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Snail2 is an essential mediator of Twist1-induced epithelial-mesenchymal transition and metastasis

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Abstract

To metastasize, carcinoma cells must attenuate cell-cell adhesion to disseminate into distant organs. A group of transcription factors, including Twist1, Snail1, Snail2, ZEB1, and ZEB2, have been shown to induce Epithelial-Mesenchymal Transition (EMT), thus promoting tumor dissemination. However, it is unknown whether these transcription factors function independently or coordinately to activate the EMT program. Here we report that direct induction of Snail2 is essential for Twist1 to induce EMT. Snail2 knockdown completely blocks the ability of Twist1 to suppress *E-cadherin* transcription. Twist1 binds to an evolutionarily conserved E-box on the proximate *Snail2* promoter to induce its transcription. Snail2 induction is essential for Twist1-induced cell invasion and distant metastasis in mice. In human breast tumors, the expression of Twist1 and Snail2 is highly correlated. Together, our results show that Twist1 needs to induce Snail2 to suppress the epithelial branch of the EMT program and that Twist1 and Snail2 act together to promote EMT and tumor metastasis.

Keywords

tumor metastasis; E-cadherin; Epithelial-Mesenchymal Transition; Snail2; Twist1

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Disclosure of Potential Conflict of interest

No potential conflict of interest was disclosed.

Introduction

For carcinoma cells to break away from neighboring cells and invade, they must lose cell-cell adhesion and gain motility (1). A highly conserved developmental program named Epithelial-Mesenchymal Transition (EMT) has been implicated in promoting dissemination of single carcinoma cells from primary epithelial tumors (2). The EMT program is activated during many developmental processes—such as mesoderm formation and neural crest development (3). During EMT, cells lose their epithelial characteristics, including cell adhesion and polarity, and acquire a mesenchymal morphology and the ability to migrate. Biochemically, cells switch off the expression of epithelial markers, such as adherens junction protein E-cadherin and turn on mesenchymal markers, including vimentin and fibronectin (4).

Functional loss of E-cadherin in an epithelial cell has been considered a hallmark of EMT. Forced expression of E-cadherin in certain invasive carcinoma cells can inhibit their ability to invade and metastasize; conversely, blocking E-cadherin function in non-invasive tumor cells activates their invasiveness and metastatic abilities (5,6). A partial loss of E-cadherin is also associated with carcinoma progression and poor prognosis in various tumor types (7).

Transcriptional modulation of the *E-cadherin* gene promoter is a key event to suppress E-cadherin expression during EMT. The human *E-cadherin* promoter contains E-box elements that are responsible for its transcriptional repression (8,9). Several Zn-finger transcription factors, including Snail1 (10,11), Snail2 (12), ZEB1 (13), and ZEB2 (14), are capable of directly binding to the E-boxes of the *E-cadherin* promoter to repress its transcription. In addition to these Zn-finger transcription factors, transcription factors belonging to other families have also been shown to be able to regulate EMT in culture and during development. In a search for genes involved in mouse mammary tumor metastasis, the bHLH transcription factor Twist1 is found to be capable of inducing EMT in human mammary epithelial cells. Our previous study also found that the Twist1 transcription factor was essential for the ability of tumor cells to metastasize from the mammary gland to the lung in a mouse breast tumor model (15).

During EMT in metastasis and in embryogenesis, many EMT-inducing transcription factors are often activated simultaneously, such as expression of Twist1, Snail1, Snail2 and ZEB2 in neural crest cells (16,17). To understand how these transcription factors coordinate the EMT program, we have used an inducible Twist1 system to address whether and how Twist1 activates other EMT-inducing transcription factors to suppress E-cadherin and promote EMT and tumor metastasis.

Materials and methods

Cell lines

HMLE cells were obtained from Dr. Robert Weinberg and cultured as described (18). SUM1315 and MCF7 cells were obtained from ATCC and cultured as described (19).

Antibodies

Primary antibodies used include Twist1 (20), E-cadherin, β -catenin, γ -catenin, fibronectin, vimentin, N-cadherin (BD Biosciences), β -actin (Abcam), as previously described (15); H-Ras (18), Snail2 (21) (Santa Cruz), and α -tubulin (22) (Abcam).

Quantitative PCR

Total RNAs were extracted using RNeasy Mini Kit (Qiagen) and reverse transcribed using cDNA Reverse Transcription Kit (Applied Biosystems). Resulting cDNAs were analyzed in triplicates using SYBR-Green PCR mix (Applied Biosystems). Relative mRNA concentrations were determined by $2^{-(Ct-Cc)}$ where Ct and Cc are the mean threshold cycle differences after normalizing to GAPDH values. Primers used for PCR are listed in Supplementary Materials.

Immunofluorescence

Cells were grown on coverslip slides for 3 days, fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 for 10 min, and blocked with 5% goat serum. Samples were incubated with primary and Alexa secondary antibodies (Invitrogen). After washing, wells were covered with SlowFade with DAPI (Invitrogen). Antibodies were used as described (15).

Chip sequencing

An Illumina sequencing library was prepared from Twist1 ChIP DNA obtained from induced HMLE-Twist1-ER cells using the ChIP-seq Sample Preparation Kit (Illumina). The DNA library was sequenced on the Illumina Genome Analyzer. The resulting sequence reads were mapped to the human genome using the Illumina software suite. The data are displayed on the UCSC genome browser 2006 assembly.

Chromatin Immunoprecipitation

Cells were crosslinked with 1% Paraformaldehyde (PFA), lysed and sonicated. Nuclear lysates were incubated with Protein G Dynabeads (Invitrogen) conjugated with an anti-Twist1 or anti-estrogen receptor antibody overnight. DNA was reverse crosslinked and purified. Primers used for PCR were described in Supplementary Materials.

Luciferase Reporter assay

MCF7 cells were transfected with Snail2prom-Luc2 reporter plasmid, pGL4[RLuc] plasmid, and additional plasmids as described. 24 hours later, the cell lysates were assayed accordingly (Promega). The firefly luciferase activity was normalized to that of Renilla luciferase.

Promoter sequence alignment

Analysis of the Snail2 promoter E-box sequences across species was performed using ConTra online software (23).

Invasion and Migration Assays

40,000 cells were cultured on 8mm Transwell (Costar) for 72 hours, fixed with 4% paraformaldehyde, washed and stained with 0.1% crystal violet. The top membrane was cleaned, washed, and dried. Crystal violet was released with 10% acetic acid and the absorbency was measured at 520 nm. All assays were performed in triplicates.

Subcutaneous tumor implantation and metastasis assay

1 million of HMLER-Twist1 cells expressing shControl and 4 million of HMLER-Twist1 cells expressing shSnail2 were injected subcutaneously into Nude mice. Tumor size was measured weekly until reaching 2cm in diameter before mice were sacrificed. Lungs were imaged under a fluorescence dissection microscope. The total number of GFP positive

colonies was quantified using ImageJ. Graphed tumor weight represents total primary tumor burden per mouse.

Bioinformatics and Statistical Analysis

Three published microarray datasets were downloaded from NCBI's Gene Expression Omnibus (GEO). Obtained GEO identifiers (Study name, sample size) were GSE1456 (STOCKHOLM, n=159), GSE2034 (EMC, n=286), and GSE3494 (UPPSALA, n=236). All samples were from breast cancer patients. Quantile-normalization was performed on an integrated dataset of .CEL files (24). The Median-Polish algorithm was used to convert multiple probe-level measurement values into the gene-level aggregate value (25). The Spearman correlation coefficient was calculated between *Twist1* and individual genes and ranked in individual datasets. Measurement values of the two probe sets were plotted in Supplementary Figure 4 with a linear regression model fit.

Results

Twist1 indirectly suppresses *E-cadherin* transcription to promote EMT

To understand how Twist1 suppresses *E-cadherin* expression during EMT, we have generated a fusion protein between Twist1 and the modified hormone-binding domain of estrogen receptor (Twist1-ER), which can be activated specifically upon binding to 4-hydroxytamoxifen (26). We expressed Twist1-ER in the immortalized human mammary epithelial (HMLE) cells via retroviral infection. HMLE cells expressing Twist1-ER underwent morphological changes of EMT upon 4-hydroxy-tamoxifen treatment in 10–12 days (Figure 1A). We harvested these cells at day 18 to characterize the EMT markers and found that activation of Twist1 resulted in loss of cell adhesion and gain of mesenchymal differentiation in HMLE cells (Figure 1B), identical to the EMT program induced by Twist1 (15).

We next examined the expression of *E-cadherin* mRNA and protein following Twist1 activation. Interestingly, both *E-cadherin* mRNA and protein levels remained unchanged during the first 7 days after Twist1 activation and only started decreasing afterwards. By the end of the 12 day time course, both *E-cadherin* mRNA and protein decreased drastically (Figure 1C and 1D), which correlated with loss of cell adhesion in cell morphology (Figure 1A). Given that 4-hydroxytamoxifen activates the Twist1-ER fusion protein immediately (26), the 6–8 days required to reduce *E-cadherin* mRNA and protein levels suggest that Twist1 does not directly suppress *E-cadherin* transcription during EMT.

Induction of the transcription factor Snail2 is required for Twist1-induced EMT

Since Twist1 may not directly suppress *E-cadherin* transcription, we hypothesized that Twist1 might activate other Zn-finger transcription factors such as Snail1, Snail2, ZEB1 and ZEB2 to suppress *E-cadherin* transcription. During *Drosophila* mesoderm formation, Twist1 directly induces the expression of Snail1 to promote EMT (27). To test whether Twist1 induces the Snail family transcription factors in human cells, we examined Snail1 and Snail2 expression following Twist1 activation. Interestingly, Snail2 was induced within four hours upon Twist1 activation (Figure 2A), while Snail1 was only significantly induced at Day 12 (Supplementary Figure 1). Induction of Snail2 protein was also observed in HMLE-Twist1-ER cells two days upon Twist1 activation and in HMLE cells expressing Twist1 (Figure 2B). Since we are interested in possible direct target genes that Twist1 regulates to suppress *E-cadherin*, we hypothesized that induction of Snail2 by Twist1 could play a key role in the transcriptional repression of *E-cadherin*.

To test whether induction of Snail2 is required for the ability of Twist1 to induce EMT, we designed two shRNA lentiviral constructs specifically against human *Snail2* and expressed them in HMLE cells. Both shSnail2-1 and shSnail2-2 could efficiently suppress Snail2 mRNA and protein below the basal level in HMLE cells (Figure 2A–2C). We expressed both shRNAs against Snail2 in HMLE cells expressing the inducible Twist1-ER construct and treated the cells with 4-hydroxy-tamoxifen. Interestingly, in the absence of Snail2, Twist1 was not able to induce the morphological changes associated with EMT as observed in HMLE cells expressing a control shRNA construct. Instead, these cells with shSnail2 constructs maintained an epithelial morphology with intact cell-cell adhesions and a cobblestone-like morphology even after 18 days (Figure 2D). Also knockdown of Snail2 did not affect Twist1 expression in these cells (Figure 2B). Together, these data indicate that induction of Snail2 is required for the ability of Twist1 to promote EMT in HMLE cells.

Induction of Snail2 is specifically responsible for suppression of E-cadherin in response to Twist1 activation

To determine how suppression of Snail2 affects expression of various EMT markers, we closely followed the entire time course of Twist1 activation in HMLE-Twist1-ER cells expressing shSnail2 or a control shRNA. First, we examined the expression of E-cadherin following the induction time course, since Snail2 is a direct repressor of E-cadherin (12). Indeed, suppression of Snail2 in HMLE-Twist1-ER cells completely inhibited the progressive reduction of E-cadherin mRNA and protein upon 4-hydroxytamoxifen treatment (Figure 3A and 3B). In HMLE-Twist1-ER cells expressing a control shRNA, E-cadherin expression started decreasing at Day 7 after Twist1 activation; at Day 18, E-cadherin protein was completely lost and β -catenin disappeared from cell membranes, indicating the loss of adherens junctions upon Twist1 activation (Figure 3C). In contrast, in HMLE-Twist1-ER cells expressing shSnail2, both E-cadherin and β -catenin remained at cell-cell junctions even after 18 days of Twist1 activation (Figure 3C). These results indicate that Snail2 is critical for the ability of Twist1 to suppress E-cadherin and induce EMT.

To understand whether suppression of E-cadherin by Snail2 is the only effect of Snail2 on the entire EMT program, we examined the mRNA and protein expression of EMT mesenchymal markers in response to Twist1 activation. Interestingly, we found that unlike E-cadherin, the initial induction of other EMT markers, including fibronectin, vimentin, and N-cadherin occurred normally in the absence of Snail2 in HMLE-Twist1-ER cells upon 4-hydroxytamoxifen treatment (Figure 4A). However, at Day 7 when E-cadherin was not suppressed in HMLE-Twist1-ER cells expressing shSnail2, fibronectin, vimentin, and N-cadherin proteins failed to increase further to reach the high levels of induction observed in HMLE-Twist1-ER cells expressing a control shRNA (Figure 4A and 4B). We also performed immunofluorescence analysis to examine the expression and localization of these EMT markers and observed similar effects (Supplementary Figure 2). In addition, we attempted to overexpress Snail2 in HMLE cells and found that Snail2 alone was not sufficient to induce EMT in HMLE cells (data not shown). These results suggest that, although Snail2 is not directly responsible for the induction of fibronectin, vimentin, and N-cadherin during EMT, loss of Snail2 and E-cadherin suppression completely blocks EMT morphogenesis and attenuates the expression of mesenchymal markers, including fibronectin, vimentin, and N-cadherin.

Snail2 is a conserved direct transcriptional target of Twist1 in amniotes

Since Snail2 is induced immediately upon Twist1 activation and plays a critical role in Twist1-induced EMT, we tested whether Twist1 directly binds to the *Snail2* gene promoter to activate its transcription. Using Chromatin Immunoprecipitation (ChIP) coupled with high-throughput sequencing, we identified a distinct Twist1 binding E-box domain 306bp

from the transcription start site of the human *Snail2* promoter (Figure 5A). In contrast, there is no significant enrichment for the 10kb promoter regions of *Snail1* or *E-cadherin* (Figure 5A). To demonstrate that Twist1 specifically binds to the *Snail2* promoter, we treated HMLE-Twist1-ER cells with 4-hydroxytamoxifen for four days and performed ChIP analysis to pull-down Twist1-binding DNA fragments. Both Twist1 and ER antibodies were able to specifically enrich the E-box region of the human *Snail2* promoter, while there was no specific enrichment for the control *GAPDH* gene (Figure 5B). To test the specificity of the Twist1-binding E-box on the *Snail2* promoter, we tested the ability of Twist1 to activate the isolated 606bp human *Snail2* promoter by luciferase reporter assay. We found that Twist1 was able to activate the *Snail2* promoter-driven luciferase activity and mutation of the E-box sequence on the *Snail2* promoter reduced Twist1-induced activation (Figure 5C). These data suggest that Twist1 specifically and directly binds to the E-box on the human *Snail2* promoter to activate its transcription.

To test whether the direct regulation of *Snail2* by Twist1 is conserved throughout evolution, we searched for the consensus Twist1-binding E-box and its surrounding sequence on the human *Snail2* promoter across species. Interestingly, we found that this consensus Twist1-binding sequence (ccaCATcTGgaagcc) on the *Snail2* promoter is highly conserved among amniote genomes examined, including chimpanzee, monkey, mice, rabbit, armadillo, elephant, hedgehog, and chick (Figure 5D). However, this sequence could not be found in *Xenopus* and *Zebrafish*, indicating that the direct induction of *Snail2* by Twist1 is highly conserved in amniotes.

Induction of *Snail2* is required for the ability of Twist1 to promote invasion and metastasis

Given that induction of *Snail2* is required for activating the EMT program by Twist1, we next tested whether *Snail2* plays an essential role in Twist1-mediated tumor cell invasion and metastasis. As in HMLE-Twist1-ER cells, knockdown of *Snail2* also prevented constitutive Twist1-induced EMT in HMLE cells (Figure 6A). We tested whether *Snail2* is required for Twist1-induced cell migration and invasion. As shown in Figure 6B, suppression of *Snail2* resulted in reduced cell migration and invasion. This data indicate that cell migration and invasion induced by Twist1 require the activation of the entire EMT program. Without *Snail2*, Twist1 is not sufficient to promote cell migration and invasion.

We next tested whether *Snail2* is required for the ability of Twist1 to promote tumor metastasis in vivo. To assess the role of *Snail2* in tumor metastasis, we transformed GFP-labeled HMLE-Twist1 cells expressing sh*Snail2* or a control shRNA with oncogenic Ras (HMLER cells) (Figure 6A). When we implanted these cells subcutaneously in nude mice, HMLER-Twist1 cells expressing a control shRNA generated hundreds of tiny GFP+ micrometastatic lesions in the lung (Figure 6C). Interestingly, when *Snail2* was knocked down, HMLER-Twist1 cells failed to form GFP+ metastatic lesions in the lung (Figure 6C), even though the primary tumors reached similar weight as shControl tumors (Figure 6D). It is worth noting that knockdown *Snail2* reduced the initial primary tumor seeding rates, probably due to the difficulty for epithelial cells to establish cell-cell adhesion after being injected subcutaneously in suspension. Therefore we allowed the *Snail2* knockdown tumors to grow four more weeks in mice to reach the same primary tumor size as the control tumors (Supplementary Figure 3). Even with longer primary tumor growth time and similar final primary tumor burdens, examination of individual lung lobes revealed that suppression of *Snail2* caused a 500-fold reduction in the ability of HMLER-Twist1 cells to form micrometastasis in the lung (Figure 6D). Immunohistochemistry analysis of the resulting tumors showed that E-cadherin expression was completely absent from cell membranes in HMLE-Twist1 tumors, while suppression of *Snail2* resulted in epithelial tumors with strong E-cadherin expression at cell-cell junctions (Figure 6C). Together, these results demonstrate that activation of Twist1 is sufficient, at least in some cell types, to promote tumor cells to

disseminate from the primary site to distant organs. Furthermore, the data strongly indicate that Snail2 functions downstream of Twist1 to play a critical role in regulating tumor invasion and metastasis.

Twist1 and Snail2 are frequently co-expressed in human breast tumors

To further demonstrate that a direct link between Twist1 and Snail2 exists in human tumor cells, we expressed shRNAs against Twist1 in the human breast tumor cell line SUM1315 that expresses high levels of endogenous Twist1 and Snail2. Indeed, when we examined the expression of endogenous Snail2 in these cells, we observed a dramatic reduction of Snail2 expression upon suppression of Twist1 (Figure 7A). This result indicates that expression of endogenous Snail2 is dependent on Twist1 in human breast tumor cells.

To more directly probe the association between Twist1 and Snail2 in human breast tumor samples, we analyzed three published human breast tumor gene expression datasets summing up to 681 primary breast cancers (28–30). In each data set, we ranked all 22282 genes based on how tight their expression levels are correlated with Twist1 in individual samples. As shown in Figure 7B, in all three datasets, Snail2 was consistently ranked as one of the most correlated genes with Twist1. Furthermore, expression of Twist1 and Snail2 were positively correlated (Supplementary Figure 4). The association between Twist1 and Snail2 in human breast tumor samples provides further support for a functional link between Twist1 and Snail2 in breast tumor progression.

Discussion

Our study has identified Snail2 as the direct transcription target of Twist1 in repressing E-cadherin during the EMT program. We show that induction of Snail2 is required for the ability of Twist1 to promote EMT in culture and distant metastasis in mice. Furthermore, we identified a conserved Twist1-binding site on the proximate Snail2 promoter in amniotes. These results show that Twist1 induces Snail2 to coordinate the EMT program to promote tumor metastasis (Figure 7C).

Our study revealed an evolutionally conserved link between Twist1 and its direct transcription target Snail2 in suppressing *E-cadherin* transcription, thus promoting the loss of cell adhesion during EMT. In *Drosophila*, Twist1 directly induces Snail1 to promote EMT during mesoderm formation (27). Our study shows that in humans, Snail2, a family member, replaces Snail1 to perform this key function downstream of Twist1 during EMT. Elevated expression of both Twist1 and Snail2 have been observed in lymph node-positive breast tumors and associated with poor outcome (31). Twist1 and Snail2 are also functionally linked during early embryogenesis. In early *Xenopus* embryo, loss of Twist1 leads to a decrease in the Snail2 mRNA level, and both of them negatively regulate the expression of Cerberus, an inhibitor of Nodal, BMP, and Wnt signaling (32). During *Xenopus* embryonic vasculogenesis, knockdown of Twist1 or Snail2 provoked similar embryonic hemorrhage, and either Twist1 or Snail2 could rescue the hemorrhage defects due to Myc knockdown (33). Together, these data show that the direct link between Twist1 and Snail2 is highly conserved and functionally important during embryogenesis and tumor metastasis.

Interestingly, induction of Snail2 is not only required for the ability of Twist1 to suppress E-cadherin, but the entire EMT morphogenesis is blocked in the absence of Snail2. Further analyses of additional EMT markers following the time course of Twist1 activation indicate that genes such as N-cadherin are able to initially increase at similar rates in the absence of Snail2. The initial immediate induction of N-cadherin is possibly due to the direct action of Twist1, together with its dimerization partner E12/E47. Interestingly, N-cadherin failed to

further increase to the high induction level after 7 days of Twist1 activation, the exact time when E-cadherin is supposed to be suppressed upon Twist1 activation. This result indicates that although Snail2 is not directly responsible for the induction of N-cadherin, induction of Snail2 and suppression of E-cadherin are essential to send out a permissive signal to allow further expression of mesenchymal markers and the progression of the EMT program.

The permissive signal that allows progression of the EMT program remains unknown. The disappearance of E-cadherin from adherens junctions results in the release of its partner, β -catenin, into the cytosol, which has the potential to enter the nucleus to activate LEF/TCF-mediated transcription. Although the contribution of the adherens junction-associated β -catenin pool to transcription is still a matter of debate, several studies suggest that β -catenin-mediated transcription can further induce the expression of Snail2 (34) and Twist1 (35), thereby contributing to the EMT program. Furthermore, fibronectin and vimentin have been shown to be β -catenin target genes (36–38), which explains why suppression of Snail2 expression blocks their further induction in response to Twist1 activation. Similarly, Zeb2 has also been shown to not only suppress E-cadherin, but also partially regulate vimentin expression (39). Together, these studies suggest that suppression of E-cadherin not only breaks the physical barrier to allow cell scattering, but also could actively contribute to activation of mesenchymal traits during EMT.

We found that Snail2 was induced four hours after Twist1 activation; however, reduction of E-cadherin mRNA only became significant seven days after Twist1 activation. This observation raises the question of whether Snail2 alone is sufficient to suppress *E-cadherin* transcription during Twist1-induced EMT. Several observations could help to explain this phenomenon. First, we found that two additional E-cadherin suppressing transcription factors, ZEB1 and ZEB2, were also induced upon Twist1 activation; and suppression of Snail2 drastically inhibited induction of ZEB1 and ZEB2 upon Twist1 activation. Therefore, induction of ZEB1 and ZEB2 could be required to collaborate with Snail2 to achieve drastic suppression of *E-cadherin* transcription. A double-negative feedback loop controlling ZEB1/ZEB2 and microRNA-200 family expression could further promote the expression of ZEB1 and ZEB2 during EMT (40–43). Together, these factors strengthen the ability of cells to suppress E-cadherin expression during EMT. Second, recent studies found that Ajuba LIM proteins, also components of adherens junctions, can travel to the nucleus to serve as corepressors with the Snail family transcription factors to repress *E-cadherin* (44,45). Therefore, suppression of E-cadherin during EMT progresses in a positive feedback loop. At the beginning of EMT, a slight decrease of E-cadherin causes the weakening of cell adhesion and the release of Ajuba LIM proteins; binding to Snail2 or Snail1, Ajuba LIM proteins can then further suppress *E-cadherin* transcription. These transcription repressors, including Snail1/Snail2, Ajuba LIM proteins, ZEB1/ZEB2, and the microRNA-200 family, function as a powerful E-cadherin suppression machine to drastically suppress *E-cadherin* transcription and promote EMT progression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Twist1 indirectly suppresses E-cadherin transcription to promote EMT

A. Bright-field images of HMLE cells expressing Twist1-ER or a control vector before and after 12 days of 20 nM 4-hydroxytamoxifen (4-OHT) treatment. B. Lysates from HMLE cells expressing Twist1-ER or a control vector were collected before and after 18 days of treatment with 4-OHT, analyzed by SDS-PAGE, and probed for Twist1-ER protein, E-cadherin, β-catenin, γ-catenin, fibronectin, vimentin, N-cadherin, and α-tubulin. C. Real-time PCR analysis of E-cadherin mRNA expression in HMLE cells expressing Twist1-ER or a control vector treated with 4-OHT. D. Lysates from HMLE cells expressing Twist1-ER or a control vector were collected during 12 days of 4-OHT treatment, analyzed by SDS-PAGE, and probed for E-cadherin and α-tubulin. HMLE cells expressing Twist1 or a control vector are used as the negative or positive control.

Figure 2. Induction of the transcription factor Snail2 is required for Twist1-induced EMT
 A. Real-time PCR analysis of Snail2 mRNA expression in HMLE cells expressing Twist1-ER or a control vector treated with 4-OHT and in HMLE-Twist1-ER cells expressing shSnail2-1, shSnail2-2 or shControl during 12 days of 4-OHT treatment. B. Lysates from HMLE cells expressing Twist1 or a control vector, from HMLE cells expressing Twist1-ER, and from HMLE-Twist1-ER cells expressing shSnail2-1, shSnail2-2 or shControl during 12 days of 4-OHT treatment were collected, analyzed by SDS-PAGE, and probed for Twist1 or Twist1-ER, Snail2, and α -tubulin. C. Quantification of the Snail2 protein level shown in the bottom panel of B. The relative Snail2 protein level was calculated by normalizing the signal of Snail2 to α -tubulin, and the values at individual timepoints were compared to the value at Day 0 for each cell line. D. Bright-field images of HMLE-Twist1-ER cells expressing shSnail2-1, shSnail2-2 or shControl before and after 12 days of 4-OHT treatment.

Figure 3. Induction of Snail2 is required for suppression of E-cadherin during Twist1-induced EMT

A. Real-time PCR analysis of E-cadherin mRNA expression in HMLE-Twist1-ER cells expressing shSnail2-1, shSnail2-2 or shControl during 12 days of 4-OHT treatment. B. Lysates from HMLE-Twist1-ER cells expressing shSnail2-1, shSnail2-2 or shControl were collected during 12 days of 4-OHT treatment, analyzed by SDS-PAGE, and probed for E-cadherin and α -tubulin. C. Cells from HMLE-Twist1-ER cells expressing shSnail2-1, shSnail2-2 or shControl were immunostained for E-cadherin and β -catenin (green) and nuclei (DAPI, blue) at Day 0, 7 and 18 after 4-OHT treatment.

Figure 4. Suppression of Snail2 attenuates expression of mesenchymal markers during Twist1-induced EMT

A. Real-time PCR analysis of fibronectin, N-cadherin, and vimentin mRNA expression in HMLE-Twist1-ER cells expressing shSnail2-1, shSnail2-2 or shControl during 18 days of 4-OHT treatment. The final folds of induction are indicated in each graph. B. Lysates from HMLE-Twist1-ER cells expressing shSnail2-1, shSnail2-2 or shControl were collected during 12 days of 4-OHT treatment, analyzed by SDS-PAGE, and probed for fibronectin, N-cadherin, vimentin, and α -tubulin.

Figure 5. *Snail2* is an evolutionally conserved direct transcriptional target of *Twist1* in amniotes
A. Right panels are ChIP-Sequencing result showing the enriched *Twist1*-binding peaks at the +5kb to -5kb regions of the human *Snail2*, *E-cadherin* and *Snail1* promoters. The Y-axis represents the amplitude of the ChIP-Seq signals. Left panel is a close-up view of the enriched *Twist1*-binding E-box sequence at the -306bp from the transcription start site of the human *Snail2* promoter. B. HMLE-*Twist1*-ER cells were treated with 4-OHT for 4 days. Chromatin was immunoprecipitated using corresponding antibodies and PCR was performed using one primer set for the *Twist1*-binding region on the *Snail2* promoter and one control set for GAPDH. C. The 606bp *Snail2* promoter or the *Snail2* promoter with E-box mutation were transfected with or without *Twist1* and its dimerization partner E47 and processed for luciferase promoter assay. D. Alignment of the conserved *Twist1*-binding E-box (bold) in *Snail2* promoters. The number at the start of the sequence indicates the distance from transcription start sites.

Figure 6. Induction of Snail2 is required for the ability of Twist1 to promote invasion and metastasis

A. Right panels are brightfield images of HMLE-Twist1 cells expressing shSnail2-1 or shControl. The left panel shows that their cell lysates were analyzed by SDS-PAGE, and probed for Snail2, Twist1, Ras, and β -actin. B. HMLE-Twist1 cells expressing shSnail2-1 or shControl were plated on Transwell to assay migration and on Transwell with Matrigel to assay invasion. The percentage of cells that migrated through the insert was quantified. Error bars are standard error of the mean (SEM). C. Top two panels are representative images of lungs from mice injected with HMLER-Twist1 cells expressing shSnail2-1 or shControl. The bottom panel is immunostaining for E-cadherin (brown) in primary tumors from HMLER-Twist1 cells expressing shSnail2-1 or shControl. D. The top graph is quantification of total number of GFP positive colonies in individual lungs. The bottom graph is total primary tumor weight in individual mice carrying tumors of HMLER-Twist1 cells expressing shSnail2-1 or shControl. Error bars are SEM. N = 6 mice for the shControl group, and N = 8 mice for the shSnail2-1 group. Error bars are SEM.

Figure 7. Twist1 and Snail2 are frequently co-expressed in human breast tumors

A. Lysates from SUM1315 cells expressing shTwist1 or shControl were analyzed by SDS-PAGE and probed for Twist1, Snail2, and β-actin. B. All 22283 gene probesets are ranked based on their expression correlation coefficients with Twist1 in their human breast tumor datasets STOCKHOLM (n=159), EMC (n=286), and UPPSALA (n=236) and their distributions are shown in individual graphs. The rank of Snail2 is marked as a vertical line in each graph. C. A model describing how induction of Snail2 by Twist1 suppresses E-cadherin and promotes EMT and tumor metastasis.