Twist1 is an epithelial–mesenchymal transition (EMT)-inducing transcription factor (TF) that promotes cell migration and invasion. To determine the intrinsic role of Twist1 in EMT and breast cancer initiation, growth, and metastasis, we developed mouse models with an oncogene-induced mammary tumor containing wild-type (WT) Twist1 or tumor cell-specific Twist1 knockout (Twist1TKO). Twist1 knockout showed no effects on tumor initiation and growth. In both models with early-stage tumor cells, Twist1, and mesenchymal markers were not expressed, and lung metastasis was absent. Twist1 expression was detected in ∼6% of the advanced WT tumor cells. Most of these Twist1+ cells coexpressed several other EMT-inducing TFs (Snail, Slug, Zeb2), lost ERx and luminal marker K8, acquired basal cell markers (K5, p63), and exhibited a partial EMT plasticity (E-cadherin+/vimentin+). In advanced Twist1TKO tumors, Twist1 knockout largely diminished the expression of the aforementioned EMT-inducing TFs and basal and mesenchymal markers, but maintained the expression of the luminal markers. Circulating tumor cells (CTCs) were commonly detected in mice with advanced WT tumors, but not in mice with advanced Twist1TKO tumors. Nearly all WT CTCs coexpressed Twist1 with other EMT-inducing TFs and both epithelial and mesenchymal markers. Mice with advanced WT tumors developed extensive lung metastasis consisting of luminal tumor cells with silenced Twist1 and mesenchymal marker expression. Mice with advanced Twist1TKO tumors developed very little lung metastasis. Therefore, Twist1 is required for the expression of other EMT-inducing TFs in a small subset of tumor cells. Together, they induce partial EMT, basal-like tumor progression, invasation, and metastasis.

Twist1 | breast cancer | EMT | metastasis | mouse model

Epithelial–mesenchymal transition (EMT) is observed in mesodermal induction during embryonic development and certain disease conditions in adults such as wound healing and carcinogenesis, in which active cell migration and lineage changes are involved (1). Similarly, either experimentally induced EMT in cultured cancer cells or tissue environment-induced EMT in the cancer cell-derived xenograft tumors changes the morphology and increases the migration and invasion capability of these cancer cells (1, 2). Because the migration and invasion capability of cancer cells usually associates with their metastatic potential, EMT has been considered crucial for driving cancer metastasis (2). Indeed, EMT positively correlates with tumor cell invasiveness and metastasis in multiple mouse models. For example, Snail expression negatively correlates with E-cadherin expression, but positively correlates with mesenchymal marker expression, and knockout (KO) of Snail reduces tumor cell metastasis (3, 4). Snail-expressing tumor cells are also highly metastatic when injected i.v. (3). The mouse tumor cells expressing Fsp1, a mesenchymal marker, usually invade to the locations close to blood vessels (5). However, opposite results from mouse models have also been reported. For example, the Fsp1-expressing mouse breast tumor cells were shown unable to metastasize to the lung (6), and suppression of EMT by deleting Snail or Twist1 in the mouse pancreatic ductal adenocarcinoma is unable to inhibit metastasis (7). Furthermore, because cancer cells with mesenchymal morphology cannot be recognized by a pathological diagnosis and the cancer cells of nearly all metastatic lesions exhibit epithelial morphology, it has been a challenge to validate the clinical significance of EMT in human cancer metastasis. Therefore, the exact role of EMT in cancer metastasis remains unclear.

Twist1 is a basic helix–loop–helix domain-containing TF that either activates or suppresses genes (8). During embryonic development, Twist1 is required for cranial neural tube, somite, and limb bud development in mammals (8, 9). Heterozygous loss-of-function mutation of Twist1 causes Saethre-Chotzen syndrome in humans and a similar phenotype in mice (9–11). Homozygous KO of Twist1 results in embryonic lethality in mice, indicating its essential role in development (9). Interestingly, Twist1 is only expressed in a couple of tissues in adult mice, including fibroblasts of the mammary glands (MGs) and dermal papilla cells of the hair follicles (12). Thus, inducible KO of Twist1 in adult mice does not affect their viability and general health, suggesting its nonessential role in adult animals (12). It is conceivable that Twist1 would be a cancer-preferential drug target with little adverse effect in adult patients if Twist1 is required for cancer cells. Importantly, Twist1 is expressed in multiple types of cancer cells including some of the breast cancer (BrC) cell lines (8, 13). In BrC cells, Twist1 expression induces...
Results

Twist1 KO in the Mouse MG Tumor Cells Does Not Affect Primary Tumor Initiation and Growth. We generated 8-wk-old MMTV-TVAxTwist1
twist/F/F and MMTV-TVAxTwist1
twist/F/F female mice (12, 22, 26) and injected RCIP virus into the ducal lumens of their inguinal MGs (SI Appendix, Fig. S2A). After 6–10 wk, we detected palpable tumors in both groups with similar initiation latencies (SI Appendix, Fig. S2B). In total, 26 and 35 tumors were developed from 42 and 60 virus-infected MGs in MMTV-TVAxTwist1
twist/F/F and MMTV-TVAxTwist1
twist/F/F mice, respectively, giving a comparable tumorigenic rate of ~60% for both groups. Furthermore, the growth rates of these palpable tumors were similar in the two groups (SI Appendix, Fig. S2C). In MMTV-TVAxTwist1
twist+/+RCIP (WT/RCIP) tumors after becoming palpable for 10 wk, we detected only the WT Twist1, whereas we detected both the deleted and the floxed Twist1 alleles in MMTV-TVAxTwist1TKO/RCIP (Twist1TKO/RCIP) tumors as a result of the presence of both the tumor cells with Twist1 deletion and the nontumor cells with the floxed Twist1 (SI Appendix, Fig. S2D). Total Twist1 protein was decreased in Twist1TKO/RCIP versus WT/RCIP tumors, although its level was variable in different Twist1TKO/RCIP tumors, probably as a result of different ratios of tumor cells to stromal cells (SI Appendix, Fig. S2E). In the laser-captured WT/RCIP and Twist1TKO/RCIP tissues with enriched tumor cells, Twist1 mRNA was relatively high and barely detectable, respectively (SI Appendix, Fig. S2F).

Immunohistochemistry (IHC) and/or immunofluorescent (IF) staining detected Twist1 in subsets of both tumor and stromal cells in WT/RCIP tumors, but only in stromal cells in Twist1TKO/RCIP tumors. In WT/RCIP tumors, double Twist1-positive (Twist1+) and PyMT-HA-positive (PyMT+) tumor cells were usually observed as small clusters, which was about 6% of total PyMT+ tumor cells. In Twist1TKO/RCIP tumors, only PyMT+ tumor cells were detected (SI Appendix, Fig. S3). In addition, IHC assays only detected Twist1 in stromal cells, not in tumor cells in the 6-wk palpable early-stage WT/RCIP and Twist1TKO/RCIP tumors (SI Appendix, Fig. S4A). In the 10-wk palpable WT/RCIP and Twist1TKO/RCIP mammary tumors, we found no significant differences in cell proliferation assayed by Ki67 IHC, cell apoptosis assayed by cleaved-caspase 3 IHC, and macrophage recruitment detected by F4-80 IHC (SI Appendix, Fig. S5).

Together, these results demonstrate that mammary tumorigenesis has been effectively induced in mice by using the MMTV-TVA/RCIP system, Twist1 is only expressed in a subset of tumor cells in the late-stage advanced tumors, efficient tumor cell-specific KO of Twist1 has been achieved in Twist1TKO/RCIP tumors, the tumor cell-intrinsic function of Twist1 is nonessential for initiation and growth of the RCIP-induced primary tumors, and Twist1 expression in the tumor cells is not required for tumor cell proliferation and apoptosis and for macrophage recruitment to the tumor environment.

Twist1 Expression in the Primary Tumor Cells Induces a Partial EMT Phenotype. Using PyMT as a tumor cell marker in triple IF staining, we examined the expression relationships between Twist1 and the epithelial marker E-cadherin or the mesenchymal markers vimentin and Fsp1 in the 10-wk large WT/RCIP and Twist1TKO/RCIP mammary tumors. As mentioned, about 6% of PyMT+ tumor cells are Twist1+ cells [immunoreactive score (IRS) ≥ 1]. In these advanced WT/RCIP tumors, Twist1+ tumor cells usually appear as clusters. Most of the Twist1+/PyMT+ tumor cells express low-level E-cadherin (IRS ≤ 1), whereas most of the Twist1+/PyMT+ tumor cells express high- or moderate-level E-cadherin (IRS ≥ 2). The proportion of tumor cells with low E-cadherin is significantly increased in Twist1+/PyMT+ versus Twist1+/PyMT− tumor cells. In the same-stage Twist1TKO/RCIP tumors, we detected high or moderate levels (IRS ≥ 2) of E-cadherin in the majority (~92%) of tumor cells, although we still observed ~8% of tumor cells with decreased E-cadherin (IRS ≤ 1), which could be rescued by other E-cadherin-repressing factors (Fig. 1A and SI Appendix, Fig. S6A). These results indicate that Twist1 expression can decrease E-cadherin in the spontaneously developed mouse MG tumors.

In the large WT/RCIP tumors, the majority (~83%) of Twist1+/PyMT+ cells express different levels of vimentin (IRS ≥ 1), with ~53% of these double-positive cells containing moderate to high levels of vimentin (IRS ≥ 2). However, the majority (~96%) of Twist1+/PyMT+ cells are vimentin+; only ~4% of these Twist1+ tumor cells express vimentin at different levels. In the large Twist1TKO/RCIP tumors, in addition to the vimentin+ fibroblasts, only ~2.4% of PyMT+ tumor cells express vimentin (Fig. 1B and SI Appendix, Fig. S6B). The results indicate that Twist1 expression positively correlates with vimentin expression,
The mRNA expression levels of other EMT-inducing TFs including Zeb1, Zeb2, Snail, and Slug in the whole WT/RCIP and Twist1TKO/RCIP tumors did not show any significant differences (SI Appendix, Fig. S8A). Their mRNA levels in the RNA samples prepared from the laser-captured tissues with enriched tumor cells were variable from one tumor to another. The overall consensus was that only Slug mRNA was significantly decreased in Twist1TKO/RCIP versus WT/RCIP tumors (SI Appendix, Fig. S8 B–E). Double IF staining detected 3.9% of Snail+, 34% of Slug+, and 2.5% of Zeb2+ tumor cells from the total PyMT+ tumor cell population in WT/RCIP tumors. However, in Twist1TKO/RCIP tumors, these percentages decreased to 0.4%, 7%, and 0.2%, respectively. The Snail+/PyMT+ and Zeb2+/PyMT+ tumor cells were usually observed in the central areas of the tumor cell clusters, whereas Slug+/PyMT+ tumor cells were distributed in both central and peripheral areas of the tumor cell clusters in WT/RCIP tumors. In Twist1TKO/RCIP tumors, the numbers of Snail+, Zeb2+, and Slug+ tumor cells in the central areas were significantly reduced, but the number of Slug+ tumor cells in the peripheral area remained unchanged. We did not detect any PyMT+/Zeb1+ tumor cells in either WT/RCIP or Twist1TKO/RCIP tumors (Fig. 2A and SI Appendix, Figs. S9–S12). All these TF proteins were detected in the fibroblast-like stromal cells and showed no obvious differences in WT/RCIP and Twist1TKO/RCIP tumors (SI Appendix, Figs. S9–S12). These results indicate that Snail, Slug, and Zeb2 proteins are expressed in subsets of tumor cells, and tumor cell-specific KO of Twist1 decreases the number of tumor cells with their expression.

Interestingly, many Snail+, Slug+, and Zeb2+ tumor cells were located in the tumor cell clusters, whereas most Twist1+ tumor cells were observed in WT/RCIP tumors. Semiquantitative analysis revealed that in these areas, all the Snail+ and Zeb2+ tumor cells and 50% of the Slug+ tumor cells were also Twist1+ tumor cells. Strikingly, the clustered Snail+, Slug+, and Zeb2+ tumor cells observed in WT/RCIP tumors were not observed in Twist1TKO/RCIP
tumors. In the similar areas of clustered tumor cells, no Snail+ and very few (0.5%) Zeb2+ tumor cells were present, and the number of Slug+ tumor cells was also significantly reduced to 18% (Fig. 2R and SI Appendix, Figs. S13–S15). These results demonstrate that Twist1 protein is commonly coexpressed with Snail, Slug, and Zeb2 in a subset of tumor cells, and Twist1 KO results in a dramatic decrease in the expression of these EMT-inducing TFs in these tumor cells.

**Twist1 Expression Silences Luminal but Activates Basal Cell Marker Expression in the Tumor Cells.** ERα, a marker of ERα+ LECs, was detected in ~50% of either WT/RCIP or Twist1TKO/RCIP tumor cells at 6 wk. At 10 wk, ERα was still detected in ~50% of Twist1TKO/RCIP tumor cells, with 30% of these cells expressing low-level ERα. However, only low-level ERα was detected in ~20% of WT/RCIP tumors at 10 wk. The number of WT/RCIP tumors with high ERα Allred scores (IRS > 4) was significantly reduced compared with Twist1TKO/RCIP tumors, suggesting Twist1 KO maintained ERα expression. However, according to the Allred scoring system (27), all tumors of both groups still belonged to the category of ERα+ tumors (IRS > 2) (SI Appendix, Fig. S4).

In the whole WT/RCIP and Twist1TKO/RCIP tumors, the mRNA expression levels of multiple luminal and basal breast tumor markers including ErbB3, Muc1, PAM, E-cad, Anxa1, and Jag1 also showed no significant changes (SI Appendix, Fig. S16), suggesting Twist1 KO does not globally change a luminal tumor to a basal-like tumor.

We next performed double and triple IF staining to assess the feature of individual Twist1+ tumor cells. In WT/RCIP tumors, the cells in the Twist1+ tumor cell clusters did not express ERO and the LEC marker K8 (SI Appendix, Fig. S17). Most of these Twist1+ tumor cells, but not all, expressed basal cell markers K5 and p63 (SI Appendix, Fig. S18). Twist1+/K5+/K5+/p63+ basal-like tumor cells were rare, and were usually located in the peripheral areas of the tumor cell clusters and scattered among the K8+ luminal tumor cells (SI Appendix, Figs. S18 and S19). Twist1+/K5+/K5+/p63+ ERα+ luminal tumor cells were the main cell population in both WT/RCIP and Twist1TKO/RCIP tumors (SI Appendix, Figs. S17 and S19). In Twist1TKO/RCIP tumors, the Twist1+/K5+/K5+/p63+ tumor cell pocket was not observed (SI Appendix, Figs. S17–S19). These results suggest that most of the Twist1+ cells are basal-like tumor cells. In addition, FACS assay for counting tumor cells expressing ALDH1, a marker of mammary epithelial or cancer stem-like cells, and mammosphere (or tumorsphere) growth assay revealed no significant differences in the number of stem-like tumor cells in WT/RCIP and Twist1TKO/RCIP tumors (SI Appendix, Fig. S20).

**Twist1 Expression Promotes Tumor Cell Dissemination into the Blood Circulation.** We examined circulating tumor cells (CTCs) in the peripheral white blood cell (PBWC) preparation from 0.15 mL blood by IF staining for the tumor cell-specific markers Cre or PyMT-HA. Numerous CTCs were identified in eight of nine blood samples from mice with large WT/RCIP tumors. However, no CTC was detected from all seven blood samples from mice with large WT/RCIP tumors. The indicated percentage data were from counting 4,660 Twist1+/A,4,820 Cre+, 4,426 Snail+/A,426 Cre−, 2,986 Slug+/A,800 Cre+, 0 Zebl+/A,000 PyMT+, and 3,200 Zeb2+/A,426 PyMT+ CTCs, respectively. (A) IF staining for Cre or PyMT-HA antibody to detect tumor cells (green). The number of Cre+ or PyMT+ CTCs in each sample was counted and presented in the bar graph. (B) Double IF staining for Cre+ tumor cells and Twist1, Snail, Slug, Zebl1, or Zeb2. Cells were prepared from 1.5 mL blood of mice with large WT/RCIP tumors. The indicated percentage data were from counting 4,480 E-cad+/A,280 PyMT/Cre+, 6,186 Vim+/A,133 PyMT+, and 2,080 Fsp1+/A,640 PyMT+ CTCs, respectively. (C) Double IF staining for PyMT and E-cadherin (E-cad), vimetin (Vim), or Fsp1 in WT/RCIP CTCs. The percentage data were from counting 4,872 E-cad+/A,280 PyMT/Cre+, 6,186 Vim+/A,133 PyMT+, and 2,080 Fsp1+/A,640 PyMT+ CTCs, respectively. (D) Double IF staining for E-cad and Vim in WT/RCIP CTCs. The percentage data were from counting 5,973 CTCs with 5,400 E-cad+, 5,866 Vim+, and 5,333 E-cad+/Vim+ CTCs, respectively. (E) H&E-stained lung sections prepared from MMTV-TVA+/A, Twist1+ mice with the 10-wk WT/RCIP tumors and MMTV-TVA+/A, Twist1+ mice with the 10-wk Twist1TKO/RCIP tumors. Arrows indicate metastatic foci. A metastatic index was presented as the average percentage of metastasis tumor areas to total lung areas measured. (Magnification: A–D, 400X; E, 100X.)

62% of these CTCs. Weak Zebl2 immunostaining signals were found in 72% of CTCs, but Zebl1 was undetectable in these CTCs (Fig. 3B). Among the epithelial and mesenchymal markers, E-cadherin, vimetin, and Fsp1 were detected in 85%, 99%, and 44% of WT/RCIP CTCs, respectively (Fig. 3C). Double IF staining revealed that 2%, 9%, and 89% of WT/RCIP CTCs were positive to E-cadherin only, vimetin only, and both of them, respectively (Fig. 3D). These results indicate that multiple EMT-inducing TFs are coexpressed in the majority of WT/RCIP CTCs. Most CTCs maintain the expression of both epithelial and mesenchymal markers, exhibiting a partial EMT phenotype in the blood circulation.

**Tumor Cell-Specific KO of Twist1 Inhibits Lung Metastasis.** Lung metastasis was not observed in either MMTV-TVA+/A, Twist1+ or MMTV-TVA+/A, Twist1− mice with palpable small tumors for 6 wk or shorter. At 8 wk, lung metastases were observed in the former mice with WT/RCIP tumors, but not observed in mice with Twist1TKO/RCIP tumors. At 10 wk, lung metastatic foci were observed in 67% (12/18) of mice with large WT/RCIP tumors, with an average of 3.89 metastatic foci per lung. However, lung metastases were only observed in 39% (7/18) of mice with large Twist1TKO/RCIP tumors, with an average of only 0.83 metastatic foci per lung (SI Appendix, Fig. S21C). On the lung sections, the percentage of metastasis area to total lung area, an index for metastasis extent,
was also drastically reduced in mice with Twist1TKO/RCIP tumors versus mice with WT/RCIP tumors (Fig. 3E). In addition, only one of the examined five MMTV-TVA×Twist1+/6 heterozygous mice with RCIP-induced large MG tumors showed lung metastatic foci, suggesting a decreased Twist1 gene dosage is correlated with a reduced lung metastasis. These results demonstrate that Twist1 KO in the primary mammary tumors largely inhibits both the incidence and the extent of lung metastasis.

The lung metastatic lesions of Twist1TKO/RCIP tumors showed a slightly higher rate of cell proliferation compared with the lung metastatic lesions of WT/RCIP tumors (SI Appendix, Fig. S22A), suggesting the decreased lung metastatic lesions of the Twist1 KO tumor cells was not caused by any decrease in their proliferation in the lung. Next, we isolated genomic DNA samples from the lung sections of MMTV-TVA×Twist1+/6 and MMTV-TVA×Twist1+/6 mice with large WT/RCIP and Twist1TKO/RCIP tumors and performed genotype analysis. The strong DNA band for the tumor cell-specific PyMT was detected in the lungs of MMTV-TVA×Twist1+/+ mice, which were consistent with the extensive lung metastases of WT/RCIP tumors in these mice. Among seven examined lungs of MMTV-TVA×Twist1+/6 mice, weak PyMT DNA bands were detected in four lungs, which were consistent with the decreased metastases in these mice versus MMTV-TVA×Twist1+/+ mice. As expected, the Twist1+/+ (WT) and Twist1+/6 alleles were respectively detected in the lung sections of MMTV-TVA×Twist1+/+ and MMTV-TVA×Twist1+/6 mice, and the deleted Twist1 allele was absent in the lungs of MMTV-TVA×Twist1+/+ mice. However, the deleted Twist1 alleles were detected in the lungs of MMTV-TVA×Twist1+/+ mice where PyMT DNA was detected, suggesting the presence of Twist1TKO/RCIP tumor cells in these lungs (SI Appendix, Fig. S22B). All PyMT+ WT/RCIP and Twist1TKO/RCIP tumor cells in the lung metastases were E-cadherin+, but Twist1+ and vimentin+. Furthermore, there were no E-cadherin and vimentin double-positive tumor cells in these lung metastases (SI Appendix, Fig. S23). These results demonstrate that all tumor cells in the lung metastases have a typical epithelial cell phenotype.

### Discussion

The MMTV-TVA/RCIP mouse model system deletes the floxed Twist1 and expresses PyMT in the same RCIP virus-infected LECs that express Twist1 KO tumor cells. These tumor cells are induced from a very small subset of the TVA-expressing LECs infected by RCIP virus, which closely simulates spontaneous tumorigenesis in humans with tumor initiation and growth in a normal surrounding cellular environment. In RCIP virus, the Cre-coding sequence is located before short synthetic internal ribosome entry site and PyMT, which allows Cre to be expressed higher than PyMT to guarantee efficient Twist1 KO in tumor cells. The single virus-mediated coexpression of Cre and PyMT also saves time and resources by reducing crossbreeding of multiple mouse lines. These unique features make this system very useful for characterizing the genetic roles of floxed genes during MG tumor initiation, progression, and metastasis.

Although RCIP-mediated deletion of the floxed Twist1 alleles is complete, RCIP virus-induced initiation and growth of MG tumors with or without Twist1 KO are the same, indicating that Twist1 is not required for MG tumor formation from LECs. In the KRAS-induced pancreatic ductal adenocarcinoma mouse model, inactivation of Twist1 also does not change primary tumor burdens (7). These findings further validate the previous studies showing knockdown of Twist1 in BrC cell lines did not affect their proliferation in culture and xenograft tumors in mice (13, 15). In contrast, one study reported an essential role of Twist1 in developing skin tumors induced by carcinogens or KRAS (28). Together, these studies suggest the role of Twist1 in carcinogenesis depends on different tissue environments and/or oncogenic factors.

Twist1 is not expressed in normal LECs and early-stage tumor cells induced by RCIP. Accordingly, tumor cells with EMT markers are barely detected and lung metastasis is also not observed at this stage. Different levels of Twist1 are only detected in a small subset of late-stage WT/RCIP tumor cells. Most of these Twist1 tumor cells also express multiple EMT-inducing TFs such as Snail, Slug, and Zeb2 and coexpress both the mesenchymal marker vimentin and the epithelial marker E-cadherin, although E-cadherin is at a lower level versus Twist1 tumor cells. Importantly, Twist1 KO largely diminished Snail+, Slug+, and Zeb2+ tumor cells in Twist1TKO/RCIP tumors, suggesting Twist1 expression is tightly associated with the expression of these EMT-inducing TFs. As a consequence, Twist1 KO also largely diminished the presence of tumor cells with a partial EMT state in the late-stage Twist1TKO/RCIP tumors. These results clearly demonstrate that Twist1 expression is positively associated with and required for the expression of multiple EMT-inducing TFs, which should work together with Twist1 to program the partial epithelial-to-mesenchymal plasticity of the tumor cells. This also suggests that Twist1 is indeed a master regulator for programming breast tumor cell plasticity in vivo.

Although the expression of Twist1 and other EMT-inducing TFs in small subsets of tumor cells did not change the overall morphology and tumor subtype category of the whole tumor, they actually reprogrammed the lineage of individual tumor cells from luminal to basal-like, as indicated by the loss of ER and K8 luminal markers and the gain of K5 and p63 basal cell markers. As discussed earlier, most of these Twist1+ basal-like tumor cells also exhibit a partial EMT plasticity. We speculate that these cells may be in an unstable transition state that could go either epithelial or mesenchymal direction, depending on the tumor microenvironmental cues that induce the expression of EMT-inducing TFs. Once these cells leave their environment, the expression of these TFs would turn off. This may explain why the number of cancer stem-like cells kept unchanged in WT/RCIP tumors with these Twist1+/K5+/p63+ basal-like tumor cells and Twist1TKO/RCIP tumors without this type of tumor cells, even K5+/p63+ cells, are usually considered to contain stem-like or progenitor cells.

WT/RCIP tumors produced many CTCs, but the number of Twist1 KO CTCs was extremely rare in the mouse blood, in accordance with the results of a previous study. Using a squamous cell carcinoma model with inducible Twist1 expression, this study showed that although Twist1 expression promotes tumor cell invasation, turning off Twist1 expression is required for the disseminated tumor cells to establish metastases in a distant organ (29). Another study also showed that Snail expression in the mouse mammary tumor cells is transient, and only transient induction of Snail overexpression in the primary tumor cells promotes their metastases (4). Therefore, it is possible that Twist1 expression in primary...
tumor cells and CTCs induces partial EMT and drives local invasion, intravasation, and extravasation. After CTCs invade the lung tissue, Twist1 expression shuts down, probably because of environmental change, to allow metastatic colonization. It has been shown that silencing the expression of Prx1, a homeobox factor that induces EMT and cell migration and invasion, or both Prx1 and Twist1 significantly promotes BrC cell colony formation in culture and metastatic colonization in vivo (30). Future studies that are designed to trace Twist1+ tumor cells will help validate whether all lung metastases are derived from Twist1+ primary tumor cells and CTCs in this mouse model.

The results obtained from our genetic mouse models with competent immune system are consistent with the previous studies showing that knockdown of Twist1 in established BrC cell lines inhibits their metastases in immune-defective host mice (8, 13, 15). Our results that Twist1 expression in the primary tumor cells and CTCs induces partial EMT and promotes metastasis in mice are also consistent with the results of previous studies, which showed positive associations among Snail expression, EMT phenotype, and metastasis in another mouse MG tumor model (4). In contrast, Twist1 is shown to be nonessential for the metastasis of KRAS-induced pancreatic cancer in mice (7), suggesting Twist1 may play different roles in different cancer models. One tumor cell lineage-tracing study also showed that the initial Fsp1+ mouse MG tumor cells induced by PyMT do not metastasize to the lung, suggesting that not all tumor cells with EMT markers are metastatic (6). In our mouse model, Fsp1 and Twist1 are not coexpressed in the same fibroblasts in the mouse MGs. In the mice with the late-stage WT/RCP tumors, the number of Fsp1+ tumor cells is rare (<1%), and only 36% of Twist1+ cells are Fsp1 positive in the primary tumors. The number of Fsp1+ CTCs is less than half of the number of vimentin+ CTCs. These results suggest that Twist1+/vimentin+/Fsp1+ and Twist1+/vimentin+/Fsp1+ tumor cells may belong to different subpopulations with different metastatic potentials.

In summary, Twist1 and other EMT-inducing TFs are coexpressed in a subset of primary MG tumor cells and CTCs, which reprograms a partial epithelial-to-mesenchymal plasticity and a luminal-to-basal cell lineage change required for intravasation. After disseminating to the lung tissue, these tumor cells may return to epithelial state because of environmental change and develop macro lung metastases. KO of Twist1 largely inhibits the expression of other EMT-driving TFs and the partial EMT phenotype in the primary tumor cells and effectively blocks tumor cell intravasation and lung metastases. These findings suggest that inhibition of either expression or function of Twist1 in the primary MG tumor cells should significantly block BrC cell intravasation and metastasis.

Materials and Methods

Animal protocols were approved by the Animal Care and Use Committee of Baylor College of Medicine. Replication-competent avian sarcoma-leukosis-Cre-short synthetic internal ribosome entry site-PyMT virus was constructed, produced, and injected into the MG ducts of MMTV-TVAxRosa26R, MMTV-TVAxWT, and MMTV-TVAxTwist1+/− female mice for inducing tumorigene- sis. MG tumor initiation, growth, and lung metastases were monitored at different time points. Tissue sections prepared from MG tumors and lung sections were used for IHC and double or triple IF staining for PyMT, Twist1, E-cadherin, vimentin, Fsp1, Snail, Slug, Zeb1/2, Eru, K8, K5, p63, Ki67, CD31, and/or F4-80. CTCs were prepared together with PBMCs from blood samples by lysing red blood cells and attaching the cells to the culture chamber slides. These slides were used for IF staining for tumor cell markers Cre or PyMT, and other proteins such as Twist1, Smal, Slug, Zeb1, E-cadherin, and vimentin, to allow staining, immunoblotting, laser capture, qPCR, FACS, and mammosphere growth assay were performed by following commonly used protocols. All experimental materials and procedures are described in detail in SI Appendix, SI Materials and Methods.

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