Mammary Epithelial-Specific Ablation of the Focal Adhesion Kinase Suppresses Mammary Tumorigenesis by Affecting Mammary Cancer Stem/Progenitor Cells

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Abstract

Focal adhesion kinase (FAK) has been implicated in the development of cancers, including those of the breast. Nevertheless, the molecular and cellular mechanisms by which FAK promotes mammary tumorigenesis *in vivo* are not well understood. Here, we show that targeted deletion of FAK in mouse mammary epithelium significantly suppresses mammary tumorigenesis in a well-characterized breast cancer model. Ablation of FAK leads to the depletion of a subset of bipotent cells in the tumor that express both luminal marker keratin 8/18 and basal marker keratin 5. Using mammary stem/progenitor markers, including aldehyde dehydrogenase, CD24, CD29, and CD61, we further revealed that ablation of FAK reduced the pool of cancer stem/progenitor cells in primary tumors of FAK-targeted mice and impaired their self-renewal and migration *in vitro*. Finally, through transplantation in NOD-SCID mice, we found that cancer stem/progenitor cells isolated from FAK-targeted mice have compromised tumorigenicity and impaired maintenance *in vivo*. Together, these results show a novel function of FAK in maintaining the mammary cancer stem/progenitor cell population and provide a novel mechanism by which FAK may promote breast cancer development and progression. [Cancer Res 2009;69(2):466–74]

Introduction

The mammary gland is a dynamic organ undergoing significant developmental changes during puberty, pregnancy, lactation, and involution. Numerous studies have provided strong evidence for the existence of mammary stem cells (MaSC) capable of self-renewal and differentiation into the basal and luminal lineages comprising the functional mammary epithelium (1, 2). Recently, populations enriched in MaSCs have been isolated from adult virgin mice using cell surface markers CD24 and CD29 or CD49f (3, 4). Further analysis revealed that these populations are basal epithelial cells and are negative for steroid hormone receptor ERα (5). These studies suggest that MaSCs reside in the basal compartment of the mammary epithelium and that integrins may play essential roles in MaSCs. Indeed, a recent study has shown that deletion of β1 integrin in basal mammary epithelial cells (MaEC) significantly impaired the regeneration potential of MaSCs (6).

Breast cancer is a genetically heterogeneous disease that may arise from the malignant transformation of normal MaSCs and/or progenitor cells (7, 8). These stem cell–like cancer-initiating cells or mammary cancer stem cells (MaCSC) share several characteristics of normal MaSCs, including self-renewal and differentiation to produce the cell type heterogeneity in breast cancers. Experimental support for the cancer stem cell hypothesis was first provided by studies in human leukemia by showing that a small population of leukemic stem cells could transfer the disease to the recipient mice in transplantation (9). This concept is extended to the solid tumors by identifying a subpopulation of highly tumorigenic cells with stem cell properties from human breast cancers (10) and, subsequently, in other human cancers (7, 8). Given the critical role of MaCSCs in breast cancer development and progression, the characterization of key signaling proteins and pathways that regulate MaCSC self-renewal and maintenance will be crucial for understanding mammary carcinogenesis, as well as the development of novel treatment strategies targeting the MaCSC pool.

Focal adhesion kinase (FAK) is a cytoplasmic tyrosine kinase that plays a major role in mediating signal transduction by integrins, as well as growth factor receptors, in the regulation of cell adhesion, migration, survival, proliferation, and differentiation in a variety of cells (11, 12). FAK has been implicated in the development of breast cancer and other malignancies (13, 14). In normal human breast tissue, FAK is expressed at low levels whereas noninvasive ductal carcinoma *in situ* (15, 16) and invasive breast cancer (16–18) overexpress FAK. In a large population–based study of breast tumor samples, high FAK expression was shown to be associated with an aggressive phenotype exemplified by a high mitotic index, estrogen and progesterone receptor negativity, and HER-2/neu overexpression (18). FAK expression is required for the early phase of lung metastasis of mammary adenocarcinoma in a rat syngeneic xenograft model (19). Furthermore, intrinsic FAK activity controls orthotopic breast carcinoma metastasis through the regulation of urokinase plasminogen activator expression (20) and promotes a mitogen-activated protein kinase–associated angiogenic switch during breast tumor progression (21). Despite the accumulating evidence in strong support of a role of FAK in breast cancer, the molecular and cellular mechanisms by which FAK promotes mammary tumorigenesis *in vivo* remain to be characterized.

Genetically engineered mouse (GEM) models offer powerful tools to analyze the molecular and cellular mechanisms of breast cancer induction and progression. Numerous GEM models of human breast cancer have been developed (22). Of these, the polyomavirus middle T (PyVT) transgenic model (in which PyVT

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi:10.1158/0008-5472.CAN-08-3078

oncoprotein is driven by MMTV-LTR promoter) has been well characterized (23, 24) and showed many morphologic, histologic, and molecular biomarker similarities to those of human breast cancers that are associated with poor prognosis, including loss of estrogen and progesterone receptors and persistent expression of HER-2/neu and cyclin D1 (24). Several recent studies using different GEM models of breast cancer have strongly suggested that mammary tumorigenesis may originate from the bipotent mammary stem/progenitor cells. For example, in the MMTV-PyVT–induced mammary tumors, high content of stem-like cells labeled by markers of MaSC/progenitor cells, including CD24, CD29, and CD61, has been documented (25), suggesting that MMTV-driven oncogene expression may hit MaSCs and/or progenitor cells and lead to their malignant transformation. In a spontaneous mammary tumor model developed in the conditional *Brac1/p53* knockout mice, expansion of a subpopulation of tumor cells expressing normal MaSC markers ($CD29^{hi}CD24^{med}$) was found to correlate with cisplatin resistance (26). In another study, Zhang and colleagues has shown that a subset of tumor-initiating cells, isolated from a syngeneic p53-null mouse mammary tumor model, resides in the $Lin^{-}CD29^{hi}CD24^{hi}$ population, which closely resembles the population of mammary stem/progenitor cells (27).

To explore the potential role of FAK in MaCSCs, we introduced the MMTV-PyVT breast cancer model into the mice with FAK conditional knockout in the MaECs that were developed previously in our laboratory (28). We found that ablation of FAK leads to a reduced pool of MaCSCs. Furthermore, deletion of FAK significantly affected these cells in both self-renewal and migration *in vitro*. Finally, through transplantation in immunodeficient NOD-SCID mice, we showed that FAK-null MaCSCs exhibited compromised tumorigenicity and impaired maintenance *in vivo*. These observations provide support for the cancer stem cell hypothesis and suggest that targeting critical signaling molecules, such as FAK, in the MaCSCs could potentially be an effective therapy for breast cancer.

Materials and Methods

Mice. Floxed FAK and MFCKO mice have been described previously (28, 29). MMTV-PyVT transgenic mice (23) were obtained from the mouse repository of Mouse Models of Human Cancers Consortium at National Cancer Institute. Mice genotyping for *FAK, Cre*, and *PyVT* alleles were performed, as described previously (23, 29). Mice were palpated every 7 d after weaning, and the size of tumors was measured with a caliper and recorded. Mice were housed and handled according to local, state, and federal regulations, and all experimental procedures were carried out according to the guidelines of the Institutional Animal Care and Use Committee at University of Michigan.

Mammary gland whole mounts, histology, immunohistochemistry, and immunofluorescent labeling. Mammary glands were excised, and whole mounts stained with carmine alum were analyzed, as described previously (28). Mammary tumors or lungs were harvested from mice and subjected to analysis by histology, immunohistochemistry, or immunofluorescent labeling, as described previously (28, 30). The following antibodies were used: FAK (1:200, Santa Cruz Biotechnology), PyVT (1:500, Santa Cruz Biotechnology), keratin 8/18 (CK8/18; 1:400, American Research Products), and keratin 5 (CK5; 1:1,000, Covance). Texas Red and FITC-labeled secondary antibodies (Jackson Labs) were used at a dilution of 1:250. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole/antifade (Invitrogen).

Protein extraction, SDS-PAGE, and Western blotting. Mammary glands or primary tumor samples were harvested during necropsy and snap frozen in liquid nitrogen, grinded with a mortar and pestle, and proteins were extracted using a triple detergent buffer, as described previously (28). They were then subjected to SDS-PAGE and Western blotting analysis, as described previously (28, 30).

Preparation of mammary tumor cells. After 4 to 5 wk of tumor appearance, primary tumors or tumor transplants were removed and dissociated mechanically and enzymatically to obtain single-cell suspension. Briefly, tumor tissues were minced and dissociated in Ham's F12/DMEM (1:1, Invitrogen) supplemented with 10 mmol/L HEPES, 2% bovine serum albumin (BSA; Fraction V, Invitrogen), 5 mg/mL insulin, 0.5 mg/mL hydrocortisone, 10 ng/mL cholera toxin, 300 units/mL collagenase, and 100 units/mL hyaluronidase (all from Sigma) at 37°C for 2 to 4 h. Tumor cells were collected by centrifuging the cell suspension at 100 × *g* for 10 min followed by one wash with F12/DMEM. The resulted tumor cell pellet was further digested for 5 min in 0.05% trypsin/0.025% EDTA (Life Technologies) solution to generate a single-cell suspension. An equal volume of F12/DME-H supplemented with 5% fetal bovine serum was added to stop the digestion. The cell suspension was filtered twice through a 40-μm nylon mesh (BioDesign, Inc.). After centrifugation at 100 × *g*, the pellet was resuspended in F12/DMEM with a reduced calcium concentration (0.06 mmol/L, StemCell Technologies) supplemented with 5 units/mL dispase (Collaborative Biomedical Products). To remove RBC, the pellets were treated with ammonium chloride solution. Tumor cells were prepared from tumors developed in multiple mice and pooled for the following analyses.

Flow cytometry analysis. Freshly isolated tumor cells were subjected to flow cytometry analyses, as described previously (25, 31, 32). Lin^{-} cells were obtained by removing CD45-postive, CD31-positive, and Ter119-positive cells using the EasySep biotin selection kit (StemCell Technologies). PE-conjugated antimouse CD24, FITC-conjugated antimouse CD29, and biotin-conjugated antimouse CD61 were from BD Biosciences, Biolegend, and eBiosciences, respectively. Fluorescence-activated cell sorting (FACS) analysis was performed using a FACStarPLUS (Becton Dickinson) flow cytometer. ALDEFLUOR assay was performed using ALDEFLUOR kit from StemCell Technologies according to manufacturer's protocols.

Mammosphere culture. Mammosphere culture of sorted Aldefluor-positive ($ALDH^{+}$) and Aldefluor-negative ($ALDH^{-}$) tumor cells was performed, as previously described, with minor modifications (33). Single cells were plated in six-well ultralow attachment plates (Corning) at a density of 5,000 cells/mL in primary culture and 2,000 cells/mL in subsequent passages. Cells were grown in a serum-free mammary epithelial basal medium (MEBM; Cambrex) supplemented with B27 (Invitrogen), 20 ng/mL epidermal growth factor (BD Biosciences), antibiotic-antimycotics (1×, Invitrogen), 20 μg/mL gentamycin, 1 ng/mL hydrocortisone, 5 μg/mL insulin, and 100 μmol/L β-mercaptoethanol (Invitrogen) for 7 to 10 d. For counting mammospheres, the content of all wells was collected, pooled, and transferred to a regular 96-well flat-bottomed plate in 100 μL of completed MEBM. Mammospheres settled in these conditions were counted within 30 min under a microscope at low magnification.

Boyden chamber assay. Cell migration assays using modified Boyden chamber (Neuro Probe) were performed as described previously (29).

Tumor cell transplantation. $ALDH^{+}$ and $ALDH^{-}$ tumor cells were suspended in PBS with different concentrations. They were then mixed with Matrigel (BD Biosciences; 1:1) and implanted into the no. 4 inguinal mammary fatpads of NOD-SCID female mice (8 wk, from Jackson Laboratory). To ensure that tumor cells are implanted, the no. 4 inguinal mammary fatpad was first exposed using aseptic surgery procedure, and the tumor cells are then directly injected into the mammary fatpad. Mice with tumor cell injection were examined by palpation every week for 2 mo.

Statistical analysis. Statistical significance was evaluated by paired *t* test, using $P < 0.05$ as indicative of statistical significance. Kaplan-Meier tumor-free survival data were compared using the log-rank test.

Results

MaEC-specific deletion of FAK suppresses mammary tumor formation and progression. We recently generated MaEC-specific FAK conditional knockout mice (designated as MFCKO with $FAK^{f/f}$; MMTV-Cre genotype) by crossing the FAK floxed mice (designated as CNT with $FAK^{f/f}$ genotype) with MMTV-Cre transgenic mice (28).

To test whether deletion of FAK in MaECs could affect breast cancer development in the MFCKO mice, we crossed the MFCKO and CNT mice with the MMTV-PyVT transgenic mice, which is a widely used mouse model that develops metastatic breast cancer induced by the PyVT oncoprotein (23). Three cohorts of female mice with the genotypes $FAK^{f/f}$;MMTV-Cre;MMTV-PyVT (designated as Target mice), $FAK^{f/f}$;MMTV-PyVT (designated as PosCNT mice), and $FAK^{f/+}$;MMTV-Cre;MMTV-PyVT (designated as CreCNT mice) were established (see Supplementary Fig. S1), and mammary tumor development in these mice was monitored by physical palpation. Approximately half of PosCNT and CreCNT mice developed palpable mammary tumors by the age of 11 weeks ($T_{1/2}$ = 11 weeks), and there was no statistical difference between these two groups (Fig. 1*A*). In contrast, Target mice showed a significantly increased tumor-free interval ($T_{1/2}$ = 17 weeks) compared with the PosCNT and CreCNT mice. The average number of tumors per mouse was also decreased for the Target mice compared with PosCNT mice (Fig. 1*B*). Consistent with these results, whole-mount staining of mammary glands from Target and control mice at 5, 6, and 8 weeks of age showed significantly reduced hyperplastic nodules in the Target mice (Fig. 1*C*). By 8 weeks of age, ~50% of the epithelial surfaces were occupied by the hyperplastic nodules in the PosCNT mice, but this ratio was only ~10% for the Target mice.

We next analyzed FAK deletion in the tumors developed in the Target mice. Figure 1*D* shows a significantly diminished expression of FAK in the tumor samples from the Target mice compared with control mice, as expected. In contrast, comparable levels of PyVT oncoprotein expression were found in the Target and control mice, suggesting that the reduced mammary tumor formation is not due to changes in PyVT expression in the Target mice. These results were further verified by immunohistochemical analysis, as FAK expression was only detected in the tumor cells of the control but not the Target mice (Fig. 2*A*). As an internal control, FAK was detected in the blood vessels (*arrows*) of the same tumor sample of Target mice, confirming that FAK is specifically deleted in MaECs, but not endothelial cells. As expected, PyVT oncoprotein was expressed at comparable levels in both samples.

The expression of FAK and several downstream signaling molecules in isolated tumor cells were further analyzed (Fig. 2*B*). Similar to the results in Fig. 1*D*, FAK expression was abolished in the tumor cells from the Target mice, whereas PyVT oncoprotein was expressed at comparable levels in all tumor samples. Furthermore, we did not observe increased expression or phosphorylation of the FAK-related kinase Pyk2, although other reports suggested that Pyk2 may be up-regulated when FAK is inactivated under some conditions (34). Interestingly, expression of cyclin D1 was reduced in the tumor cells from the Target mice, which is consistent with the previous reports showing regulation of cyclin D1 by FAK in fibroblasts (35) and MaECs (28). Tyrosine phosphorylation of p130cas, one of the major downstream targets of FAK in the regulation of cell migration (36, 37), was reduced in the Target tumor cells. Together, these results showed that FAK is efficiently deleted in the mammary cancer cells of Target mice, and this deletion significantly suppresses mammary tumor formation in MMTV-PyVT mouse model.

Given the observed correlation between FAK expression and/or activation with tumor progression and metastasis in other studies (13, 14), we examined whether deletion of FAK in the Target mice could affect tumor growth and lung metastasis. Measurements of the average size of the mammary tumors at weekly intervals for the three cohorts of the mice showed that tumors in the Target mice grew at a slower rate compared with those in the control mice, as measured by either tumor volume or weight (Fig. 2*C*). At 5 weeks after the initial detection of primary tumors, lung metastases were detected in ~60% of the PosCNT and CreCNT mice but not in the Target mice, suggesting that lung metastasis was decreased in the Target mice. By 8 weeks, many Target mice also developed lung metastasis. However, quantitation of the metastatic nodules in lung sections revealed a significant reduction of the number of metastatic nodules in the Target mice compared with the controls (Fig. 2*D*). It should be noted, however, that the reduced tumor growth may contribute, at least in part, to the decreased number of lung colonies observed in the Target mice and that future studies will be necessary to show a direct effect of FAK deletion on suppression of metastasis. Together, these results suggest that deletion of FAK reduces tumor growth and, possibly, also suppresses metastasis *in vivo*.

Ablation of FAK leads to the depletion of a subset of bipotent cells expressing both luminal and basal epithelial markers. One important feature of PyVT-induced mammary tumors is that they have many characteristics similar to those of human breast cancer classified as poorly differentiated invasive ductal carcinoma (23, 24). To determine if ablation of FAK in mammary tumor changes its differentiation status, we stained tumors of CreCNT and Target mice using markers of MaECs, including luminal marker CK8/18 and basal epithelial marker CK5. Both the CreCNT and Target tumors showed strong CK8/18 labeling for the vast majority of tumor cells, with no apparent difference between each genotype observed (Fig. 3*A*, *top*). However, significant differences in CK5 labeling were found in mammary tumors of CreCNT and Target mice. In tumor nodules of early carcinoma stage (Fig. 3*A*, *middle*), we found that CK5 was present mainly at the basal cell lining that surrounds tumor nodules. Interestingly, clusters of CK5-positive cells, probably resulted from cellular proliferation, are frequently found at the periphery of tumor nodules of CreCNT (*left, arrows*) but not of Target mice (*right*). In some tumor samples, basal cells inside the tumor nodules are also found in CreCNT mice (*left, arrow heads*) but not Target mice (*right*). In tumor nodules of CreCNT mice at late carcinoma stage, the CK5-positive cells were widely present and comprised a significant portion of tumor whereas this subset of CK5-positive cells was relatively low in the tumors of Target mice (Fig. 3*A*, *bottom*). These results suggest that targeted deletion of FAK leads to the depletion of basal cells and a more differentiated pattern of luminal cells in mammary tumors. To further characterize this subset of CK5-positive cells, we performed double immunofluorescent labeling using both CK5 and CK8/18 antibodies. As shown in Fig. 3*B*, coexpression of both markers was detected for many of the basal cells in the tumors of CreCNT mice ($\sim 5.05 \pm 1.00\%$; Fig. 3*B-g*, *arrows*). However, in tumors of Target mice, cells with coexpression of both markers were significantly decreased ($\sim 0.31 \pm 0.12\%$; Fig. 3*B-h*, *arrows*), suggesting a role of FAK in maintaining the bipotent progenitor population.

Ablation of FAK reduces the content of MaCSCs. The loss of a bipotent progenitor population in Target mice suggests that deletion of FAK may affect MaCSCs (6). To test this, we first examined the content of MaCSCs in tumors of CreCNT and Target mice using identified markers of MaSC/progenitor, including CD24, CD29 (3), and CD61 (5). In MMTV-PyVT mouse model, the cancer stem–like population has been recently shown as $Lin^{-}CD24^{+}CD29^{+}CD61^{+}$ (25).

Examination of the tumor cells for these markers revealed a significant decrease of the $Lin^-CD24^+CD29^+CD61^+$ subpopulation (gated on viable Lin^-CD24^+ tumor cell population) in the Target mice (32%; Fig. 4*A*, *right*) compared with that from the CreCNT mice (67%; Fig. 4*A*, *left*). We also examined tumor cells isolated from the two types of mice for aldehyde dehydrogenase (ALDH) activity, as the $ALDH^+$ cell population has been shown to be enriched for MaCSCs (31). As shown in Fig. 4*B*, under the similar gating criteria, the $ALDH^+$ population in freshly isolated tumor cells was significantly decreased from 15.5% in CreCNT mice to 4.6% in the Target mice. Together, these studies showed that ablation of FAK in mammary cancer cells reduces the content of MaCSCs.

Ablation of FAK leads to impaired self-renewal and migration of MaCSCs *in vitro*. To investigate the potential role of FAK in the regulation of MaCSCs, we used mammosphere formation assay as a measure for self-renewal of MaCSCs *in vitro* (31, 33, 38, 39). We first isolated $ALDH^+$ and $ALDH^-$ cells from tumors of CreCNT mice and cultivated primary and secondary mammospheres in suspension culture. As shown in Fig. 5*A*, the $ALDH^+$ but not $ALDH^-$ cells, have significantly higher capacity to generate mammospheres at the first and second passages, confirming the nature of $ALDH^+$ cells as the subset of cells possessing stem/progenitor activities. Next, we examined mammosphere formation of $ALDH^+$ cells isolated from tumors of CreCNT and Target mice. As shown in Fig. 5*B*, the $ALDH^+$ cells in Target mice have significantly lower capacity to generate mammospheres than those isolated from CreCNT mice. In addition, the size of mammospheres produced from Target cells is also smaller than that of CreCNT tumor cells (see Supplementary Figs. S3*A* and S3*B*). The reduction in both the number and size of mammospheres derived from Target mice suggests that FAK deletion results in impaired self-renewal of MaCSCs.

In MMTV-PyVT tumor model, MaCSCs, isolated based on markers of CD24, CD29, and CD61, have been shown to have higher migratory activity compared with corresponding non–stem-like cells (25). To evaluate if MaCSCs isolated on ALDH activity have similar property, we sorted $ALDH^+$ and $ALDH^-$ cell populations from freshly isolated tumor cells of CreCNT mice and assessed their migration using a transwell migration assay. Figure 5*C* shows a significantly higher migration for $ALDH^+$ cells compared with unsorted and $ALDH^-$ cells. Such high migratory activity of $ALDH^+$ tumor cells supports ALDH as a marker of MaCSCs in mice. In Fig. 5*D*, we further explored if FAK deletion affects the migration of MaCSCs. We found that the migration of Target $ALDH^+$ cells is decreased by ~70% relative to CreCNT $ALDH^+$ cells. These results suggest an important role of FAK in the regulation of migration of MaCSCs.

Ablation of FAK impairs tumorigenicity and maintenance of MaCSCs *in vivo*. To assess if deletion of FAK affects MaCSCs autonomously, we isolated $ALDH^+$ and $ALDH^-$ tumor cells from CreCNT and Target mice and transplanted them into NOD-SCID recipient mice. We found that $ALDH^+$ tumor cells of CreCNT displayed tumorigenicity at a dilution ranging from 100 to 50,000 cells per injection (Supplementary Table S1). However, for $ALDH^+$ cells of Target tumor sample, only 50,000 cells per injection, but not 5,000 or 500 cells per injection, generated tumors after 2 months of observation, suggesting that ablation of FAK in MaCSCs significantly compromised their tumorigenicity *in vivo*. The capacity of $ALDH^-$ tumor cells to generate tumors in NOD-SCID mice was also tested. Whereas $ALDH^-$ tumor cells isolated from the Target mouse displayed no tumorigenicity for dilution up to 50,000 cells per injection, $ALDH^-$ tumor cells from the CreCNT mouse displayed tumorigenicity at 50,000 and 5,000, but not 1,000, cells per injection. This low tumorigenicity of CreCNT

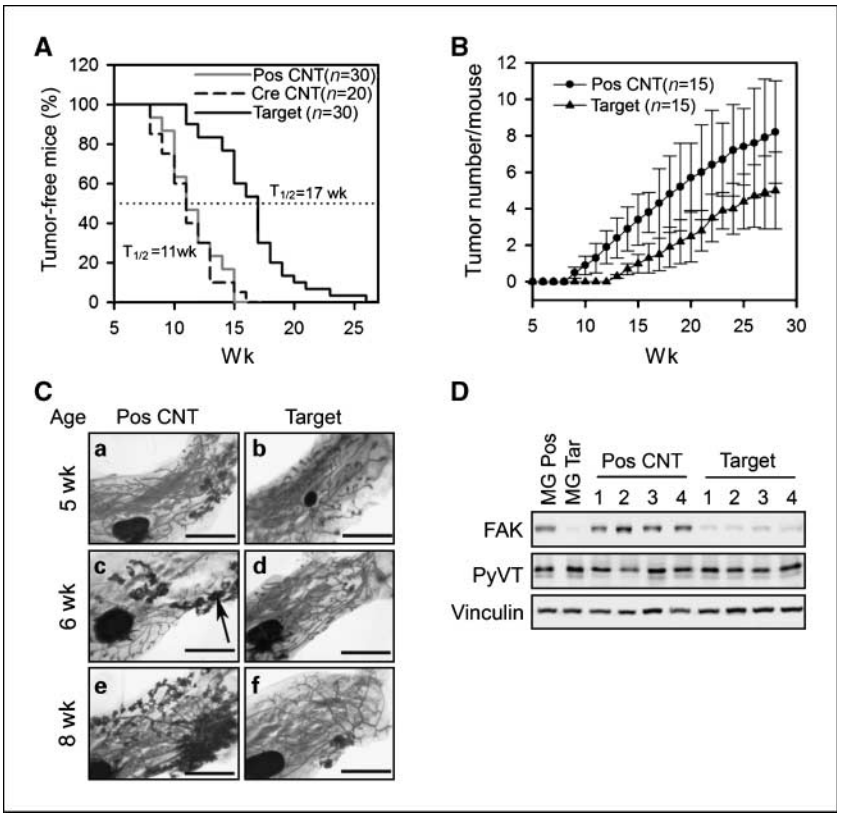

Figure 1. Targeted disruption of FAK in the mammary epithelium suppresses mammary tumor formation. *A,* Kaplan-Meier analysis of mammary tumor development in the PosCNT ($n = 30$), CreCNT ($n = 20$), and Target ($n = 30$) mice. Target versus PosCNT or CreCNT, $P < 0.01$ by the log-rank test. *B,* average number (±SD) of palpable mammary tumors per mouse per genotype at the indicated ages. Target versus PosCNT, $P < 0.05$ by the two-way ANOVA. *C,* representative mammary gland whole mounts from 5-wk-old, 6-wk-old, and 8-wk-old PosCNT (*a, c*, and *e*) and Target (*b, d*, and *f*) mice. The arrow marks hyperplastic nodules. *Scale bar,* 5 mm. *D,* immunoblotting analysis of the lysates from primary tumors of four different (1–4) PosCNT and Target mice, as well as mammary glands from the mice using antibodies against FAK, PyVT, and vinculin.

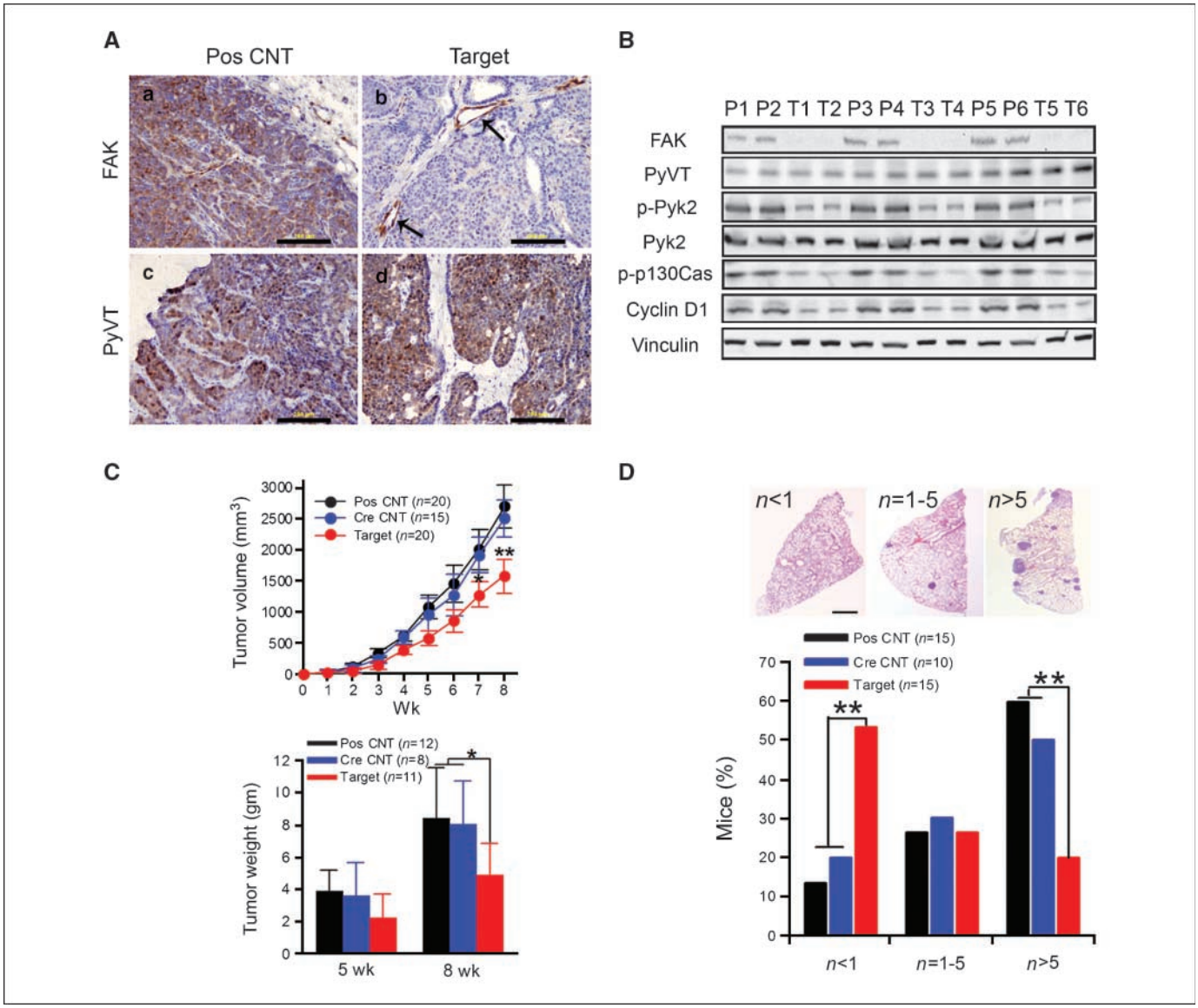

Figure 2. Analyses of protein expression, tumor growth, and metastasis in Target mice. *A,* sections from the primary tumors of the PosCNT (*a, c*) and Target (*b, d*) mice were analyzed by immunohistochemistry using antibodies against FAK (*a, b*) or PyVT (*c, d*). The arrows mark blood vessels in *b. Scale bars,* 200 μm. *B,* lysates of six tumors in three different PosCNT (*P1–P6*) and Target (*T1–T6*) mice were prepared and analyzed by immunoblotting using antibodies against various proteins, as indicated. *C,* mean cumulative mammary tumor volume (±SD; *top*) and weight (±SD; *bottom*) for each genotype at indicated times after primary tumor appearance were plotted and analyzed. *, $P < 0.05$; **, $P < 0.01$, when Target mice is compared with either PosCNT or CreCNT mice. *D,* lung sections (four to six sections per mouse) were prepared at 8 wk after the primary mammary tumor onset and stained with H&E, and the micrometastatic nodules were quantitated under a microscope. *Top,* representative images with <1, 1-5, or >5 metastases per lung section; *bottom,* percentages of mice of the indicated genotype with <1, 1-5, or >5 metastases per lung section. *Scale bar,* 2 mm. **, $P < 0.01$, when Target mice is compared with either PosCNT or CreCNT mice.

$ALDH^-$ cells may be caused by contamination of $ALDH^+$ cells during cell sorting. This possibility is confirmed by FACS analysis of tumor cells from recipient mice with 50,000 $ALDH^-$ cells injected, as 13% of these cells were found to be $ALDH^+$ (Supplementary Fig. S5). We then measured the growth of tumors in the recipient mice. Figure 6*A* shows that 500 $ALDH^+$ tumor cells of CreCNT generated large tumors (*arrow on the right*) whereas 500 $ALDH^+$ cells of Target tumor samples failed to generated palpable tumors at 3 months after injections (*arrow on the left*). Figure 6*B* shows that $ALDH^+$ tumor cells from CreCNT mice at 50,000, 5,000, and 500 per injection all developed palpable tumors earlier than that of 50,000 $ALDH^+$ cells from Target mice. Together, these results confirm that ablation of FAK in MaCSCs leads to increased tumor latency and decreased capacity to recapitulate tumors.

Lastly, to determine if deletion of FAK impairs the maintenance of MaCSCs, we isolated cells from the secondary tumors derived from 500 CreCNT and 50,000 Target $ALDH^+$ tumor cells, respectively, and determined the corresponding MaCSC content. Figure 6*C* shows that the tumor developed in recipient mouse from injection of 500 CreCNT $ALDH^+$ tumor cells contained 37.3% $ALDH^+$ population whereas that from injection of 50,000 Target $ALDH^+$ cells contained only 13.0% $ALDH^+$ cells, suggesting that deletion of FAK in mammary tumors impaired the maintenance of MaCSCs. In a complementary experiment, we also examined the tumors in the recipient mice using CD24, CD29, and CD61 as

markers. Figure 6*D* shows that the $Lin^-CD24^+CD29^+CD61^+$ subpopulation gated on viable Lin^-CD24^+ tumor cells in tumors derived from 50,000 Target $ALDH^+$ cells dropped to ~33% compared with 98% in tumors derived from 500 CreCNT $ALDH^+$ cells. All together, these results support the idea that deletion of FAK impairs the maintenance of MaCSCs, resulting in a reduced MaCSC pool that contributes to the decreased growth and metastasis of mammary tumors in the Target mice.

Discussion

Although previous studies has implicated a role for FAK in breast cancer and other malignancies (11–14), the molecular and cellular mechanisms by which FAK stimulate mammary tumorigenesis *in vivo* are not well understood. Our studies presented here identify a novel function for FAK in the regulation of MaCSCs, providing significant and new insights into the mechanisms of FAK promotion of breast cancer initiation and progression. Our finding that FAK plays a role in the maintenance of MaCSCs is supported by a number of previous observations. Dontu and colleagues have shown that human MaSCs and progenitor cells can form mammospheres in suspension culture and be propagated using this *in vitro* system (33). Because the majority of primary MaECs undergoes apoptosis in suspension (a process termed anoikis), the ability of MaSCs to propagate through mammospheres suggests that they possess the ability to survive and proliferate in an anchorage-independent manner. Interestingly, expression of constitutively active FAK in Madin-Darby canine kidney cells rendered them resistant to anoikis (40), suggesting that activation of FAK in MaCSCs may be important for their self-renewal and maintenance *in vitro* and, possibly, *in vivo*. Our findings are also consistent with the studies that identified CD49f (α_6-integrin; ref. 4) and CD29 (β_1-integrin; ref. 3) as specific markers of MaSCs in mice, and a more recent study demonstrating that ablation of β_1-integrin in the basal compartment affects MaSCs (6).

The most important prediction of cancer stem cell hypothesis is that malignant tissue stem/progenitor cells, such as MaCSCs, are the main culprit of cancers that drive tumorigenicity, recurrence, and metastasis (7, 8). Our results showing the reduced content and impaired maintenance of MaCSCs in the Target mouse, which has significant suppression of mammary tumorigenesis, lend support for this hypothesis. McLean and colleagues have shown that inactivation of FAK in the epidermis significantly suppressed both tumor formation and malignant progression in the skin (41). It would be interesting to determine whether deletion of FAK in the epidermis also affects epidermal stem cells as a mechanism of suppression of tumor formation and progression. While this possibility has not been directly tested, it is worthwhile to note that disruption of FAK in keratinocytes did not affect their survival and proliferation *in vitro* (41). This is in contrast to the findings from us and others that FAK deletion in MaECs significantly decreased proliferation of MaECs and mammary tumor cells both *in vitro* and *in vivo* (28, 42). Thus, it

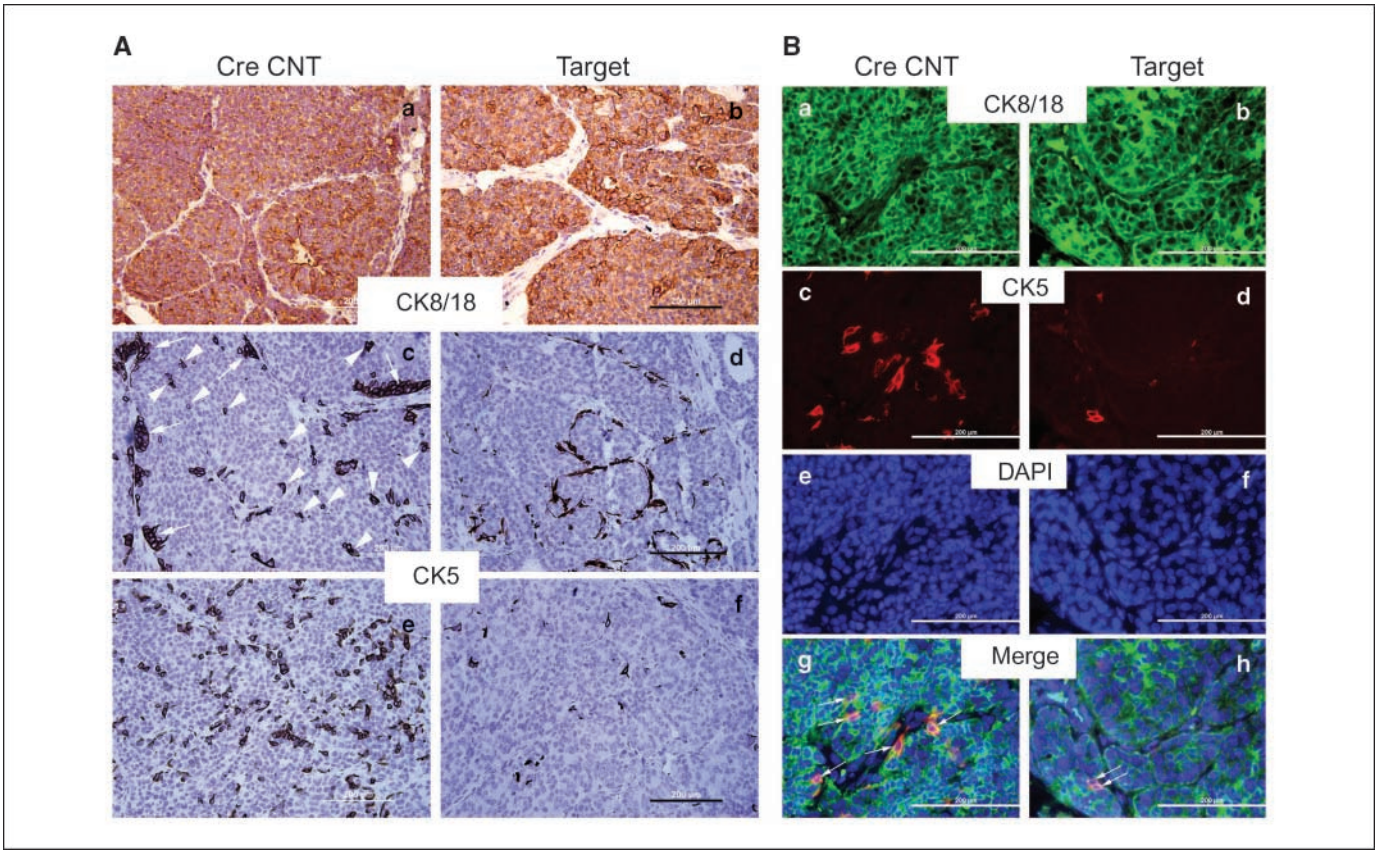

Figure 3. Ablation of FAK depleted a subset of basal cells that coexpress luminal and basal markers in the tumors of Target mice. *A,* sections from the primary tumor of CreCNT (*a, c, e*) and Target (*b, d, f*) mice were analyzed by immunohistochemistry using antibodies against CK8/18 (*a, b*) and CK5 (*c–f*). *c,* arrows mark clusters of basal cells in the periphery of tumor nodules of CreCNT mice in early carcinoma stage and arrowheads mark basal cells present in the tumor nodules. *e* and *f*, sections of tumor of CreCNT and Target mice, respectively, in late carcinoma stage. *Scale bars,* 200 μm. *B,* sections from the primary tumor of CreCNT (*a, c, e, g*) and Target (*b, d, f, h*) mice were analyzed by double labeling immunofluorescence using antibodies against CK8/18 and CK5. *g* and *h,* arrows indicate bipotent cells with coexpression of both markers. *Scale bars,* 200 μm.

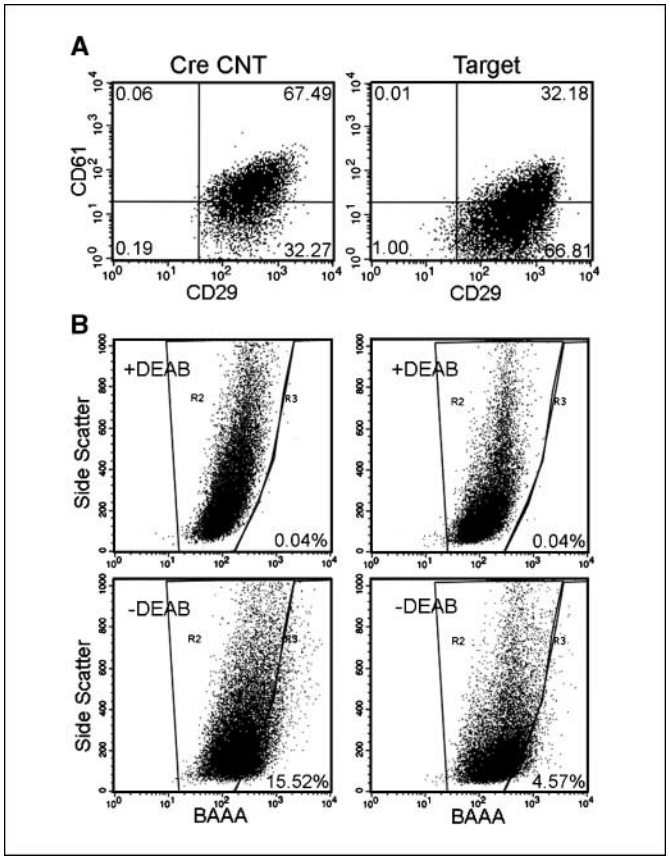

Figure 4. Decreased MaCSC content in the primary tumors of Target mice. *A,* freshly isolated tumor cells from CreCNT (*left*) and Target (*right*) mice were depleted of CD45-positive and CD31-positive cells and labeled with CD24, CD29, and CD61 antibodies. The MaCSC population in each strain of mice was gated as $Lin^-CD24^+CD29^+CD61^+$ (*top right quad*) under the same gating criteria. Results are representative of two separate experiments. In the second experiment, 58.32% and 28.08% Lin^-CD24^+ cells are $CD29^+CD61^+$ for CreCNT and Target tumor cells, respectively. *B,* Aldefluor assay of freshly isolated tumor cells of CreCNT (*left*) and Target (*right*) mice. The percentage of $ALDH^+$ cells was determined under similar gating criteria (resided in gate R3). Results are representative of two separate experiments. In the second experiment, 23.37% and 8.28% tumor cells are $ALDH^+$ for CreCNT and Target tumor cells, respectively (see Supplementary Fig. S2).

remains possible that integrin signaling through FAK may play a preferential role in MaSCs and MaCSCs in breast cancer while affecting the formation and/or progression of cancers through other mechanisms in the skin and other tissues.

Lahlou and colleagues reported very recently that deletion of FAK in MaECs blocked breast cancer formation and progression (42). The conclusion drawn from this paper is in agreement with our studies. However, it is important to note that the conclusion was reached from a different and, perhaps, complementary set of data using independent mouse models. Lahlou and colleagues observed only a slight decrease in the tumor development and no difference in the metastasis in the FAK conditional knockout mice. However, they showed that all malignant primary tumors and metastatic nodules in the FAK conditional knockout mice expressed FAK, which were derived from those cells in which FAK was not deleted (called escapees) due to the estimated 65% deletion efficiency in their model. These observations led the authors to conclude that both initial formation and metastasis of breast tumor require FAK. In contrast, we obtained almost 100% deletion of FAK in both luminal and basal epithelial cells in our mouse models (Supplementary Fig. S6). These models allowed us to show more directly a role for FAK in mammary tumorigenesis in the Target mice. Furthermore, the absence of FAK in the primary tumors in the Target mice allowed us to further investigate the molecular and cellular mechanisms involved, which were not possible in the model of Lahlou and colleagues because all tumors were derived from the escapees that expressed FAK (42).

Our results showing a role of FAK in maintaining MaCSCs raise the possibility that integrins or growth factor receptor tyrosine kinases, the upstream activators of FAK, may also be involved in breast cancer through their regulation of MaCSCs. Indeed, previous studies have shown that blocking antibodies to β1-integrin prevented the malignant phenotype of human breast carcinoma cells *in vitro* (43), and ablation of β1-integrin abolished the development and progression of breast cancer in the MMTV-PyVT mouse model (44). A recent study has revealed that ablation of β1-integrin in the basal compartment of mammary epithelium impaired the regenerative potential of MaSCs (6), suggesting that a role of β1-integrin in breast cancer could be explained by its function in maintaining MaCSCs. In addition to β1-integrin,

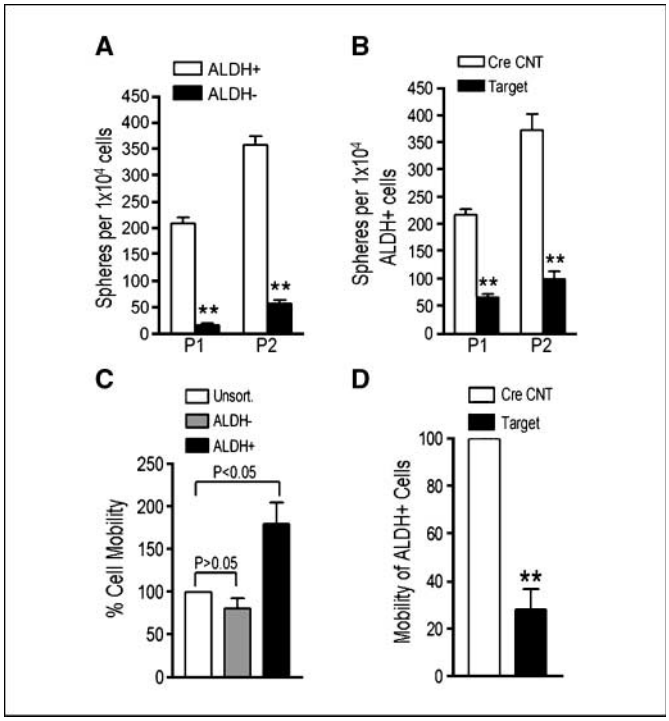

Figure 5. MaCSCs isolated from the primary tumors of the Target mice have reduced capacity for self-renewal and migration *in vitro. A,* freshly isolated tumor cells of a CreCNT mouse were sorted for $ALDH^+$ and $ALDH^-$ cells and analyzed for primary (*P1*) and secondary (*P2*) mammosphere formation under suspension culture conditions. Results are generated from eight separated incubations for each sample and are representative of two independent experiments. **, $P < 0.001$ compared with values for $ALDH^+$ cells. *B,* $ALDH^+$ tumor cells of CreCNT and Target mice were analyzed for primary (*P1*) and secondary (*P2*) formation of mammospheres. Results are generated from six separated incubations for each sample and are representative of two independent experiments. Data for a separate experiment, as well as images of mammospheres, are shown in Supplementary Fig. S3C. **, $P < 0.001$ compared with values for cells isolated from CreCNT mice. *Bar,* 200 μm. *C,* unsorted, $ALDH^-$, and $ALDH^+$ tumor cells of CreCNT mice were analyzed using Boyden chamber assay for their migratory capacity. Results are drawn from three independent experiments. *D,* $ALDH^+$ tumor cells of CreCNT and Target mice were analyzed in Boyden chamber assay to compare their migratory capacity. Results are drawn from three independent experiments. **, $P < 0.001$ compared with values for cells isolated from CreCNT mice. Representative images of migrated cells are shown in Supplementary Fig. S4.

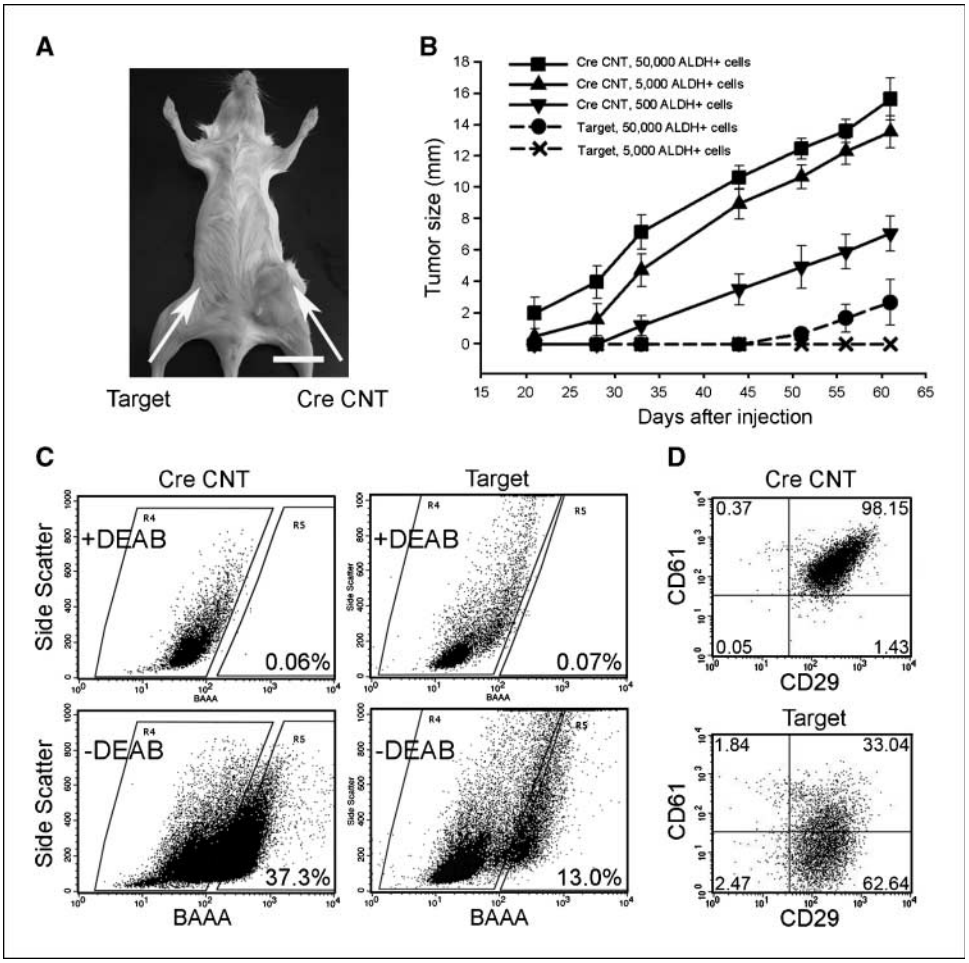

Figure 6. MaCSCs isolated from the Target mice have reduced tumorigenicity and impaired maintenance in NOD-SCID mice after transplantation. *A,* tumor grown in a NOD-SCID mouse from 500 CreCNT ALDH$^+$ tumor cells after 3 mo of observation (*arrow on the right*) whereas 500 Target ALDH$^+$ tumor cells injected in the no. 4 inguinal mammary fatpad (*arrow on the left*) failed to generate tumor at the same time point. *Bar,* 1 cm. *B,* tumor growth curves resulted from ALDH$^+$ tumor cells of CreCNT and Target mice at different dilutions. *C,* Aldefluor assay of freshly isolated tumor cells from secondary tumors (at 1 mo after their appearance) developed from 500 CreCNT (*left*) and 50,000 Target ALDH$^+$ tumor cells (*right*). The percentage of ALDH$^+$ cells for each tumor transplant was determined under similar gating criteria. Results are representative of two separate experiments. *D,* tumor cells dissociated from secondary tumors derived from 500 CreCNT (*top*) or 50,000 Target ALDH$^+$ tumor cells (*bottom*) were depleted of CD45-positive and CD31-positive cells and labeled with CD24, CD29, and CD61 antibodies. The MaCSC population in each tumor transplant was gated as Lin$^-$CD24$^+$CD29$^+$CD61$^+$ under the same gating criteria. Results are representative of two separate experiments.

β4-integrin and its downstream signaling pathways have been shown to promote mammary tumorigenesis through transactivation of HER2/neu signaling (45). Interestingly, HER2 has recently been shown to regulates the MaCSC population to drive tumorigenesis and invasion (46). FAK has not been found as a major mediator of β4-integrin signaling, and additional studies will be necessary to clarify whether β4-integrin might also influence breast cancer development through its effects on MaSCs, either directly or indirectly, via the HER2/neu receptor tyrosine kinase. In this regard, it is interesting to note that expansion of MaSCs was observed in the MMTV-Wnt1, but not the MMTV-HER2/neu mice, in a recent report (3). Whereas a number of markers have been identified for MaSCs and MaCSCs, it is not known whether these markers function to promote self-renewal of the stem cells (i.e., as a functional marker). It is possible (and even likely) that β1-integrin (or other integrins that also activate FAK) would serve as such a functional marker. The integrin-FAK signaling pathway may play an essential role in mediating regulation of MaSCs and MaCSCs by the mammary stroma and the tumor microenvironments, respectively, which may provide the niches crucial for the self renewal of the stem cells.

We noted that MaCSCs isolated from primary tumors of Target mice have severely impaired tumorigenicity in NOD-SCID mice compared with those isolated from primary tumors of CreCNT mice. However, deletion of FAK, although substantially inhibited, did not completely eliminate tumor regeneration potential of MaCSCs. It is likely that, although it is important, integrin-FAK signaling is only one of the contributing pathways in the regulation of MaSCs and MaCSCs in breast cancer. Several developmental signaling pathways, including Notch, Wnt, and hedgehog signaling, have been shown to play critical roles in the regulation of various stem cells (47–50). Abnormal functions and regulations of components of these signaling pathways are often associated with different cancers, implicating potential roles of these signaling pathways in the cancer stem cells derived from different tissue origin (7, 8). It would be interesting to determine the relative contributions and potential cross-talks of integrin-FAK signaling with other signaling pathways. These studies also suggest that the use of a combination of inhibitors for multiple signaling pathways might be more effective than blockade of single pathway regulating MaCSCs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 8/8/2008; revised 9/24/2008; accepted 10/8/2008.

Grant support: NIH grant GM48050 and University of Michigan Comprehensive Cancer Center Weatherwax Foundation grant (J-L. Guan).

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We thank the University of Michigan Cancer Center Flow Cytometry core and C. Bian for technical assistance and Drs. Steve Weiss, Yang Liu, Christophe Ginestier, and members of Guan laboratory for the critical reading of the manuscript and helpful comments.

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Mammary Epithelial-Specific Ablation of the Focal Adhesion Kinase Suppresses Mammary Tumorigenesis by Affecting Mammary Cancer Stem/Progenitor Cells

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Cancer Res 2009;69:466-474.