Transient SNAIL1 Expression is Necessary for Metastatic Competence in Breast Cancer

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Conflicts of Interest: The authors declared no conflicts of interest related to this work.
ABSTRACT

SNAIL1 has been suggested to regulate breast cancer metastasis based on analyses of human breast tumor transcriptomes and experiments using cancer cell lines and xenografts. However, in vivo genetic experimental support for a role for SNAIL1 in breast cancer metastasis that develops in an immunocompetent tumor microenvironment has not been determined. To address this question, we created a genetic SNAIL1 model by coupling an endogenous SNAIL1 reporter with an inducible SNAIL1 transgene. Using multiple genetic models of breast cancer, we demonstrated that endogenous SNAIL1 expression was restricted to primary tumors that ultimately disseminate. SNAIL1 gene deletion either during the premalignant phase or after primary tumors have reached a palpable size blunted metastasis, indicating that late metastasis was the main driver of metastasis and that this was dependent on SNAIL1. Importantly, SNAIL1 expression during breast cancer metastasis was transient and forced transient, but not continuous, SNAIL1 expression in breast tumors was sufficient to increase metastasis.

Running Title

SNAIL1 is required and sufficient for metastasis.

Key words: SNAIL1; metastasis; EMT.
INTRODUCTION

The transcription repressor SNAIL1 has been shown to be one of the earliest Epithelial-Mesenchymal Transition (EMT) regulators during development [1], critical for cancer EMT [1], and its expression in primary human breast tumors strongly associated with metastasis [2-4]. Despite these observations, the role of SNAIL1-initiated EMT during tumor metastasis remains contentious [5, 6]. In humans, metastatic cells have been isolated from the periphery and in most instances they appear epithelial, not mesenchymal [7, 8]. These results have been used to argue against a significant role for EMT in tumor metastasis. However, most available isolation methods are biased toward epithelial cells as they often target epithelial markers (e.g. EpCAM) to capture circulating tumor cells (CTCs). In addition, metastatic cells isolated from the periphery may not reflect their cellular status in primary tumors where the initiation of invasion leading to metastasis occurs. In fact, cancer EMT is thought to be a transient process [9, 10]. In recent studies, transient, not continuous, TGFβ stimulation can result in reversible induction of SNAIL1 and EMT [4, 7]. Similarly, in human breast cancer SNAIL1 expression in primary tumors correlates with overt metastasis, whereas its expression in bone marrow disseminated tumor cells (BM DTCs) does not [4].

To date, most SNAIL1 animal models have utilized cancer cell lines induced to express SNAIL1 before being injected intravenously, resulting in more lung metastases [11, 12]. While these suggest that SNAIL1 contributes to extravasation, colonization, or reactivation of growth of DTCs, they do not address whether SNAIL1 is required in primary tumors to initiate invasion and intravasation. Other approaches employed tumor xenografts that overexpress SNAIL1, leading to increased metastasis [4, 7]. However
xenograft models do not account for the critical interaction between a tumor and its microenvironment and immune system. Without genetic evidence, what have remained unclear are whether: 1) SNAIL1 expression in primary tumors that develop *de novo* in immunocompetent hosts is necessary and sufficient to promote tumor cell invasion and metastasis; and 2) SNAIL1 downregulation in metastatic tumor cells after initial invasion is required for DTCs to form overt metastases.

Here we describe the generation of a mouse model harboring an endogenous SNAIL1 reporter coupled with a tetracycline (tet)-inducible SNAIL1 transgene. By crossing these mice and previously characterized floxed SNAIL1 mice [13] with multiple genetic breast cancer mouse models, we demonstrate that conditional deletion of the SNAIL1 gene in primary breast tumors resulted in a significant reduction in both DTCs and lung metastases, indicating that breast cancer metastasis is highly dependent on SNAIL1. In addition, SNAIL1 expression during breast cancer metastasis was transient. Forced, continuous overexpression of SNAIL1 starting in primary tumors led to a decrease in lung metastasis despite an increase in DTC number. In contrast, transient overexpression of SNAIL1 increased both DTC number and lung metastasis.

**MATERIALS AND METHODS**

**Generation of SNAIL1-CBR mice**

B6/129/SvJ hybrid XY ES cells were electroporated with the targeting construct, and 200 G418-resistant clones screened by PCR to obtain four recombinants. Two recombinants were transiently transfected with CRE, and 100 ganciclovir-insensitive
clones screened by PCR to obtain five CRE-excised recombinants. Two recombinants were laser-injected into 8 cell-staged blastomeres, producing 100% F1 XY mice [14], which were crossed with wild-type females to produce the SNAIL1-CBR line.

**Antibodies and Reagents**

The following antibodies were used: rabbit anti-SNAIL1 (Cell Signaling); mouse anti-E-cadherin (BD Biosciences); mouse anti-Vimentin (Cell Signaling); mouse pan-keratin (Cell Signaling); mouse anti-NeuNT (Fisher); mouse anti-CD45 (Cell Signaling); mouse anti-FLAG (Sigma); mouse anti-actin (Sigma); all secondary antibodies were from Jackson ImmunoResearch. The following reagents were used: neutral buffered formalin (Fisher); hematoxylin (Sigma); eosin (Fisher); carmine alum buffer (StemCell Technologies); RBC lysis buffer (Sigma); HistoMouse Broad Spectrum Kit (Invitrogen); DAPI (Sigma); donkey serum and Fab fragment for blocking for IF in DTCs (Jackson ImmunoResearch); Feket’s solution (Fisher); Indian ink (Fisher); doxycycline (Sigma); CCl₄ (Sigma); d-luciferin (Sigma).

**Histological analyses**

Breast whole mount was performed with carmine alum as previously described [15]. Picrosirius stain of liver sections was performed as previously described [16]. Immunohistochemistry (IHC) was performed per the manufacturer’s instructions (Invitrogen). For immunofluorescent staining (IF) of DTCs, total lung cells were obtained by dissociating a section of lung without visible metastases with collagenase and BM cells isolated from both femurs and tibias and depleted of red blood cells, then adhered
to poly-L-Lysine treated slides, fixed, permeabilized, and stained with indicated antibodies per standard protocol.

**Lung metastasis analysis by Indian ink stain**

Lungs were removed and inflated with 2 ml of 15% Indian ink by intra-tracheal injection. Ink-injected lungs were washed with Feket's solution and then bleached overnight with fresh Fuket's solution [17]. The numbers of surface lung metastases were quantified using a magnifying glass.

**BM DTC enrichment and Her2Neu FACS analyses**

Bm DTCs were enriched from total erythrocyte-depleted BM cells by CD45 negative selection using CD45 MicroBeads (Miltenyi Biotec) and an autoMACS Separator per the manufacturer's instructions. Both CD45⁺ and unlabeled CD45⁻ fractions were collected, fixed with 0.01% formaldehyde, permeabilized with 0.1% Triton X-100, stained anti-Her2Neu mAb and fluorescently labeled secondary antibody, and analyzed using a standard FACS protocol.

**Spatiotemporal analyses of SNAIL1 and EMT phenotypes**

For primary tumors, individual tumors were obtained from independent mice and sections stained for SNAIL1, E-cadherin and Vimentin by IHC and the percentage of tumors that had detectable SNAIL1 expression determined. For BM and lung DTCs, spreads of pooled whole BM cells or pooled dissociated whole lung cells from the same mice were coimmunostained for Her2Neu and SNAIL1, E-cadherin or Vimentin. The
percentage of cells that was positive for SNAIL1 in 150 Her2Neu-positive DTCs was determined. For lung metastases, lung sections from the same mice were stained for SNAIL1, E-cadherin and Vimentin by IHC and the percentage of metastases that were positive for SNAIL1 in 150 individual metastatic foci determined.

**Statistical Analysis**

Mean values were compared using a Student’s unpaired, two-tailed t-test.

**RESULTS**

**Creation and characterization of a coupled endogenous SNAIL1 reporter and inducible SNAIL1 mouse (SNAIL1-CBR)**

To determine the spatiotemporal expression and role for SNAIL1 during cancer metastasis *in vivo* we created a multifunctional SNAIL1 mouse model: SNAIL1-CBR mouse (Figs. 1A and S1A-B). To generate a SNAIL1 reporter that would allow for *in vivo*, real time detection of endogenous SNAIL1 expression, we introduced a cDNA encoding a clic beetle red luciferase (CBR) in frame with the third exon of the endogenous SNAIL1 locus. In addition, we inserted a tet-inducible SNAIL1 transgene (TRE-SNAIL1-3FLAG) 3’ to the SNAIL1-CBR gene so as to be able to inducibly overexpress SNAIL1 *in vivo* in a tissue specific manner. Both fusion constructs SNAIL1-CBR and SNAIL1-3Flag retain SNAIL1’s function as an EMT-inducing factor as measured by their ability to downregulate E-cadherin, upregulate Vimentin, and induce classical morphological changes of EMT [18, 19] (Fig. S1C-D).
To determine whether SNAIL1-CBR expression was properly regulated in cells, we determined protein and mRNA expression of SNAIL1 and SNAIL1-CBR in the targeted ES cells induced to differentiate by removing the leukemia-inhibitory factor (LIF) from culturing media. Consistent with previous reports [20, 21], expression of both wild-type SNAIL1 and SNAIL1-CBR was detected 3 days after LIF withdrawal (Fig. 1C). This indicated that the introduction of CBR did not alter the transcriptional regulation of the SNAIL1 locus. To determine the inducibility of the TRE-SNAIL1-3FLAG transgene in cells, we introduced into targeted ES cells a tet-on DNA transactivator (rtTA). In the resultant cells, SNAIL1-3Flag protein and mRNA were detected only in the presence of doxycycline (dox) (Fig. 1D).

SNAIL1-CBR heterozygous mice were fertile and phenotypically indistinguishable from wild-type mice. A survey of organs from these mice revealed low bioluminescence signals in mammary glands and most internal organs (Fig. S1E-F), possibly due to physiologic expression of SNAIL1 in activated resident fibroblasts [13]. To demonstrate the utility and reliability of the SNAIL1-CBR reporter in vivo, we measured tissue-specific bioluminescence intensity during both physiologic and pathologic EMT processes known to be SNAIL1-dependent. Placental development is a robust EMT process and SNAIL1 has been implicated in trophoblast differentiation and uterine invasion [22, 23]. At 7.5 days post coitus (dpc) when trophoblast EMT is high [24], uterine SNAIL1-CBR signal was 5 fold higher compared to the basal signal (Fig. 1E). We cannot exclude the possible contribution of increased SNAIL1-CBR signal from embryonic gastrulation – another contemporaneous SNAIL1-dependent EMT event [25] – to the uterine signal. Pathologically, SNAIL1 has been implicated in carbon
tetrachloride (CCl₄)-induced liver fibrosis [26]. SNAIL1-CBR mice treated with intraperitoneal CCl₄ developed liver fibrosis, as evidenced by the increased collagen deposition (Fig. 1G), and hepatic SNAIL1-CBR signal concurrently increased ~5 fold compared to the vehicle control (Fig. 1F). These control experiments indicated that SNAIL1-CBR expression is properly regulated, readily detectable during in vivo EMT, and thus, a reliable reporter of endogenous SNAIL1 expression in vivo.

The SNAIL family of transcription factors (SNAIL1 and SNAIL2) has been suggested to play a role in mammary development [27, 28]. Therefore to determine the functionality of the TRE-SNAIL1-3FLAG transgene in vivo, we crossed SNAIL1-CBR mice with mice carrying the mouse mammary tumor virus promoter driving expression of rtTA (MMTV-rtTA or MTA) [29]. TRE-SNAIL1-3FLAG transgene expression was detected in mammary tissue only in the presence of both MTA and dox (Fig. 1I) and resulted in increased ductal branching compared to control SNAIL1-CBR mice lacking MTA (Fig. 1H). This result indicated that the TRE-SNAIL1-3FLAG transgene was expressed and functional in vivo.

**SNAIL1 expression in primary breast tumors strongly correlates with metastasis.**

Since SNAIL1 expression in primary human breast tumors correlates with higher rates of metastasis [4], we asked whether a similar association also holds true in mouse breast cancer models. To do so, we first crossed SNAIL1-CBR mice with the MMTV-NeuNT breast cancer model (MMTV-neuNT; SNAIL1-CBR mice), which produces breast tumors similar to ER-negative, Her2Neu-positive human breast tumors [30, 31], and determined SNAIL1 expression within emerging breast tumors through SNAIL1-
CBR bioluminescence and correlated the signal intensity with the development of lung metastasis (Fig. 2A-C). A remarkably strong positive correlation between SNAIL1-CBR expression in primary tumors and incidence of lung metastasis was observed. No mouse with primary breast tumors that all exhibited SNAIL1-CBR intensity of $\leq 3 \times 10^6$ photons/cm$^2$ (mean of 2.5x10$^6$) had detectable lung metastasis, whereas in mice with at least 1 primary breast tumor that exhibited an intensity of $\geq 10^7$ photons/cm$^2$ (mean of 1.75x10$^7$), 100% developed lung metastasis (Figs. 2C and S2A). In all subsequent experiments we defined primary breast tumors exhibiting $<3 \times 10^6$ photons/cm$^2$ as SNAIL1-CBR negative and $>10^7$ photons/cm$^2$ as SNAIL1-CBR positive (Fig. S2A).

In human breast cancer SNAIL1 expression in primary tumors predicts for distant metastasis, however its expression in BM DTCs does not [4], suggesting that SNAIL1 may be important in primary tumors to initiate invasion and migration of cancer cells that ultimately give rise to DTCs, as opposed to playing a role in the outgrowth of DTCs to form overt metastases. If so, then SNAIL1 expression in primary tumors might be associated with higher numbers of DTCs. We chose to focus on DTCs instead of blood-borne CTCs because DTCs are considered to have completed the metastatic cascade (i.e. local invasion, intravasation, extravasation and survival in secondary organs). In addition, it is unclear what fraction of CTCs eventually gives rise to DTCs as the majority fail to complete the metastatic process [32]. To test this possibility, we collected total dissociated lung cells and total erythrocyte-depleted BM cells, then quantified the number of DTCs through Her2Neu IF using a mouse monoclonal antibody specific for human and rat, but not mouse, Her2Neu [33]. In mice with SNAIL1-CBR positive primary tumors there was a 10 fold greater number of BM DTCs compared to SNAIL1-
CBR negative primary tumor-bearing mice (Fig. 2D-E). A similar strong positive correlation was also observed between the number of lung DTCs and SNAIL1 expression in primary tumors (Fig. S2B). In addition, the numbers of BM and lung DTCs correlated well with each other (Fig. S2C). Since the BM afforded greater reliability of enriching for DTCs and there is less chance of contamination with tumor cells from metastatic foci than in the lung, we focused on BM DTCs for the remainder of the experiments. We could not exclude the possibility that some DTCs could have lost Her2Neu expression; however, the contribution of Her2Neu-negative DTCs, if any, to the total number of DTCs would be expected to be similar in animals with either SNAIL1-CBR positive or negative primary tumors because the ratio of Her2Neu-positive and negative cells in primary tumors were similar between SNAIL1-CBR positive and negative primary tumors (Fig. S2D).

In summary, these results indicated that SNAIL1-CBR (i.e., SNAIL1) expression in primary mouse breast tumors was a strong predictor for the production of DTCs and subsequent overt lung metastases.

Next we asked whether SNAIL1-CBR positive primary tumors were associated with a more invasive phenotype that is associated with increased metastatic disease. Grossly, SNAIL1-CBR positive primary tumors (confirmed by RT-PCR - Fig. S3A) were irregular in shape with multiple protrusions penetrating into and tightly attached to underlying fascia and muscles, whereas SNAIL1-CBR negative tumors were round and loosely adhered to the fascia (Fig. 2F-G). Microscopically, SNAIL1-CBR positive tumors exhibited multiple invasive projections, in which numerous cells expressed SNAIL1 and lacked E-cadherin expression (Figs. 2H, left panel, black arrows; 2I, red arrowheads;
and S3B, left panel). In contrast, a smooth contour distinguished SNAIL1-CBR negative tumors from surrounding ECM and in these tumor cells E-cadherin expression was present (Figs. 2H, right panel; 2J; and S3B, right panel). Thus expression of SNAIL1 in primary breast tumors is associated with an aggressive carcinomatous phenotype, characterized by local invasion, while primary tumors not expressing SNAIL1 were adenoma-like.

**SNAIL1 expression in primary breast tumors precedes detection of DTCs, but SNAIL1 is not expressed by DTCs and lung metastases.**

If SNAIL1 expression in primary tumors drives EMT and thereby invasion and intravasation, then SNAIL1 expression in primary tumors might be predicted to precede the detection of DTCs. To test this hypothesis, we made use of the tet-inducible activated rat Her2Neu mouse breast cancer model (MMTV-rtTA; TetO-neuNT or MTA; TAN) because of its predictable and reproducible tumor penetrance and latency [34], two key characteristics necessary for a temporal analysis (Fig. 3A).

SNAIL1 mRNA expression in primary tumors and the number of BM DTCs were determined in mice after 5 to 8 weeks of dox treatment, a time well before evident lung metastases. BM DTCs were scored by Her2Neu IF. Low-level SNAIL1 mRNA was present after 5 weeks of dox treatment, a time when primary tumors were barely palpable (Fig. 3B). This increased 3-5 fold by weeks 6-8 of dox treatment (Fig. 3B). BM DTCs were barely detectable after 6 weeks of dox treatment but rose sharply beginning at weeks 7 and 8 (Fig. 3C). It was unlikely that the initial upsurge in both primary tumor SNAIL1 mRNA levels and BM DTCs was due to growth of primary tumors, as there was
no significant difference in these parameters between weeks 7 and 8 despite a simultaneous 3-fold growth in total primary tumor burden (Fig. 3D). Thus, SNAIL1 expression in primary tumors preceded the detection of BM DTCs.

In another approach testing this hypothesis, we crossed the SNAIL1-CBR mouse with the MMTV-PyMT breast cancer model, which, like the MTA; TAN model, also has a predictable and uniform, albeit more accelerated, course of tumor development [30, 35]. Again, a similar temporal relationship between SNAIL1 expression in primary tumors and generation of DTCs was observed in MMTV-PyMT; SNAIL1-CBR mice: SNAIL1-CBR reporter and SNAIL1 mRNA expression followed first palpable tumors (Fig. S4A-C) and preceded the increase in BM DTCs (Fig. S4D).

In sum, these two experiments indicated that SNAIL1 expression within primary tumors immediately preceded the detection of DTCs.

To determine whether SNAIL1 contributed to later stages of metastasis when DTCs reactivate growth to form overt metastases, we measured SNAIL1 mRNA expression in sets of primary tumors, BM DTCs, and isolated lung metastases from the same mice. MTA; TAN mice were treated with dox for 12 weeks (from 6 to 18 weeks of age) when nearly all had developed lung metastasis (Fig. 3A), then tissues harvested. BM DTCs were enriched >100 fold by depleting CD45-positive hematologic BM cells (not shown). Relative to primary tumors, SNAIL1 mRNA level was >4 and >3 fold less in BM DTCs and lung metastases, respectively (Fig. 3E). This decrease was also apparent at the protein level as assessed by SNAIL1 IHC and IF (Table 1). Of 25 primary tumors, 13 (52%) exhibited detectable SNAIL1 expression at invasive fronts where there was decreased E-cadherin and increased Vimentin. In contrast, SNAIL1
expression was rarely found in Her2Neu-positive BM DTCs (4%) and lung DTCs (not shown), and lung metastases (~5%). Histologically, the majority of lung metastases appeared more epithelial than mesenchymal (Fig. 3F). Similar results were observed in MMTV-PyMT; SNAIL1-CBR mice (Table S1 and Fig. S4E).

Taken together, these results indicated that SNAIL1 expression was restricted to primary tumors and associated with a mesenchymal phenotype, whereas overt lung metastases were more epithelial appearing, suggesting a reversal of EMT, possibly through the Mesenchymal-Epithelial Transition or MET, in metastases. The possibility that some lung metastases were formed through SNAIL1-independent events cannot be excluded, however.

**SNAIL1 is required for breast cancer metastasis.**

To determine whether SNAIL1 was necessary for breast cancer metastasis, we made use of a previously characterized floxed SNAIL1 mouse [13]. Although SNAIL1 expression in primary tumors of MMTV-PyMT mice was first detected when primary tumors reached ~0.5cm in diameter (Fig. S4B-C), it is possible that earlier low level or transient SNAIL1 expression occurs and if so this could be important to promote invasion. In humans and mouse models, early dissemination from premalignant lesions such as DCIS has been described [33, 36-38]. Whether metastatic cells derived from early dissemination possess the same potential to develop overt metastases as those from later dissemination remains unclear. Therefore, to test the requirement for SNAIL1 in breast cancer metastasis we decided to delete the SNAIL1 gene either early (MMTV-Cre) or late (TetO-Cre) during PyMT-induced breast cancer development (Fig. 4A).
The MMTV-Cre transgene expresses CRE in the mammary epithelia concurrently with oncogenic MMTV-PyMT expression. We confirmed CRE expression from MMTV-Cre in the breast epithelium using ROSA26-LSL-tdTomato mice [39]. In 8 week-old mice, tdTomato was present in most, but not all, cells in the mammary ductal epithelia, consistent with known mosaicism of the MMTV promoter, but not in surrounding stromal cells (not shown) [40]. For late deletion of SNAIL1, we treated SNAIL1\(^{fl/fl}\); MMTV-PyMT; TRE-Cre; MTA with dox when the size of primary tumors had reached 0.5cm [age ~7 weeks, a time prior to the initial upsurge of SNAIL1 expression (Figs. 4A and S4B)]. In both early and late SNAIL1 KOs, deletion of SNAIL1 in primary tumors and the resultant reversal of EMT were verified by IHC for SNAIL1, E-cadherin and Vimentin (Figs. 4B and S5A-B). Both early and late SNAIL1 KO resulted in a profound reduction in numbers of lung metastases as measured by Indian ink stain (Fig. 4C, D and F) and BM DTCs as measured by cytokeratin (panCK) IF (Fig. 4E and G), compared to age and background-matched control mice. Primary tumor burden was not higher in control mice compared to KOs. The number of superficial lung metastases was a reliable surrogate marker for total lung metastatic burden since it correlated with that of internal metastases as determined histologically (not shown).

The few lung metastases present in early SNAIL1 KO mice could have arisen from metastatic cells that had escaped CRE-mediated SNAIL1 deletion due to the mosaic MMTV promoter or through a SNAIL1-independent process. To differentiate between these two possibilities, we determined the status of CRE-mediated SNAIL1 gene rearrangement in primary tumors and lung metastases from the same mice. The rearranged SNAIL1 allele was readily amplified in primary tumors but not in lung
metastases. In contrast, a control genomic sequence proximal to the floxed segment was comparably amplified in both (Fig. S5C). This result indicated that the few lung metastases present in early KO mice likely arose due to incomplete deletion of the floxed SNAIL1 gene in primary tumor cells.

In summary, both early and late SNAIL1 deletion in the MMTV-PyMT metastatic breast cancer model indicated that SNAIL1 was required for breast cancer metastasis.

**Transient, but not continuous, SNAIL1 overexpression in primary breast tumors increases metastasis.**

The presence of SNAIL1 expression in primary tumors yet absence in DTCs and lung metastases suggested that SNAIL1 expression during metastasis could be transient, or alternatively, needs to be turned off for effective metastasis to occur. To test this hypothesis we overexpressed SNAIL1 either transiently or continuously in MMTV-neuNT mice, as this model has modest metastatic potential (~25%) and asked if these manipulations affected the extent of metastasis (Fig. 5A).

We generated MMTV-neuNT; SNAIL1-CBR; MTA and control MMTV-neuNT; SNAIL1-CBR mice (unresponsive to dox). Upon detection of palpable primary breast tumors (~0.5 cm in diameter and SNAIL1-CBR bioluminescence not detected), mice were fed 1mg/ml dox-containing water, either continuously (12 weeks; Continuous SNAIL1) or transiently (4 weeks followed by 8 weeks of dox-free water; Transient SNAIL1), then the number of BM DTCs and incidence of lung metastasis determined for all groups (Fig. 5A). The selection of 4-week dox treatment as the transient treatment...
window was based on the time course from initial SNAIL1 expression in primary breast tumors to initial appearance of lung metastases in MTA; TAN mice (Fig. 3A).

Tumor latency was similar between Transient and Continuous SNAIL1 and control mice with means of 23 and 24 weeks, respectively. Primary tumor burden was not higher in SNAIL1-overexpressing mice compared to Controls. Continuous SNAIL1 overexpression in breast tumor cells increased the number of BM DTCs 3 fold over that in controls (Fig. 5C). Surprisingly, however, the percentage of mice that developed lung metastasis was reduced 3 fold (8.7% versus 25% in controls) (Fig. 5B). When SNAIL1 was transiently overexpressed the number of BM DTCs also increased but now 15 fold higher than in controls (Fig. 5C; 5 fold higher than in Continuous SNAIL1). More than 60% of Transient SNAIL1 mice developed lung metastasis compared to only 25% of controls and 8.7% of Continuous SNAIL1 mice (Fig. 5B).

The number of primary tumors expressing SNAIL1 and exhibiting a mesenchymal phenotype (i.e. low E-cadherin and high Vimentin), at the time of analysis, in Transient SNAIL1 mice was comparable to controls and 4 fold lower than that in Continuous SNAIL1 mice. In both Transient and Continuous SNAIL1 mice there were few lung metastases that expressed SNAIL1 and in contrast to primary tumors in Continuous SNAIL1 mice, most lung metastases in mice of all groups exhibited an epithelial-like phenotype (i.e. low SNAIL1, high E-cadherin and low Vimentin) (Table 2 and Fig. S6).

Taken together, these results indicated that SNAIL1 was necessary and sufficient to induce DTCs and overt lung metastases. Transient SNAIL1 overexpression increased both the number of BM DTCs and the incidence of lung metastasis whereas
continuous SNAIL1 overexpression decreased the incidence of lung metastasis. This suggested the possibility that SNAIL1 expression needs to be downregulated after tumor cells exit the primary tumor for overt lung metastases to form.

DISCUSSION

Using a novel SNAIL1-CBR reporter mouse model and a previously characterized conditional SNAIL1 KO mouse [13] crossed to several genetic models of breast cancer we have demonstrated that SNAIL1 plays a critical role in breast cancer metastasis in vivo. Specifically SNAIL1 expression: 1) is restricted to metastatic primary breast tumors, particularly at invasive fronts; 2) immediately precedes detection of DTCs; and 3) is required and sufficient for efficient breast cancer metastasis.

Multiple EMT factors are often present simultaneously in the same tumors [4, 12]. Recent studies suggest a spatiotemporal hierarchy among these factors in developmental and cancer EMT with SNAIL1 considered one of the earliest master regulators of EMT initiation [4, 41]. This was validated in the genetic models detailed herein. SNAIL1 expression was detected in primary tumors but only rarely in DTCs and lung metastases. The spatiotemporal expression of SNAIL1 appears to be important for effective metastasis as continuous SNAIL1 overexpression decreased while transient overexpression greatly increased the incidence of lung metastasis. This could be due to the growth inhibitory properties of SNAIL1 and EMT factors in general and is consistent with recent reports on inhibitory effects of prolonged SNAIL1 expression on the renewal capacity of tumor initiating cells [42] and on spatiotemporal regulation of other EMT
factors such as Twist1 and Prrx1 [1, 4, 9, 10, 12]. We cannot exclude the possibility that some lung metastases, especially those exhibiting detectable SNAIL1 expression, could have developed from a SNAIL1-independent mechanism. However, the contribution of such a pathway to metastasis is likely to be insignificant as lung metastasis was severely impeded when SNAIL1 was deleted or overexpressed continuously.

What maintains metastatic cells in EMT after SNAIL1 downregulation remains incompletely understood. Other EMT factors could cooperate with SNAIL1 to maintain EMT in DTCs until they reactivate growth to form overt metastases. In human breast cancer patients, Twist1’s expression in BM DTCs has been shown to correlate with subsequent development of overt metastases [4]. In these metastatic breast cancer models the EMT state appears to persist, at least partially, in DTCs as evidenced by absence of E-cadherin expression in these cells while only a few exhibited Vimentin expression. Whether other EMT factors, such as Twist1, replace SNAIL1 in DTCs in these models remains to be determined.

Although we have not conclusively established that SNAIL1 downregulation in metastatic cells is mechanistically linked to subsequent MET, the current results suggest that SNAIL1’s primary site of function is at invasive fronts of primary tumors and not at metastatic sites and therefore therapeutic targeting of SNAIL1 is unlikely to exert significant effects on DTCs and may have clinical benefit in inhibiting cancer EMT initiation and invasion in the first place. However, a note of caution to such an approach is warranted given the possibility that low SNAIL1 expression in DTCs may be required – perhaps in cooperation with Twist1 – to promote EMT and dormancy, and that inhibiting SNAIL1 in preexistent DTCs may inadvertently induce them to exit EMT to
undergo MET resulting in increased metastatic disease, as has been suggested by other recent studies with other EMT factors [9, 10].

Metastasis has been proposed to occur early in breast tumorigenesis. In several human breast cancer series, up to 19% of patients with DCIS had detectable CTCs. Yet the risk of developing overt metastases was <1% among these patients [36, 37], suggesting that the vast majority of detected CTCs in these patients are highly inefficient at colonizing secondary organs and being reactivated to form metastases. In both NeuNT and PyMT-induced breast cancer mouse models, dissemination of cancer cells was also observed in the premalignant phase [33]. Our results in MMTV-PyMT mice suggest that late dissemination contributes the greatest to overall metastasis and is highly dependent on SNAIL1. Although these results do not address whether early dissemination requires SNAIL1 function, early dissemination does not appear to be the main driver of metastasis, perhaps due to the need for early DTCs to acquire additional genetic and/or epigenetic changes and stimuli that promote efficient migration, colonization and reactivation of growth. In fact, Husemann et al. demonstrated that early DTCs from premalignant lesions required further stimulation from growth factors and a supportive BM environment to form apparent metastases [33].

ACKNOWLEDGEMENTS

We thank Drs. Stephen Weiss, David DeNardo, Ron Bose, Raphael Kopan and David Piwnica-Worms for providing floxed SNAIL1, MMTV-PyMT, MTA; TAN, ROSA26-LSL-tdTomato mice, and the CBR cDNA, respectively. We thank Jennifer Gill for help with
ES cell differentiation assay, Lynne Collins and Julie Prior for help with bioluminescence imaging of mice, and members of our labs for their helpful comments. This research was supported in part by the National Cancer Institute 1K08CA160824, the Pardee Foundation (D.D.T.), and the Komen Race for the Cure KG110889 (G.D.L.).

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of mammary adenocarcinoma in transgenic mice bearing the activated c-neu


Table 1. Spatiotemporal expression of SNAIL1 in MTA; TAN mice

<table>
<thead>
<tr>
<th>Tumor Site</th>
<th>Total number of tumors, DTCs &amp; lung metastases examined from 4 animals</th>
<th>Number of SNAIL1-positive tumors, DTCs &amp; lung metastases</th>
<th>Percent SNAIL1 positive</th>
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<td>Primary Tumors</td>
<td>25</td>
<td>13</td>
<td>52</td>
</tr>
<tr>
<td>BM DTCs</td>
<td>150</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>Lung metastases</td>
<td>150</td>
<td>7</td>
<td>4.7</td>
</tr>
</tbody>
</table>

Three 10-μm sections each of primary tumors and lungs with metastases and BM DTC spreads from 4 animals were subjected to IHC for SNAIL1 (primary tumors and lungs) or co-IF for Her2Neu and SNAIL1 (BM DTCs) and the SNAIL1-positive percentage in each tissue type determined. Related to Figure 3E-F.

Table 2. Spatiotemporal expression of SNAIL1 in MMTV-neuNT mice

<table>
<thead>
<tr>
<th>Duration of exogenous SNAIL1 Expression in Primary Tumors</th>
<th>Tumor Site</th>
<th>Number of tumors &amp; lung metastases examined from 5 mice (#)</th>
<th>Number of SNAIL1-positive tumors &amp; lung metastases</th>
<th>Percent SNAIL1 positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Endogenous SNAIL1)</td>
<td>Primary Tumors</td>
<td>25</td>
<td>4</td>
<td>16</td>
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<tr>
<td></td>
<td>Lung metastases</td>
<td>50</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Continuous SNAIL1 (#)</td>
<td>Primary Tumors</td>
<td>15</td>
<td>12</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>Lung metastases</td>
<td>24</td>
<td>4</td>
<td>17</td>
</tr>
<tr>
<td>Transient SNAIL1</td>
<td>Primary Tumors</td>
<td>25</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Lung metastases</td>
<td>50</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

Three 10-μm sections each of primary tumors and lungs with metastases from 5 Control, 2 Continuous SNAIL1 (#: only 2 in 23 mice had lung metastasis) and 5 Transient SNAIL1 mice were subjected to IHC for SNAIL1, E-cadherin and Vimentin and the SNAIL1-positive percentage in each tissue type determined. Related to Figures 5 and S6.
TABLE AND FIGURE LEGENDS

Table 1. Spatiotemporal expression of SNAIL1 in MTA; TAN mice

Three 10-μm sections each of primary tumors and lungs with metastases and BM DTC spreads from 4 animals were subjected to IHC for SNAIL1 (primary tumors and lungs) or co-IF for Her2Neu and SNAIL1 (BM DTCs) and the SNAIL1-positive percentage in each tissue type determined.

Table 2. Spatiotemporal expression of SNAIL1 in MMTV-neuNT mice

Three 10-μm sections each of primary tumors and lungs with metastases from 5 Control, 2 Continuous SNAIL1 (#: only 2 in 23 mice had lung metastasis) and 5 Transient SNAIL1 mice were subjected to IHC for SNAIL1, E-cadherin and Vimentin and the SNAIL1-positive percentage in each tissue type determined.

Figure 1. Generation and characterization of SNAIL1 reporter and tet-inducible SNAIL1 mice (SNAIL1-CBR)

A, A diagram of the modified SNAIL1 locus in SNAIL1-CBR mice.

B, Targeted ES cells were screened by PCR using indicated primers.

C, SNAIL1-CBR was properly regulated in targeted ES cells. Representatives of 3 experiments are shown.
D, The TRE-SNAIL1-3Flag transgene was expressed in ES cells only when both rtTA and dox were present.

E, Uterine SNAIL1-CBR signal in a pregnant female at 7.5 dpc _in vivo_ (left panel), _ex vivo_ (middle 2 panels) and after uterine removal (right panel). N=5 mice.

F, SNAIL1-CBR was upregulated in CCl₄-induced liver fibrosis as seen _in vivo_ (left panel) and _ex vivo_ (right panel). N=5 mice

G, CCl₄-induced bridging fibrosis (arrows) as revealed by picrosirius stain of liver sections. N=5 mice.

H, Whole breast mount stain of SNAIL1-CBR and SNAIL1-CBR; MTA mice treated with dox for 1 week starting at age 4 weeks. N=5 mice.

I, Mammary SNAIL1-3Flag mRNA expression was detected by RT-PCR only when both MTA and dox were present.

**Figure 2: SNAIL1 expression in primary breast tumors strongly correlates with increased metastasis and an invasive phenotype in MMTV-neuNT mice**

N=23 mice without and 15 mice with lung metastasis for all panels.

A, A representative image of SNAIL1-CBR bioluminescence in MMTV-NeuNT mice with SNAIL1-CBR positive or negative tumors.

B, Representative images of superficial lung metastases as seen grossly (top panel, black arrow) and after Indian ink stain as white lesions on a black background (bottom panel, white arrow).
C, A histogram of incidence of lung metastasis in mice with SNAIL1-CBR positive or negative primary tumors.

D, Representative images of BM DTCs as detected by Her2Neu IF in mice with SNAIL1-CBR positive or negative primary breast tumors.

E, A histogram of number of BM DTCs per $10^6$ BM cells in mice with SNAIL1-CBR positive or negative primary breast tumors. *p=7.4x10^{-7}.

F, Representative images of SNAIL1-CBR positive and negative tumors as seen in situ (upper panels) and ex vivo (lower panels).

G, A histogram of distribution of invasive carcinomas and adenomas by gross appearance of SNAIL1-CBR positive and negative tumors.

H, Representative images of H&E stained primary tumor sections revealing a typical protrusion into surrounding stroma in a Snail1-CBR positive tumor (black arrow) compared to the smooth contour in a Snail1-CBR negative tumor.

(I and J), Representative images of SNAIL1 and E-cadherin IHC of sections from SNAIL1-CBR positive (I) and negative (J) primary tumors, revealing SNAIL1-positive cells (red arrowheads) in an invasive front of SNAIL1-CBR positive tumor where E-cadherin expression was decreased. Mus denotes Muscle.

**Figure 3: SNAIL1 expression in primary breast tumors precedes detection of DTCs, but SNAIL1 is undetectable in DTCs and lung metastases in MTA; TAN mice.**

A, Important milestones for the MTA; TAN breast cancer model.
(B-D), Histograms of relative SNAIL1 mRNA levels in developing primary breast tumors as determined by RT-PCR and normalized to GAPDH mRNA (B; *p=0.01), number of BM DTCs per 10^6 BM cells as quantified by Her2Neu IF (C; **p=0.03), and tumor burden (D) at indicated times. N=5 mice at each time point.

E, A histogram of relative SNAIL1 mRNA levels in a set of primary tumor, BM DTCs and isolated lung metastasis from the same mouse as determined by RT-PCR and normalized to GAPDH mRNA (***p=0.0001; ****p=0.002). Representatives of 3 experiments are shown.

F, Representative images of the same H&E and IHC stained sections as in Table 1, revealing SNAIL1-positive cells (green arrows) at an invasive front of a primary tumor where E-cadherin was low and Vimentin high (top panel). SNAIL1 was undetectable in BM DTCs, which exhibited mixed phenotype (middle panel), and lung metastasis, which appeared epithelial (bottom panel).

Figure 4: SNAIL1 is necessary for breast tumor metastasis in MMTV-PyMT mice

A, A diagram of breast cancer progression and genetic mouse models used.

B, Representative images of SNAIL1 IHC stained sections of primary tumors from Early SNAIL1 KO and control mice, confirming efficient SNAIL1 deletion.

C, Representative images of lungs as seen grossly (upper panel) and after Indian ink stain (lower panel) of Control and Early SNAIL1 KO mice.

(D and E), Histograms of number of lung metastases as measured by Indian ink stain (D; *p=0.0038), and number of BM DTCs per 10^6 total BM cells as measured by panCK
IF (E; *p=0.00087) in Control and Early SNAIL1 KO mice. N=8 for Control and 6 for Early SNAIL1 KO.

(F and G), Histograms of number of lung metastases as measured by Indian ink stain (F; *p=0.04), and number of BM DTCs per 10^6 total BM cells as measured by panCK IF (G; *p=0.03) in Control and Late SNAIL1 KO mice. N=10 for Control and 6 for Late SNAIL1 KO.

Figure 5: Transient, not continuous, overexpression of SNAIL1 in primary breast tumors increases breast cancer metastasis in MMTV-NeuNT mice

A, A diagram of breast cancer progression and genetic mouse models used.

(B and C), Histograms of incidence of lung metastasis as determined by Indian ink stain and histology (B) and number of BM DTCs per 10^6 total BM cells as determined by Her2Neu IF (C; *p=0.0013; **p=5.28x10^{-8}; ***p=2.5x10^{-5}) in Control, Continuous SNAIL1 and Transient SNAIL1 mice. N=32, 23 and 8 for Control, Continuous and Transient SNAIL1, respectively.
Author Manuscript Published OnlineFirst on August 27, 2014; DOI: 10.1158/0008-5472.CAN-14-0923
Figure 5
Transient SNAIL1 Expression is Necessary for Metastatic Competence in Breast Cancer

Hung D Tran, Krishna Luitel, Michael Kim, et al.

Cancer Res  Published OnlineFirst August 27, 2014.

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