-naive breast tumors, have a markedly higher capacity to form mammospheres compared with $CD44^+CD24^-$-depleted cells. Transduction with lenti-*let-7*, but not lentivector, reduces mammosphere generation. *, $p < 0.001$ compared with untransduced cells. Mammosphere formation by *let-7*-transduced BT-IC is also significantly reduced on serial passage but is stable in untransduced cells.

(E) SK-3rd cells infected with lenti-*let-7*, and to a lesser extent RAS-shRNA lentivirus, proliferate less during in vitro differentiation than untransduced or control cells as measured by [^{3}H]-incorporation *, $p < 0.01$; #, $p < 0.05$ compared with untransduced SK-3rd.

SK-3rd cells enhanced mammosphere formation ~6-fold (Figure 3C). *let-7* ASO also greatly increased the percentage of SP and $CD44^+CD24^-$ cells in differentiated SK-3rd and SKBR3 (Figure S10).

Enforced *let-7* expression also reduced mammospheres formed by 1° patient BT-IC. When sorted $lin^-CD44^+CD24^-$ cancer cells from chemotherapy-naive patients were infected with lenti-*let-7*, the percentage of 1° mammospheres from single-cell cultures declined >3-fold (Figure 3D, $p < 0.001$). Moreover, although the untransduced $lin^-CD44^+CD24^-$ cells maintained stable sphere-forming capacity upon in vitro passage, the number of 2° and 3° spheres formed by the corresponding *let-7*-expressing cells declined with each passage (Figure 3D, $p < 0.05$).

Although other tumor-related miRNAs (i.e., *miR-15a* and *miR-16*) were reduced in mammospheric SK-3rd (Figures 2A and S6), exogenously expressing these miRNAs to SKBR3 levels did not alter mammosphere formation ($p > 0.05$, Figure S6). Moreover, although BCL-2 has been identified as a target of *miR-15a/16* (Cimmino et al., 2005), transduction of *miR-15a* or *miR-16* in mammospheric SK-3rd did not reduce BCL-2 protein in these cells, suggesting that effective targeting may vary with the cellular context (Figure S6).

Reduced *let-7* Maintains Proliferation but Inhibits Differentiation

Another property of self-renewing cells is the potential to expand under differentiating conditions. Mammospheric

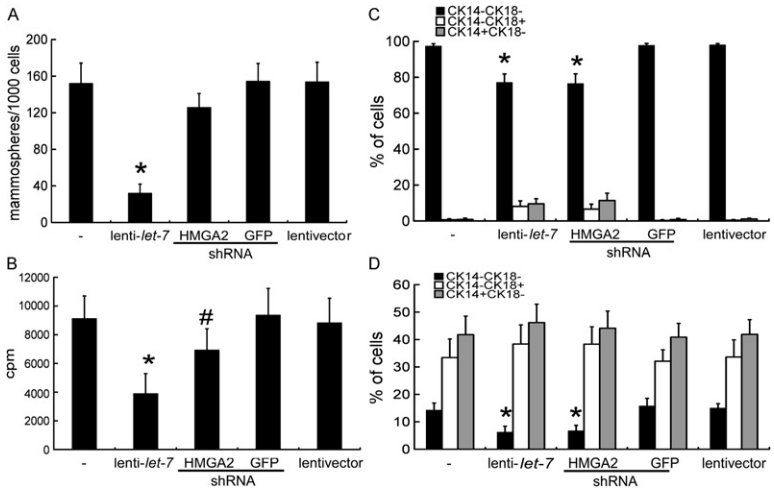

Figure 4. Silencing *HMGA2* Reduces the Undifferentiated Subpopulation and Proliferation of SK-3rd Cells but Does Not Significantly Alter Mammosphere Formation

(A) Single-cell cultures of dissociated SK-3rd cells, infected with lenti-HMGA2-shRNA, form a comparable number of mammospheres as uninfected cells or cells infected with lenti-GFP-shRNA or lentivector. Lenti-*let-7* was used a positive control. *, $p < 0.01$ as compared with untransduced SK-3rd.

(B) Silencing *HMGA2* with lenti-HMGA2-shRNA reduces proliferation of SK-3rd cells on day 4 of in vitro differentiation in adherent cultures (peak of proliferation), but not as much as lenti-*let-7* transduction. Cell proliferation was measured by [^{3}H]-incorporation *, $p < 0.01$; #, $p < 0.05$ compared with untransduced SK-3rd.

(C and D) Transduction with lenti-HMGA2-shRNA or lenti-*let-7*, but not with lenti-GFP-shRNA or vector, similarly reduces the proportion of lin$^-$ cells in SK-3rd cells cultured in mammospheres (C) or under adherent differentiating conditions for 10 days (D). *, $p < 0.01$ compared with vector transduced cells. Error bars correspond to mean ± SD.

SK-3rd proliferated at half the rate of SKBR3 as measured by [^{3}H] incorporation (Figure 3E). Under differentiating conditions, SK-3rd proliferation increased ~7-fold from baseline to a peak on day 4 and then fell by day 8 to a level somewhat higher than that of SKBR3 ($p < 0.01$). When SK-3rd cells were transduced to express *let-7,* peak [^{3}H]-incorporation declined by 58%, demonstrating that *let-7* reduces the proliferative potential of differentiating precursor cells.

Another T-IC hallmark is their undifferentiated state and potential for multilineage differentiation. Mammospheric SK-3rd expressed neither myoepithelial CK14 nor luminal epithelial CK18, while SKBR3 was 70% CK14$^+$CK18$^-$ and 30% CK14$^-$CK18$^+$ (Figures S11A and S11B). After 10 days of differentiation, most SK-3rd cells expressed differentiation markers (44% ± 4% CK14$^+$CK18$^-$, 28% ± 7% CK14$^-$CK18$^+$), but 15% ± 3% remained lin$^-$. *let-7a* overexpression significantly ($p < 0.001$) reduced the proportion of lin$^-$ cells to 78% ± 6% in mammospheric SK-3rd and to 6% ± 2% in differentiated SK-3rd. Control lentiviruses, including a lentivirus expressing RAS-shRNA (see below), had no effect on maintaining the lin$^-$ population under differentiating conditions. Conversely, *let-7* ASO greatly increased the percentage of residual CK14$^-$CK18$^-$ cells in both differentiated SK-3rd and SKBR3 ($p < 0.001$, Figure S11C). Similarly, *let-7* transduction of lin$^-$CD44$^+$CD24$^-$ cells, isolated from patient cancers, reduced the proportion of CK14$^-$CK18$^-$ cells 2-fold ($p < 0.001$, Figure S11D). Therefore, low *let-7* helped maintain the undifferentiated status and proliferative potential of mammospheric cells from a cell line and of 1° tumor BT-IC.

Silencing *RAS* or *HMGA2* Partially Recapitulates the Effect of *let-7*

We next examined whether the effects of reduced *let-7* on promoting self renewal and multilineage differentiation could be attributed to RAS or HMGA2. RAS-shRNA or HMGA2-shRNA reduced H-RAS or HMGA2, respectively, in SK-3rd to the level in SKBR3 or differentiated SK-3rd and comparably to that by *let-7a*-lentivirus (Figures 2E and 2F). SK-3rd with silenced *H-RAS* formed about half as many mammospheres as untransduced or vector-transduced SK-3rd but about three times more than cells infected with lenti-*let-7a* (Figure 3A). Moreover, the mammospheres were intermediate in size (465 ± 94 cells versus 745 ± 155 cells for untransduced SK-3rd and 277 ± 82 cells for *let-7a*-transduced SK-3rd on day 20, Figure 3B). Silencing *RAS* also reduced SK-3rd proliferation under differentiating conditions, but much less than expressing *let-7a* (Figure 3E; $p < 0.001$ on day 4 of differentiation, the peak of proliferation). Silencing *RAS*, unlike overexpressing *let-7a*, in either mammospheric or differentiated SK-3rd did not reduce the proportion of undifferentiated cells lacking CK (Figure S11).

By contrast, silencing *HMGA2* did not alter 2° mammosphere formation by mammospheric SK-3rd cells ($p > 0.05$ versus control vector, Figure 4A), but slightly reduced SK-3rd proliferation ($p < 0.05$, Figure 4B), although not as much as *let-7a* transduction. However, HMGA2-shRNA reduced the proportion of undifferentiated lin$^-$ cells in SK-3rd, grown either as spheres or under adherent conditions ($p < 0.001$, Figures 4C and 4D). Moreover, the reduction in lin$^-$ cells by HMGA2-shRNA in both mammospheric and differentiated SK-3rd was comparable to that mediated by expressing *let-7a* ($p > 0.05$, Figures 4C and 4D). Therefore, reduced *let-7* regulates different aspects of "stemness" by silencing multiple genes. *let-7* inhibits self renewal in part by regulating *RAS*, while it causes BT-IC differentiation by silencing *HMGA2*.

let-7 Expression Inhibits Tumor Formation in NOD/SCID Mice

We next assessed the effect of enforced *let-7* expression on tumor formation. Although eight out of ten mice

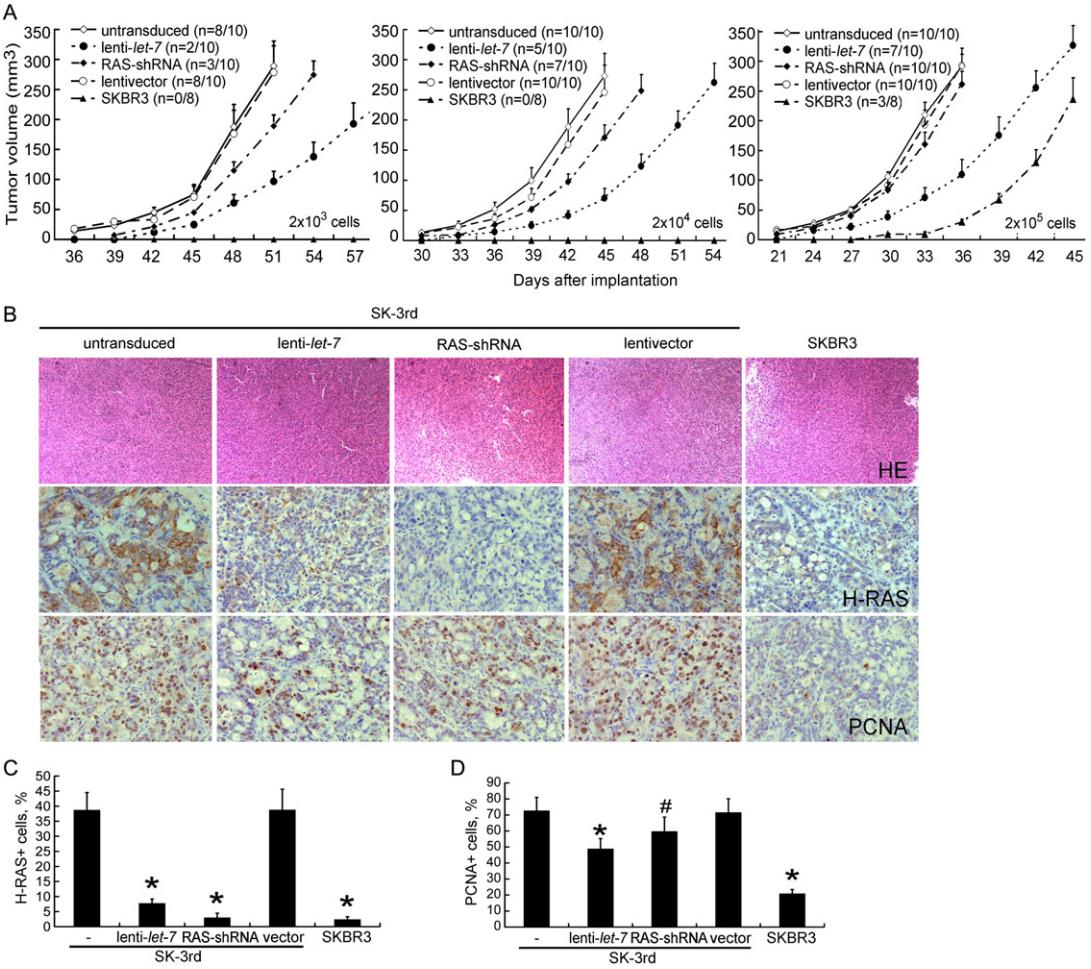

Figure 5. Mammospheric SK-3rd Cells Are More Tumorigenic Than SKBR3 in NOD/SCID Mice, and *let-7* Expression by SK-3rd Cells Suppresses Tumor Outgrowth

(A) Tumor volume was measured after mammary fat pad inoculation of 2×10^3 (left), 2×10^4 (middle) or 2×10^5 (right) SKBR3 cells or mammospheric SK-3rd cells that were untransduced or transduced with vector or to express *let-7* or RAS-shRNA. The number in the legend indicates the number of mice who developed tumors. Mammospheric SK-3rd cells are more tumorigenic than SKBR3 cells. Overexpression of *let-7a*, and to a lesser extent RAS-shRNA, led to fewer tumors, and the tumors that arose grew more slowly. Error bars correspond to mean ± SD.

(B) Tumors that grew in mice inoculated with 2×10^5 cells had similar histology by hematoxylin and eosin staining (HE, magnification 200×), but the SK-3rd tumors, either untransduced or transduced with vector, had higher expression of H-RAS (400×, and C) and a higher proliferative index by PCNA staining (400×, and D), than SKBR3 tumors. Infection of SK-3rd with lenti-*let-7* reduced RAS expression almost as much as silencing *RAS*, but *let-7* more effectively reduced proliferation. #, $p < 0.05$; *, $p < 0.001$ compared with untransduced tumors. Error bars correspond to mean ± SD.

inoculated with 2×10^3 SK-3rd cells and all mice injected with greater numbers of SK-3rd cells formed tumors, significantly fewer tumors developed from *let-7*-expressing mammospheric SK-3rd cells (Table 1A). Moreover, the *let-7a*-expressing tumors grew more slowly than the untransduced or control SK-3rd tumors; the *let-7*+ tumors reached 2 cm in diameter 25–33 days after they became palpable, while the control SK-3rd tumors reached that size in ~12 days (Figure 5A). Similarly, *let-7*-expressing mammospheric cells gave rise to fewer tumors when they were serially passaged in vivo (Table S2), suggesting that *let-7* inhibits self-renewing capacity in vivo, as well as in vitro. RAS-shRNA slightly reduced tumorigenesis, but not as much as *let-7a* (Table 1A and Figure 5A).

The tissue structure and cell morphology of tumors generated from SKBR3, mammospheric SK-3rd or SK-3rd expressing *let-7a* or RAS-shRNA were not grossly different (Figure 5B). However, H-RAS was more highly expressed in mammospheric SK-3rd xenografts than in SKBR3 tumors. Transduction of SK-3rd with lenti-*let-7a* or RAS-shRNA, but not lentivector, significantly reduced H-RAS in the tumors, to the level of SKBR3-derived tumors (Figures 5B and 5C). In keeping with their faster growth, a higher proportion of SK-3rd-derived than

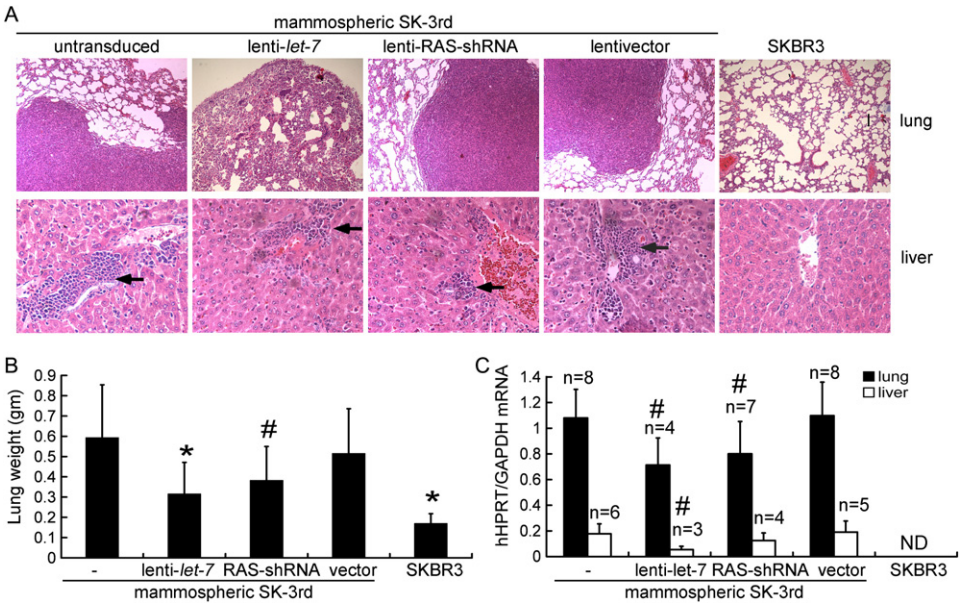

Figure 6. Mammospheric SK-3rd Cells Metastasize to the Lung and Liver (unlike SKBR3), and Infection with Lenti-*let-7* Inhibits Their Metastasis

(A) HE staining of the lung (×200) and liver (×400) of mice implanted with 2×10^5 SK-3rd cells (either untransduced or transduced with lentivector, lenti-*let-7*, or lenti-RAS-shRNA) or SKBR3. Arrows indicate focal metastasis.

(B) Mean ± SD wet lung weight in tumor-bearing mice (n = 10/group).

(C) Expression of *hHPRT* mRNA relative to mouse *GAPDH*, by qRT-PCR. The numbers indicate the number of animals in each group of ten with lung or liver metastasis. The analysis for (B) and (C) excludes animals without metastases to the relevant organ. ND, not detected. #, $p < 0.05$; *, $p < 0.001$ compared with untransduced tumors.

SKBR3-derived tumor cells stained for proliferating cell-associated antigen PCNA (Figures 5B and 5D). Transduction of SK-3rd with *let-7a*-lentivirus also significantly reduced PCNA staining in the xenografts ($p < 0.01$), although not to that of the SKBR3-derived tumor. RAS-shRNA also significantly reduced the PCNA index ($p < 0.05$), but not as effectively as lenti-*let-7*. Thus, lack of *let-7* enhanced SK-3rd cell tumorigenicity, in part by modulating *HRAS*.

let-7 Expression Inhibits Tumorigenesis by $lin^-CD44^+CD24^-$ 1° Breast Cancer Cells

To assess whether *let-7* reduction is also important for tumorigenesis by 1° cancer cells, sorted $lin^-CD44^+CD24^-$ cells from eight chemotherapy-naive patient cancers were evaluated for xenograft formation. Injection of 2×10^3 1° $lin^-CD44^+CD24^-$ cells generated tumors in six out of eight mice, while no tumors developed in mice injected with the cells that were not $CD44^+CD24^-$ from the same patients (Table 1B). Increasing the number of injected $CD44^+CD24^-$ cells to 5×10^3 resulted in tumors in eight out of eight mice but still no tumors in mice that received cells lacking this phenotype. Moreover, when cancer cells passaged in NOD/SCID mice were FACS-sorted again for $CD44^+CD24^-$ cells they could be serially transplanted for two further passages without reduced tumorigenicity (Table 1B). Lenti-*let-7* transduction not only significantly reduced tumorigenicity but also reduced tumor formation upon serial transplantation (Table 1B). Therefore, enforced *let-7* expression in 1° BT-IC interferes with both tumor initiation and in vivo self renewal.

let-7-Expressing Tumors Are Less Likely to Metastasize

We also evaluated whether enforced *let-7* or RAS-shRNA expression in SK-3rd affected metastasis by examining the lungs and livers when xenografts reached ~2 cm in diameter, the same size used to assess metastasis of untransduced xenografts. Lenti-*let-7* infection of SK-3rd reduced both the numbers of mice with lung metastases to two out of ten mice (Table 1A) and the average lung weight by 44% ($p < 0.01$, Figure 6B). Metastases were not only smaller but also dispersed among alveoli (Figure 6A), suggesting reduced clinical severity. The number of lung tumor cells, quantified by qRT-PCR for *hHPRT* in mice with metastases, was also 30% less in animals injected with *let-7a*-expressing SK-3rd compared with mice inoculated with vector-transduced cells ($p < 0.05$, Figure 6C). Expressing RAS-shRNA in SK-3rd modestly, but significantly, reduced lung metastasis, but was less effective than *let-7* (Table 1A and Figure 6).

Similarly in the liver, *let-7* expression or *RAS* silencing reduced the numbers of mice with liver metastasis by 50% and 33%, respectively (Table 1A and Figure 6A). This was confirmed by measuring 72% less *hHPRT* mRNA in the livers of mice inoculated with *let-7*-transduced cells who had micrometastases (three out of ten mice) and

a 30% reduction in mice injected with RAS-shRNA-transduced cells (four out of ten mice), respectively, as compared with those implanted with vector-transduced cells (5/10 mice, $p < 0.01$; Figure 6C). Therefore, *let-7* reduced mammospheric SK-3rd cell metastasis to both lung and liver. This reduction was only partially due to changes in RAS. Reduced metastasis by *let-7*-transduced cells was not due to a difference in 1° tumor size because all tumor-bearing animals were sacrificed when the tumor diameter reached ~2 cm but could result from the slower growth of the 1° tumor or altered metastatic potential of *let-7*-expressing cells. This study cannot distinguish between these possibilities.

DISCUSSION

Evidence for the cancer stem cell hypothesis has grown as T-IC have been identified in an increasing number of malignancies. However, because T-IC are rare within tumors, they are difficult to study, and little is known about what regulates their critical ability to self renew and initiate tumors. We found that breast tumors from chemotherapy-treated patients are highly enriched for cells with the properties of BT-IC. We took advantage of BT-IC chemotherapeutic resistance (Al-Hajj, 2007) to generate a highly malignant breast cancer cell line (SK-3rd) by sequential in vivo passage in epirubicin-treated NOD/SCID mice. SK-3rd is enriched for cells that display all the tentatively defined properties of BT-IC, including enhanced mammosphere formation, multipotent differentiation, chemotherapy resistance, and BT-IC phenotype ($OCT4^+CD44^+CD24^-lin^-$) (Clarke et al., 2006). Based on in vitro mammosphere forming assays and the proportion of SP cells, we estimate that ~16% of SK-3rd cells are T-IC and that the remaining cells, which are also $CD44^+CD24^-$, are largely EPC. Mammospheric SK-3rd cells are ~100-fold more tumorigenic in NOD/SCID mice than the parent line, metastasize, and are capable of serial xenotransplantation. The key properties of mammospheres from SK-3rd coincide with those of BT-IC obtained by sorting $lin\text{-}CD44^+CD24^-$ cells from 1° breast cancers. SK-3rd cells could provide virtually unlimited numbers of cells for studying BT-IC. A similar approach of in vivo chemotherapy might also be used to select T-IC from other breast cancer lines or possibly for other tumors. Because of the potential for accumulating specific idiosyncratic mutations in any line, the relevance of any results obtained with such BT-IC enriched cells would, however, need to be confirmed using sorted 1° cancer cells.

Chemotherapy-exposed patients should provide a means for isolating large numbers of 1° BT-IC to study what regulates stemness. Our finding that neoadjuvant chemotherapy treatment selects for the survival of $CD44^+CD24^-$ BT-IC and EPC, although not surprising in light of their known resistance to chemotherapy, is somewhat disturbing. The selective outgrowth of less differentiated cells may be one reason that neoadjuvant or adjuvant chemotherapy is not always effective at preventing late tumor recurrence.

One contributing factor for chemotherapy selection of BT-IC, whether in neoadjuvant chemotherapy patients or epirubicin-treated xenotransplanted mice, is increased expression by BT-IC of the drug efflux transporter *ABCG2*, which provides BT-IC with a selective survival advantage. Increased *ABCG2* expression in BT-IC supports earlier studies that took advantage of drug efflux to enrich for BT-IC in SP cells (Patrawala et al., 2005). Multiple mechanisms likely influence selective survival under chemotherapy, including preferential activation of DNA repair pathways in T-IC, as was shown for glioma (Bao et al., 2006).

We took advantage of our ability to obtain large numbers of self-renewing cells to study changes in miRNA expression during in vitro differentiation. Mammospheric SK-3rd cells express substantially less miRNA than differentiated SK-3rd or the parent cells. A global reduction of miRNA expression has previously been noted for both ES and cancer cells relative to normal tissue (Shcherbata et al., 2006). We did not investigate the mechanism for the global reduction in miRNAs in BT-IC. One possible contributing factor might be reduced miRNA processing, as has been described in mouse embryonic development (Thomson et al., 2006). Differences in miRNA expression are not an immediate consequence of anchorage-independent growth, since adherence for 8 hr does not substantially alter miRNA expression. Within a day of being placed under differentiating conditions, however, most miRNAs expressed in the parental line are induced, although some take longer to be expressed. Virtually absent expression of *let-7* distinguishes the tumor-initiating SK-3rd cells from their differentiated progeny and the parent line. Moreover, lack of *let-7* is required for self renewal in vitro and tumorigenicity in vivo. Overexpression of *let-7a* in SK-3rd reduces self renewal and proliferative capacity and converts highly malignant and metastasizing T-IC into less malignant cells. Conversely, antagonizing *let-7* with ASO in differentiated SK-3rd or SKBR3 enhances sphere formation. In contrast, overexpressing other oncomirs differentially underexpressed in SK-3rd mammospheres (*miR-15a*, *miR-16*, and *miR-21*) has no effect on in vitro self renewal. Although *let-7* appears to play a major role in regulating stemness, regulated expression of other differentially expressed oncomirs, including those listed above as well as *miR-17* and *miR-222*, will undoubtedly contribute to regulating the distinct pathways required for tumor initiation, transformation, proliferation, invasion, metastasis, apoptosis, and chemo/radiotherapy resistance. This will be a good system to study the role of other oncomirs in breast cancer initiation.

These results with SK-3rd and SKBR3 suggested that reduced *let-7* might regulate the fate of BT-IC more generally. Because cell lines expanded in vitro and/or in vivo might accumulate mutations that might make them different from 1° tumors, we tested the importance of *let-7*

reduction in enriched BT-IC obtained from 1° breast cancers by sorting or mammosphere culture. Regardless of how BT-IC are isolated, 1° BT-IC express substantially less *let-7* than corresponding populations of cells depleted of BT-IC. Moreover, expressing *let-7* in $lin^-CD44^+CD24^-$ cells from 1° breast cancers reduces mammosphere formation and proliferation in vitro and tumor xenograft formation. Because mammospheres and $lin^-CD44^+CD24^-$ cells contain both BT-IC and EPC, we are unable to define the exact point at which *let-7* begins to be expressed. Our results suggest that *let-7* expression begins at a very early stage of differentiation. Reduced *let-7* is an intrinsic property of BT-IC (and possibly EPC) and not a consequence of exposure to chemotherapy or anchorage-independent growth. Both unexposed SKBR3 mammospheres and enriched BT-IC from chemotherapy-naive patients, generated either by sorting or growth under sphere-forming conditions, had similarly reduced *let-7* as comparably derived cells that had been exposed to chemotherapy.

let-7 has been postulated to work as a tumor suppressor by silencing *RAS* (Johnson et al., 2005) and *HMGA2* (Mayr et al., 2007). We confirmed these findings in SK-3rd and SKBR3 cells, where H-RAS (other RAS homologs were not expressed) and HMGA2 protein, but not mRNA, correlated inversely with *let-7* expression. HRAS and HMGA2 were high in SK-3rd but low in differentiated SK-3rd and SKBR3. Moreover, exogenous *let-7a* significantly knocked down H-RAS and HMGA2. Increased H-RAS (Downward, 2003) and HMGA2 (Langelotz et al., 2003) have been reported in breast cancer and correlate with poor prognosis. H-RAS is increased in ~60% of human breast cancers, but mutations are rare (Downward, 2003), suggesting the possibility of posttranscriptional regulation. HMGA2 overexpression in tumors was thought to be primarily secondary to chromosomal translocations that delete the *HMGA2* 3′UTR with its multiple *let-7* recognition sites (Mayr et al., 2007). Our study suggests that HMGA2 overexpression can also be secondary to reduced *let-7* expression.

RAS and HMGA2 each regulate different aspects of stemness. RAS appears to be important for self renewal, since silencing *RAS* reduces mammosphere formation, clonal expansion, and tumorigenicity but has no effect on cell differentiation. HMGA2 on the other hand appears to help maintain multipotency, since silencing *HMGA2* reduces the proportion of undifferentiated cells but does not affect in vitro self renewal. In support of this, HMGA2 is overexpressed in embryos (Rogalla et al., 1996) and poorly differentiated tumors (Shell et al., 2007). *let-7* thus acts as a master regulator of multiple aspects of stemness, presumably by silencing multiple targets, some of which remain to be identified. Other direct *let-7* targets include genes implicated in cell-cycle regulation, including CDC25a, CDK6, and cyclin D (Johnson et al., 2007). Comparing miRNA and mRNA expression by $CD44^+CD24^-$ and non-$CD44^+CD24^-$ cells may help define miRNA networks and additional *let-7* targets in BT-IC. The mRNA comparison was recently reported (Shipitsin et al., 2007).

Because *let-7* regulates multiple oncogenes and more than one T-IC pathway, therapeutic use of *let-7* mimics might be attractive for differentiating resistant T-IC within breast cancer and possibly other tumors. Mimicking *let-7* could be more potent than specifically silencing one or a few oncogenes with siRNA drugs. *let-7* mimics could potentially be used as single agents or combined with conventional chemo/radiotherapy. Since *let-7* is expressed in normal breast tissue and other differentiated cells, introducing it exogenously should not trigger unintended toxicity to noncancerous cells.

A corollary of the cancer stem cell hypothesis is that metastases may also arise from T-IC. In support, we found that cells with the BT-IC phenotype ($lin^-CD44^+CD24^-$) are prevalent in metastatic pleural effusions. Moreover, xenograft metastases are reduced by *let-7* expression in parallel with other BT-IC properties. An alternate hypothesis is that metastatic tumors originate when cells in a 1° tumor undergo an epithelial-mesenchymal transition (EMT) (Mani et al., 2007). Our results suggest that these two seemingly contrary ideas are not mutually exclusive. In fact, *let-7* is poorly expressed in mesenchymal, but highly expressed in epithelial, tumors (Shell et al., 2007). The opposite is true of the *let-7* target *HMGA2*, which is highly expressed in embryos, mesenchymal cells, and mesenchymal tumors (Lee and Dutta, 2007). Therefore, reduced *let-7* may link T-IC and EMT.

let-7 genes map to sites with frequent chromosomal instability in cancer (Calin et al., 2004), and *let-7* is poorly expressed in lung (Takamizawa et al., 2004) and colon cancer (Akao et al., 2006). Downregulation of *let-7* in breast tumors compared to normal breast has been reported in a previous microarray analysis (Iorio et al., 2005). This is confirmed by our present study where *let-7* was reduced by ~35% in 1° cancers compared with adjacent normal breast. However, this difference is small when compared to the ~4-fold reduction in *let-7* in BT-IC compared to BT-IC-depleted 1° cancer cells. T-IC and EPC within tumors might contribute to the moderate reduction of *let-7* in the bulk of breast cancer cells. Measuring *let-7* reduction in breast tumors might serve as a surrogate for the frequency of BT-IC or other poorly differentiated cells in the tumor and provide useful prognostic information about the likelihood of chemotherapy response or relapse. In fact, low *let-7* and high *HMGA2* expression strongly correlates with poor prognosis in advanced ovarian cancer (Shell et al., 2007).

Despite evidence for T-IC in an increasing array of cancers, the cancer stem cell hypothesis remains a hypothesis. Our results need to be considered in light of evidence for possible phenotypic heterogeneity of T-IC, as recently suggested for glioma (Beier et al., 2007) and of the heterogeneity of breast tumor cells that develop after tumor initiation secondary to additional mutations or epigenetic changes (Shipitsin et al., 2007). An alternate possibility to the cancer stem cell hypothesis also needs to be kept

in mind. A recent study suggests that T-IC might be an artifact of xenotransplantation, consisting of the minority of cells capable of surviving with the support of mouse, rather than human, growth factors supplied by the xenogeneic tumor microenvironment. In fact, when mouse lymphoma cells generated in E_{μ}-c-*myc* transgenic mice are transplanted into congenic mice, lymphoma cells lacking stem cell markers also form tumors (Kelly et al., 2007). However, the stronger adaptability of the tentatively defined T-IC to a xenogeneic microenvironment may also be an indicator of their ability to seed congenic tumor sites. It may be that the multipotency of the T-IC provides a means to generate not only the tumor but the supporting niche cells necessary to sustain it, as has recently been shown for human ES cells (Bendall et al., 2007).

EXPERIMENTAL PROCEDURES

Detailed procedures are provided in the Supplemental Data.

1° Tumors

Tumors were obtained from 25 female patients with biopsy-diagnosed breast carcinomas, including 20 cases of 5° cancer and 5 cases of recurrent breast cancer with pleural metastasis (Table S1). Twelve 1° breast cancer patients received neoadjuvant chemotherapy followed by modified radical mastectomy, and eight 1° cancer cases were treated with resection without chemotherapy. Specimens were obtained by ultrasound-guided vacuum-assisted Vacora biopsy (Bard Biopsy System, Tempe, AZ) in seven patients before neoadjuvant chemotherapy. Pleural fluid was obtained by pleural puncture from five cases of recurrent cancer with pleural metastasis. All samples were immediately mechanically disaggregated, digested with collagenase as described (Al-Hajj et al., 2003) and filtered through a 30 μ filter. Tumor cells were sorted after staining with FITC-anti-CD44, PE-anti-CD24 and nontumor cells were depleted with a cocktail of lineage marker antibodies.

Generation of SK-3rd and SK-4th Cell Lines

SKBR3 cells (ATCC) were passaged in NOD/SCID mice by injecting 2×10^6 cells into the mammary fat pad of 5-week-old mice. Epirubicin (8 mg/kg, Pharmacia and Upjohn) was injected into the tail vein weekly. Single-cell suspensions of tumor xenografts, removed when tumors reached ~2 cm in diameter, were isolated as described (Ponti et al., 2005). Dissociated cells were passaged in epirubicin-treated NOD/SCID mice as above.

Mammosphere Culture

Cells (1000 cells/mL) were cultured in suspension in serum-free DMEM-F12 (BioWhittaker), supplemented with B27 (1:50, Invitrogen), 20 ng/mL EGF (BD Biosciences), 0.4% bovine serum albumin (Sigma), and 4 μg/mL insulin (Sigma) (Dontu et al., 2003). To propagate spheres in vitro, spheres were collected by gentle centrifugation, dissociated to single cells as described (Dontu et al., 2003), and then cultured to generate mammospheres of the next generation.

Differentiation

Cells dissociated from spheres were plated at 1×10^5 cells/mL on 6-well plates precoated with Collagen IV (BD BioScience) in DMEM supplemented with 10% FCS without growth factors and passaged when they reached ~95% confluence.

Supplemental Data

Supplemental Data include 11 figures, 3 tables, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at http://www.cell.com/cgi/content/full/131/6/1109/DC1/.

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