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miR-155 promotes macroscopic tumor formation yet inhibits tumor dissemination from mammary fat pads to the lung by preventing EMT

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Abstract

miR-155, a micro-RNA, is over-expressed in many types of cancer cells, including breast cancer, and its role(s) in tumor metastasis has been studied on a very limited basis. Tumor metastasis is a multi-step process with the last step in the process being formation of macroscopic tumor in organs distant from the primary tumor site. This step is the least studied. Here, we report that stable expression of miR-155 in 4T1 breast tumor cells reduces significantly the aggressiveness of tumor cell dissemination as a result of preventing epithelial-to-mesenchymal transition (EMT) of tumor cells *in vivo*. Further, miR-155 directly suppresses the expression of the transcription factor TCF4, which is an important regulator of EMT. However, when tumor cells are injected directly into the bloodstream, miR-155 remarkably promotes macroscopic tumor formation in the lung. Analysis of gene expression profiling identified a group of genes that are associated with promoting macroscopic tumor formation in the lung. Importantly, most of these genes are over-expressed in epithelial cells. Our findings provide new insight into how miR-155 modulates the development of tumor metastasis. This study suggests that the location of tumor cells over-expressing miR-155 is a critical factor: in mammary fat pads miR-155 prevents tumor dissemination; whereas in the lung miR-155 apparently maintains the epithelial phenotype of tumor cells that is critical for macroscopic tumor formation.

Introduction

MicroRNAs (miR) are a class of evolutionarily conserved non-coding single-stranded RNAs with a length of ~22 nucleotides that regulate gene expression negatively at the posttranscriptional level by repressing translation or inducing mRNA degradation in a sequence specific manner. Thus miRs control a wide variety of biological processes

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including development, differentiation, proliferation, cell death, immune response, and tumor progression (Winter *et al.*, 2009). Recent studies have identified a number of miRs that are dysregulated in tumor when compared to normal tissues (Gallardo *et al.*, 2009; Li *et al.*, 2009; Ryu *et al.*) and non-metastatic versus metastatic tumors (Gibbons *et al.*, 2009; Park *et al.*, 2008). miR-155 is up-regulated in many types of human cancers, including breast cancers (Iorio *et al.*, 2005; Jiang *et al.*; Kong *et al.*; Ma *et al.*), lung cancers (Yanaihara *et al.*, 2006), hepatocellular carcinoma (Wang *et al.*, 2009), and many types of malignancies of B cell origin (Costinean *et al.*, 2006; Eis *et al.*, 2005), including pediatric Burkitt lymphoma and Hodgkin's lymphoma. The roles of miR-155 in metastasis have been investigated on a very limited basis.

Tumor metastasis is a complex, multi-step process that includes local invasion, intravasation, survival in the circulation, extravasation, and colonization (Nguyen *et al.*, 2009). One of the first steps of metastasis requires that epithelial derived cancer cells undergo epithelial-to-mesenchymal transition (EMT) in which the tumor cells lose their epithelial marker, E-cadherin, and gain the mesenchymal marker, vimentin. Tumor cells having undergone EMT acquire the ability to move, allowing them to be disseminated to organs distant to the primary tumor site. Transcription factors, such as Twist1, Zeb1, Zeb2, Snai1, Snai2, and TCF4, have been shown to play a critical role in EMT (Peinado *et al.*, 2007).

Clinical observations that macroscopic metastatic tumors were frequently composed of more differentiated epithelial cells rather than EMT cells (Polyak and Weinberg, 2009) did not appear to correlate with the research findings on the molecular mechanisms underlying EMT (Aokage *et al.*; Hugo *et al.*, 2007; Katoh, 2005; Logullo *et al.*; Soltermann *et al.*, 2008; Thompson and Williams, 2008; Tsuji *et al.*, 2009b). This caused a controversy as to whether EMT truly occurred and whether EMT was a requirement for metastasis to occur. This discrepancy is mostly due to a poor understanding of the last step or process required for metastasis to occur, which is the establishment of macroscopic tumor in distant organs. As an explanation of the clinical findings it has been proposed that mesenchymal-to-epithelial transition (MET) occurs at distant sites. MET is a process where disseminated mesenchymal cells regain the features of epithelial cells probably due to local selective pressure for the growth of cancer cells with more epithelial features or due to a lack of EMT inducing signals at distant sites (Polyak and Weinberg, 2009). Other scientists, based on their data, propose that EMT and non-mesenchymal cells cooperate during the metastatic spread of a cancer (Tsuji *et al.*, 2009a; Tsuji *et al.*, 2008).

We report that 4T1 mouse breast tumor cells, which are epithelial cells before being implanted into syngeneic mice, undergo EMT *in vivo*, thus initiating their dissemination to distant organs. Stable-expression of miR-155 prevented 4T1 cells from undergoing EMT and reduced dissemination of the tumor cells to the lung. However, when tumor cells are directly injected into the bloodstream, miR-155 remarkably promoted macroscopic tumor formation in the lung. Analysis of gene expression profiling led to identification of a group of genes associated with promoting macroscopic tumor formation in the lung. Interestingly, these genes are expressed in epithelial cells. These results suggest that formation of macroscopic tumors in the lung is associated with features of epithelial cells.

Results

A subset of 4T1 cells undergo EMT *in vivo* and are disseminated to the lung

EMT is linked to metastatic propensity (Polyak and Weinberg, 2009). We tested whether EMT is involved in tumor metastasis using the 4T1 mouse mammary model (Pulaski and Ostrand-Rosenberg, 2001). Twenty-five days post-tumor cell injection in the mammary fat pad of syngeneic BALB/c mice, 4T1 tumor cells from primary tumors and a secondary metastatic site, i.e., the lung, were recovered and placed in tissue culture. To eliminate the possibility of potential contamination with other types of cells, isolated cells were cultured in RPMI medium 1640 containing 60 μM thioguanine for 10 days (4T1 cells are resistant to thioguanine) (Pulaski and Ostrand-Rosenberg, 2001). Colonies of tumor cells were evident after 5-10 days of incubation. In comparison with cultured 4T1 cells, there were two types of distinct 4T1 cells recovered from primary tumors based on morphological appearance. One type of cells had typical cobblestone-like epithelial morphology, and the other type of cells was bright, round and loosely touching surrounding cells (Figure 1a). Surprisingly the majority of 4T1 cells recovered from the lung were bright, round and loosely touching surrounding cells (Figure 1a, left and upper panel).

Using real-time PCR we examined whether there was a change in the gene profile associated with EMT in tumor cells recovered from the lung. In agreement with the observation of cell morphology, Real-time PCR results indicated that 4T1 tumor cells recovered from the lung expressed significantly higher levels of TCF4, *Twist1*, *Vimentin-1, and Zeb2*, and lower levels of *cadherin-1(E-cadherin*) than those from primary tumors (Figure 1b), suggesting that tumor cells recovered from the lung had undergone EMT. Besides genes associated with EMT, the genes ANGPTL4, Cox2, HMGA2, MMP9, and Socs3 having functions in stem cells, inflammation, and metastasis were also changed significantly in 4T1 cells recovered from the lung.

miR-155 expressed in 4T1 cells does not affect primary tumor growth, but prevents EMT *in vivo*

MicroRNAs have been shown to play a role in EMT transition (Gibbons *et al.*, 2009; Park *et al.*, 2008); therefore, we quantified the expression of a number of primary microRNAs of 4T-1 cells recovered from primary tumor and metastatic tumor in the lung. Transcription of *BIC*, which is the primary microRNA of *miR-155* (Figure 2a, left panel), as well as the mature miR-155 (Figure 2a, right panel), was decreased in tumor cells recovered from the lung when compared with tumor cells derived from primary tumors.

miR-155 is up-regulated in breast tumors (Jiang *et al.*; Kong *et al.*); however, the role(s) of miR-155 in tumor progression is/are not well defined. Therefore, we determined if stable-expression of miR-155 in 4T1 cells affects tumor progression. A retrovirus vector expressing miR-155 was constructed (supplementary Figure 1). The expression of mature miR-155 in 4T1-miR-155 cells was confirmed by real-time PCR (Fig 2b, left panel). Stable expression of miR-155 in *in vitro* cultured 4T-1 cells did not have an effect on the expression of genes tested (Figure 2b, right panel) or on the proliferation rate as measured by incorporation of EdU (Figure 2c).

The effect of stable-expressing miR-155 on the growth of 4T1 tumors was tested next. Six-week-old female BALB/c mice were inoculated with 4T1-control or 4T1-miR-155 cells in a mammary fat pad and tumor size was measured twice a week over a 25 day period. Tumor growth curves revealed that 4T1-miR-155 cells formed mammary tumors at the same rate over a 25 day period as did the 4T1-control cells (Figure 2d). These results suggest that stable-expression of miR-155 in 4T1 cells did not have a significant effect on primary tumor growth when tumor cells were injected in a mammary fat pad. However, from a morphological perspective more mesenchymal-like cells were found in the 4T1-control than 4T1-miR-155 cells recovered from tumor tissues (Figure 2d, right panel). The fact that miR-155 prevents 4T1 cell EMT was further supported by real-time PCR results (Figure 2e). The expression levels of TCF4, Twist1, vimentin1, and Zeb2 were significantly lower and cadherin1 was higher in 4T1-miR-155 cells compared to 4T1-control cells. A number of genes related to inflammation were also quantified. Among those tested, the expression of MMP9 was significantly higher in 4T1-miR-155 cells than in 4T1-control cells (Figure 2e). High levels of E-cadherin in recovered 4T1-miR-155 cells were further verified by western blotting (Figure 2e, right panel).

Since EMT plays a critical role in tumor metastasis, the affects of stable-expression of miR-155 on the ability of 4T1 cells to metastasize was determined. Twenty-five days post-tumor cell injection, histological sections of lung tissue were examined for lung metastatic lesions using light microscopy and H& E staining. Macroscopic metastatic lesions in the lungs were not observed in the test or control groups of mice. At this point our explanation for these results is that macroscopic metastatic lesions had not developed in the lungs at this time point. We then quantified the frequency of recovering tumor cells from the lungs. In mice injected with 4T1-control cells, tumor cells were recovered from lungs in more than 80% of mice examined (Figure 2f). In contrast, no tumor cells were recovered from the lungs of mice injected with 4T1-miR-155 cells. These data indicate that stable expression of miR-155 reduced the ability of tumor cells to disseminate to a secondary metastatic site. These results indicate that 4T1-miR-155 tumor cells recovered from tumor tissues have a much higher percentage of non-mesenchymal cells.

miR-155 targets TCF4 directly

Our real-time PCR results indicate that the expression level of TCF4 is higher in 4T1-control cells than 4T1-miR-155 cells (Figure 2e). TCF4 is a newly identified transcription factor that promotes EMT (Sobrado *et al.*, 2009). The results of western blot analysis of E-cadherin indicate that knockdown of TCF4 in 4T1 cells led to an increase expression of E-cadherin (Figure 3a), suggesting that TCF4 indeed regulates EMT in 4T1 cells. Using TargetScan (http://www.targetscan.org) three conserved miR-155 targeting sites located at 3′-UTR of TCF4 mRNA were predicted *in silico*. We tested whether miR-155 directly regulates TCF4 expression. To determine if TCF4 is regulated by miR-155, a mouse TCF4 3′ -UTR fragment containing three predicted miR-155 targeting sites was fused downstream of the Renilla luciferase gene in a dual luciferase reporter vector (psiCHECK™-2, Promega). Dual luciferase reporter vectors with each of the miR-155 targeting sites mutated were also constructed (Figure 3b, 3c). The luciferase reporter vectors were co-transfected with a vector expressing miR-155 or miR-223 (Chen *et al.*, 2004) into 293T cells. The 3′ -

UTR of TCF4 mRNA did not contain the miR-223 target site, so a vector expressing miR-223 was used as a control. Following 48 h of incubation, cells were subjected to a dual-luciferase assay. After Renilla luciferase activity was normalized to firefly luciferase activity, results from triplicate experiments showed that the luciferase activity of the reporter containing the wild type TCF4 3′ -UTR was repressed significantly by the ectopic expression of miR-155 (Figure 3d). 293T cells transfected with reporters with either target site mutant1 or mutant2 led to a partial reversing of the luciferase activity, suggesting that each site was active. The third 3′ -UTR of TCF4 sequencing for potential miR-155 binding is too long to be cloned into a dual luciferase reporter vector, and we are unable to test whether miR-155 also regulates the expression of TCF4 through binding the third TCF4 sequencing. Results from western blotting also showed that TCF4 protein level was decreased in 4T1-miR-155 recovered from primary tumors (Figure 3d). These data indicate that miR-155 directly regulates TCF4 expression through 3′ -UTR interaction.

Stable-expression of miR-155 promotes significant macroscopic tumor formation in the lung when tumor cells are injected directly into the bloodstream

As noted previously metastasis is a multi-step process. To further explain the process we tested whether stable-expression of miR-155 affected the last step of metastasis; that is, the formation of macroscopic tumor lesions in the lung. Tumor cells injected directly into the bloodstream effectively bypassed the initial steps of the metastatic process. Surprisingly, three weeks after intravenous injection of tumor, all of the mice injected with 4T1-miR-155 cells had an astonishing overgrowth of tumor in the lung. Almost all of the lung surface was covered with tumor nodules (Figure 4a). In contrast, there were significantly fewer tumor nodules on the lung surface of mice injected with 4T1-control cells (Figure 4a). H&E staining of lung sections also showed significantly increased numbers of macroscopic tumor lesions in mice injected with 4T1-miR-155 cells (Figure 4b). These results indicate that miR-155 promotes macroscopic tumor formation in the lung in a significant manner when tumor cells are injected directly into the bloodstream.

We next sought to determine if 4T1-miR-155 cells injected intravenously into mice would retain that genetic phenotype or transition to a mesenchymal cell genetic phenotype in the lung. Tumor cells were recovered from lung tissues and gene expression was quantified using real-time PCR, western blotting analyses, and immunohistological staining of E-cadherin, a maker of EMT. Recovered 4T1-miR-155 cells had a typical epithelial cell morphology, whereas most of the 4T1-control cells were EMT-like cells (Figure 4c) although the cells were epithelia type cells before they were injected intravenously. Results of real-time PCR (Figure 4d) and western blotting (Figure 4e) are also correlated to the morphological data in that the 4T1-miR-155 cells having higher levels of mature miR-155 expressed (Fig. 4d, right panel) were epithelia cells with significantly higher levels of cadherin-1, and lower levels of TCF4, Twist1, vimentin-1, and Zeb2. To further verify that E-cadherin was highly expressed in tumors formed by 4T1-miR-155 cells, fixed lung sections were subjected to immunofluorescent staining with an antibody specific to E-cadherin. Most of the tumors formed by 4T1-miR-155 cells were more intensively stained for E-cadherin (Figure 4f). These data confirmed that 4T1-miR-155 cells injected were epithelial upon metastasis to the lung.

Genes associated with promoting macroscopic tumor formation in the lung

Our results have revealed two very interesting phenomena. First, 4T1-control tumor cells exhibit a high level of plasticity and are able to transition from an epithelial state to a mesenchymal state allowing them to disseminate to the lung. miR-155 prevents EMT. Second, stable-expression of miR-155 in 4T1 cells promotes significant macroscopic tumor lesion formation in the lungs when tumor cells are injected directly into the bloodstream. To gain insights into the molecular mechanisms of EMT and macroscopic tumor formation in the lungs, we generated 4 sets of cDNA microarray data. These included 1) 4T1-control cells injected intravenously and recovered from lung (control lung); 2) 4T1-miR-155 cells injected intravenously and recovered from lung (miR-155 lung); 3) 4T1-control cells injected subcutaneously and recovered from tumor (control tumor); and 4) 4T1-miR-155 cells injected subcutaneously and recovered from primary tumors (miR-155 tumor).

To identify a group of genes that was differentially expressed in 4T1-miR-155 epithelial cells and in 4T1-control mesenchymal cells, we enriched genes that were significantly over- or under-expressed in 4T1-miR-155 cells compared to 4T1-control cells regardless of where the tumor cells were recovered. One hundred twenty-two genes were identified (Supplemental table 1). Among the genes, 89 genes (in bold) included markers of epithelial cells, i.e., cadherin-1, Epcam, claudin4, and claudin7, that are highly expressed in 4T1-miR-155 cells and represent genes that are over-expressed in 4T1-miR-155 epithelial cells. Other genes, Erbb3, Grb7, Grhl2, MMP9, IL1α, IL23a, Jag1 and ST14 were also highly expressed in the 4T1-miR-155 cells. Thirty-three genes are over-expressed in 4T1-control mesenchymal cells.

To identify genes that were associated with promoting macroscopic tumor lesion formation in the lung, we first enriched a list of genes that were differentially expressed in control lung or miR-155 lung by subtracting genes commonly expressed in control or miR-155 tumor. We then narrowed the list down to those genes that were significantly over- or under-expressed in miR-155 lung when compared with control lung. Seventy genes were identified as being up-regulated (in bold), while 26 genes were down-regulated in 4T1-miR-155 cells (Supplemental table 2). Some of the known EMT related genes identified as being over- or under-expressed in our cDNA array data were further confirmed by real-time PCR (Figure 5).

Noticeably, among the up-regulated genes, all 70 genes overlapped the genes listed in supplemental table 1 and these 70 genes are also identified as being over-expressed in 4T1 epithelial cells. Among the down-regulated genes, all 26 genes are identified as being over-expressed in 4T1-control mesenchymal cells. These data suggest that genes associated with macroscopic tumor formation in the lung are linked to “gene imprinting” of epithelial cells. These genes are directly or indirectly regulated by stable-expression of miR-155 in 4T1 cells.

To identify genes directly targeted by miR-155, we first identified a candidate target gene list of miR-155 using TargetScanMouse release5.1 (http://www.targetscan.org/mmu_50/). Next the expression data of these genes from cDNA micro-array data generated from 4T1-miR-155 cells and 4T1-control cells was extracted. Some of the genes suppressed in 4T1-

miR-155 cells may be targeted directly by miR-155 and these include Satb1, Rheb, Ets1, Adam10, TCF4, Nfe2l2, Hhip, and Cebpb (Figure 6, Supplemental table 3). We selected Satb1 as an example to further verify the data generated from cDNA arrays since Satb1 has been identified as an important regulator of metastasis. Additionally, knockdown of Satb1 in MDA-MB-231 cells has been shown to cause the up-regulation of a number of genes, including cadherin-1 and claudin1 (Han *et al.*, 2008a). The real-time PCR results showed that the expression level of Satb1 was down-regulated about 50% in 4T1-miR-155 cells (Figure 7a). To further determine whether miR-155 regulates the expression of Satb1 by binding Sabt1 UTR sequences, mouse Satb1 3′ -UTR fragment containing a predicted miR-155 targeting site was fused downstream of the Renilla luciferase gene in a dual luciferase reporter vector (psiCHECK™-2, Promega) (Figure 7b). The luciferase reporter vectors were co-transfected with a vector expressing miR-155 (Chen *et al.*, 2004) into 293T cells. A dual luciferase reporter vector with the miR-155 targeting site mutated was also constructed. Results from dual luciferase assay showed that over-expressing miR-155 significantly repressed luciferase activity of Renilla reporter fused with wild-type by about 50% but not mutant Satb1 UTR when compared to the control vector (Figure 7c). These data suggest that expression of Satb1 is negatively regulated by miR-155 through interaction with its 3′ -UTR. To test if Satb1 plays a role in EMT in the 4T-1 model, we analyzed the effects of shRNA knock-down of Satb1 on E-cadherin expression. Retroviral vectors expressing shRNA specific to mouse Satb1 (Lanes 2, 3, 4, and 5) or to the luciferase gene (lane 1) as a control was constructed. 4T1 cells were infected with each retrovirus for 3 days. GFP^+ cells were then sorted. Significant down-regulation of expression of Satb1 in the shRNA knock-down Satb1 4T1 cells (c, middle panel), but not E-cadherin (c, right panel) was observed by real-time PCR. However, down regulation of E-cadherin protein was also not observed (Supplementary figure 2). Thus, inhibition of Satb1 expression does not appear to alter the E-cadherin expression in 4T1 cells *in vitro*.

Discussion

In this study, we investigated the roles of miR-155 before and after tumor dissemination. Stable expression of miR-155 significantly inhibits cells from undergoing EMT. miR-155 inhibits the expression of a number of genes related to the induction of EMT, including twist1, which is well known to play a crucial role in EMT (Yang *et al.*, 2004). Among these genes, we demonstrated that miR-155 directly suppresses the expression of TCF4, which plays a role in EMT (Sobrado *et al.*, 2009). Our data also show that miR-155 promotes significant macroscopic tumor formation in the lung when tumor cells are injected directly into the bloodstream. Based on these results, gene profiling demonstrated differential expression of genes in epithelial cells compared to mesenchymal cells. Coincidentally, most of the genes associated with macroscopic tumor formation in the lung are over-expressed in epithelial-like 4T1-miR-155 cells. These results suggest that formation of macroscopic tumor lesions in the lung are linked to features associated with epithelial cells.

EMT has been considered a critical step in tumor metastasis (Polyak and Weinberg, 2009). However, clinical samples of metastatic tumors are frequently composed of more differentiated epithelial cells. This finding has resulted in questioning whether EMT actually occurs and whether it plays a role during metastasis (Polyak and Weinberg, 2009; Tsuji *et*

al., 2009a). Most studies have demonstrated that EMT is a necessary step for tumor cells to be disseminated from the primary tumor site to distant organs (Gibbons *et al.*, 2009; Peinado *et al.*, 2007; Yang *et al.*, 2004). How these disseminated mesenchymal cells develop into macroscopic tumors in distant organs is largely unknown. The factors driving a reversing process, that is MET, in the organs with metastatic cells has not been identified. In this study, we found that miR-155 promoted significant macroscopic tumor formation in the lung when tumor cells were injected into bloodstream.

Gene expression profiling analysis revealed that a number of epithelial cell specific genes including Erbb3, Grb7, Grhl2, Elf3, Epcam, cadherin-1 and claudin4 were over-expressed in 4T1-miR-155 cells. These data suggest that over-expression of miR-155 leading to formation of macroscopic tumor lesions in the lung is linked to induction of genes favoring the tumor cells retaining an epithelial cell phenotype. Our results, supported by others (Tsuji *et al.*, 2008), indicate that non-mesenchymal cells injected intravenously are capable of establishing lung metastasis, and that after the subpopulations of the metastatic (mesenchymal-appearing) tumor cells have arrived in distant sites, they have undergone MET to reacquire their epithelial phenotype (Acloque *et al.*, 2009; Bukholm *et al.*, 2000; Cheng *et al.*, 2001; Wells *et al.*, 2008). It is interesting to note that the reversibility of the process (MET) is also observed during embryonic development once the migratory cells have reached their destination (Johnson, 2009; Nakaya *et al.*, 2004). It is therefore tempting to speculate that situations that unlike in the tumor, microenvironment of lung is naturally more suitable for epithelial cells growth. Therefore, tumor cells with a MET phenotype, e.g., 4T-1-miR-155, have a growth advantage in the lung environment.

The current research demonstrated that prevention of EMT by over-expression of miR-155 in tumor cells is not advantageous for tumor growth locally but was shown to result in fewer microsatellite metastasic tumors forming in the lung of mice. The data published by another group indicated that the majority of human tumor tissue has unregulated miR-155 that is likely associated with the invasiveness of tumors (Kong *et al.*, 2008). Tumor tissue consists of many types of cells including tumor-associated macrophages, immature myeloid cells and lymphocytes. Upregulation of miR-155 in macrophages or lymphocytes leads to promotion of tumor growth and metastasis, but the role of over-expression of miR-155 in tumor cells in immune competent humans is not known and needs to be investigated further.

EMT is regulated by TGF-β through upregulation of miR-155 in normal NMuMG cells (Kong *et al.*, 2008). The results of western blot analysis indicated that, like the data generated from normal NMuMG cells by another group, treatment with TGF-β leads to a down-regulation of E-cadherin in both 4T1-control and 4T1-miR155 breast tumor 4T1 cells (Supplementary Figure 3) including a number of other tumor cell lines, e.g., TA-3 breast tumor cells (Stewart and Abrams, 2007) (data not shown). However, expression of miR-155 in 4T1 cells appears to provide no further enhancement of TGF-β regulated down-regulation of E-cadherin (Supplementary Figure 3). The same is true for the TA-3 breast tumor cell line (data not shown). These data suggest that the pathway regulated by miR-155 does not intersect with the pathway regulated by TGF-β in terms of expression of E-cadherin in 4T1 tumor cells. These findings raise further questions as to what other signaling pathways (wnt, MAPK, and NF& kappa;B) involved in EMT are regulated by miR-155 (Thiery *et al.*, 2009)

Our results indicate that miR-155 prevents EMT of 4T1 tumor cells *in vivo*. miR-155 directly suppresses the expression of TCF4 and Satb1 through an interaction with 3′ -UTR. We have identified sites located in TCF4 and Sabt1 3′ -UTRs that play a role in the miR155 mediated suppression of TCF4 and Satb1 expression. TCF4 is a newly identified EMT regulator (Peinado *et al.*, 2007; Sobrado *et al.*, 2009). When over-expressed, TCF4 suppresses E-cadherin expression and promotes EMT. In addition to demonstrating miR-155 regulation of TCF4, our data show that expression of miR-155 leads to a lower expression of other EMT markers, including Twist1, Zeb2, Twist1, and Vimentin-1, and a higher expression of miR-200 and E-cadherin.

Data from our cDNA array, real-time PCR, and shRNA knockdown of Satb1 suggest that expression of Satb1 is negatively regulated by miR-155. An earlier study demonstrated that Satb1 played an important role during tumor progression and metastasis (Han *et al.*, 2008b) by regulating genes involving EMT including E-cadherin. But these results have been questioned recently by others with data indicating that expression of Satb1 in breast cancer cells did not affect tumor formation and metastasis (Iorns *et al.*). Our data suggest that the inhibition of Satb1 expression does not appear to alter the E-cadherin expression in 4T1 cells *in vitro*.

In summary, we demonstrated that miR-155 contributes in regulating EMT in 4T1 breast cancer. Macroscopic tumor formation in the lung is dominated by epithelia cells and promoted by over-expression of miR-155 whereas tumor dissemination from tumor to lung is dominated by mesenchymal cells and inhibited by miR-155. Therefore, our study presents evidence that miR-155 could be a critical therapeutic target after, but not before, metastasis has occurred.

Material and Methods

Mice and tumor model

Female BALB/c mice, 6- to 8-weeks-old, were obtained from The Jackson Laboratory (Bar Harbor, ME). All procedures were approved by the University of Louisville Institutional Animal Care and Use Committee. The 4T1 tumor cell line was obtained from ATCC and maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. Cells (2×10^5) were injected in the mammary fat pads of mice in a volume of 50 μl of saline. Tumors were measured every three days and volume was calculated using a method as described (Grizzle *et al.*, 2002). For experimental macroscopic tumor formation in the lung, 1×10^5 4T1 cells were injected into mice via the tail vein. Mice were sacrificed after 3 weeks and tumors and lungs were removed. Lungs were placed in saline solution to visualize or stained with hematoxylin and eosin (H&E) for counting metastases. Photographs of the lungs were taken and the number of nodules and the surface occupied by the metastasic lesions were quantified.

Generation of 4T1-miR-155 cell line—A retrovirus vector, MDH1-PGK-GFP 2.0, was obtained from Addgene. To construct retrovirus vector expressing miR-155, fragment 99-554 of the *BIC* gene (Based on accession AY096003) was PCR amplified using a CS2-BIC vector as a template (Chung *et al.*, 2006). All PCR primers sequences are listed in

supplemental table 4. The amplified fragment was subsequently inserted downstream of the H1 promoter of MDH1-PGK-GFP 2.0 vector to generate the plasmid MDH1-PGK-GFP-BIC (Supplemental figure 1). The activity of miR-155 in the MDH1-PGK-GFP-BIC vector was verified by its repressing luciferase activity in 293 cells transfected with UAS-luc-miR-155as (Chung *et al.*, 2006), (supplemental figure 1). UAS-luc-miR-155as contains a sequence complementary to miR-155 after the stop codon of the luciferase gene. To generate a retrovirus, 5 μg of DNA from MDH1-PGK-GFP-BIC or the MDH1-PGK-GFP 2.0 empty vector, and 5 μg of DNA from the retroviral package vector pCL (Addgene) were co-transfected into 293T cells using a transfection reagent (FUGENE, Roche). Twenty-four hours after transfection, cells were provided fresh media. Forty-eight hours post-transfection, culture supernatants containing retrovirus were collected and centrifuged at 3000 rpm for 10 minutes, supernatants were aliquoted, and stored at -80°C until used.

To make a stable cell line, 4T1 cells were seeded into a 12-well plate, cultured overnight, and the media replaced with 1 ml of retrovirus carrying GFP/control or GFP/miR-155. Cells were infected repeatedly the following day and then returned to regular culture media containing 60 μj,M thioguanine. One week after this process, GFP^+ cells were sorted using a FACSVantage sorter (BD Biosciences). Over-expression of miR-155 in sorted GFP^+ cells was verified by RT-PCR.

The other biological effects of the miR-155 were determined employing a number of techniques: 1) Isolation and culture of 4T1 from BALB/c mouse lung and tumor, 2) Cell proliferation assay, 3) Cell proliferation assay, Reverse transcription-PCR, and 4) Luciferase reporter assay. Details of each method used in this study are described in the supplemental experimental procedures.

Quantitative real-time PCR (QPCR)

Relative quantification of select mRNA was performed using a CFX96 Realtime System (BioRad) and SsoFast evagreen supermixture (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. All primers were purchased from Eurofins MWG Operon (Huntsville, AL). Fold changes in mRNA expression between treatments and controls were determined by the ΔCT method (Zhao and Fernald, 2005). Fluorescence threshold cycle (C_t) values were calculated using SDS 700 System Software (Bio-Rad). Results were normalized to the average C_t for the GAPDH and β-actin housekeeping genes contained on the QPCR. $\Delta\Delta C_t$ values were calculated to determine expression changes. Differences between groups were determined using a two sided Student's t-test and one-way ANOVA. Error bars on plots represent +/- standard error (SE), unless otherwise noted.

MicroArray and data analysis

In three independent experiments, total RNA from 4T1-control or 4T1-miR-155 cells recovered from lung or tumor was isolated and subsequently processed for microarray hybridization using Affymetrix Murine GeneST Arrays according to the manufacturer's instructions (Affymetrix). Raw data were preprocessed by the robust multi-array analysis method and differentially expressed genes were determined using the significance analysis of microarrays algorithm (Tusher *et al.*, 2001). Results were normalized and analyzed for

differences in $\log_2$ expression values. For further verification of genes of interest, real-time PCR was performed to verify the microarray results. Gene clustering was analyzed using MeV v4.5.1 software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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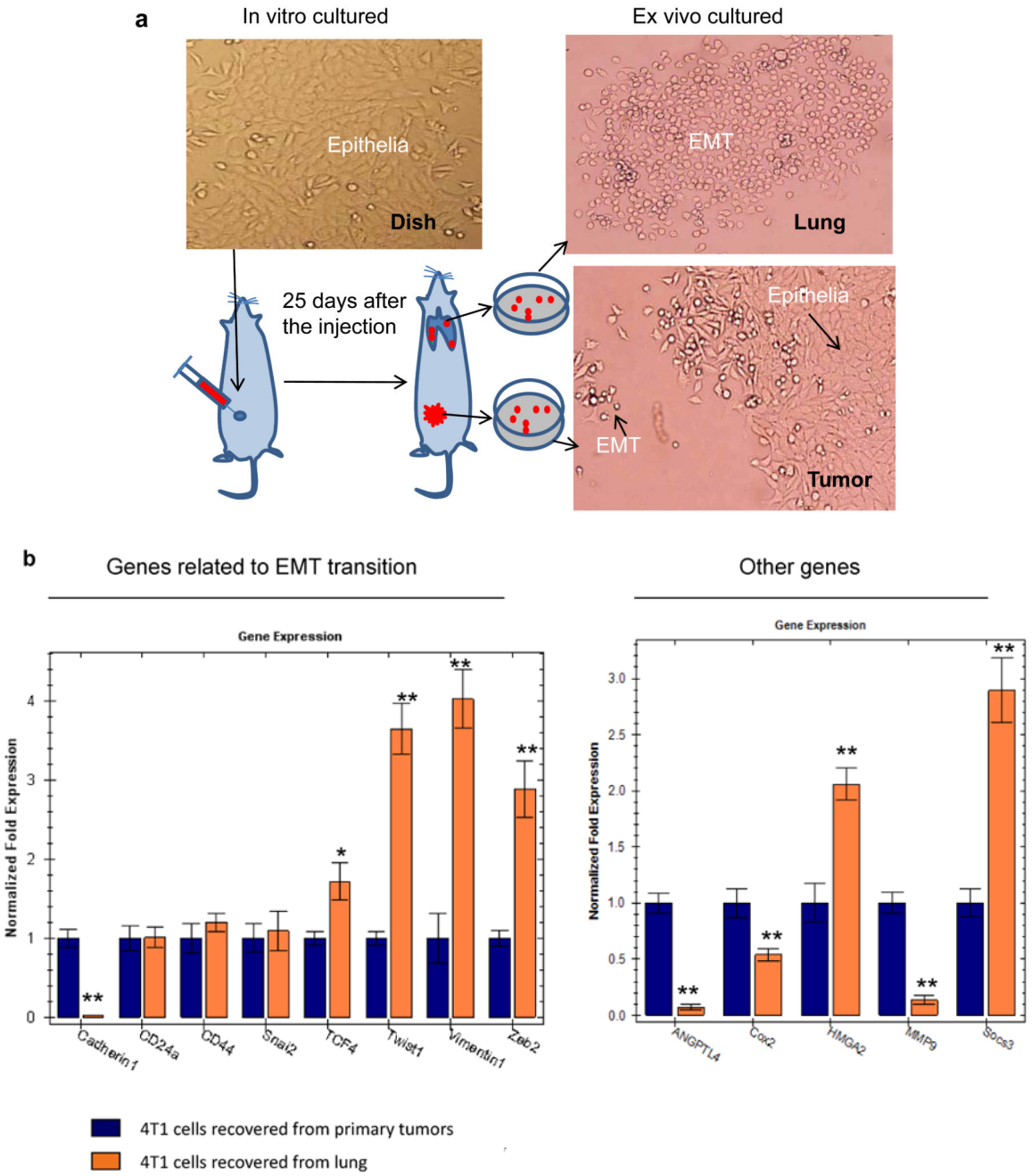
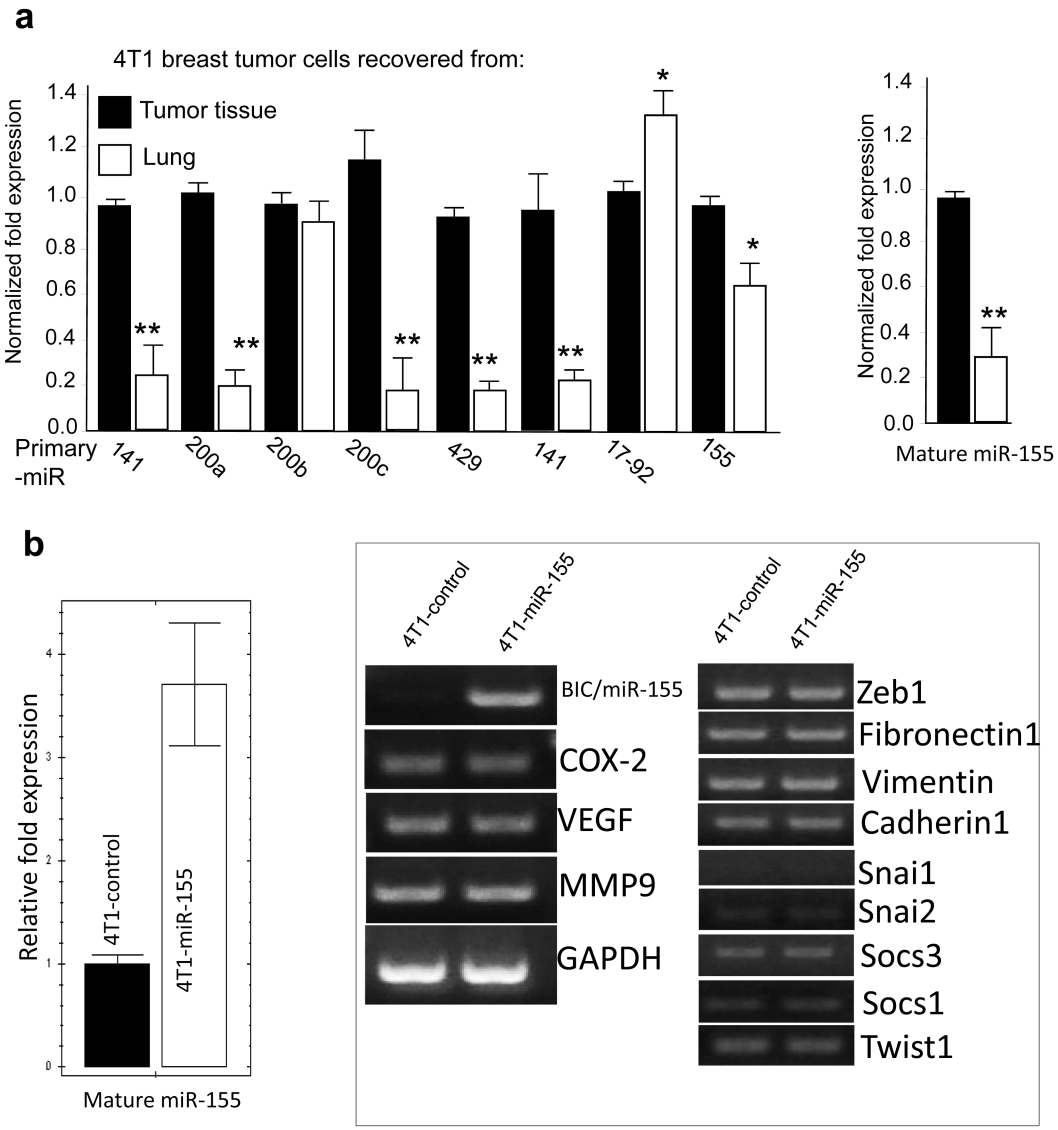
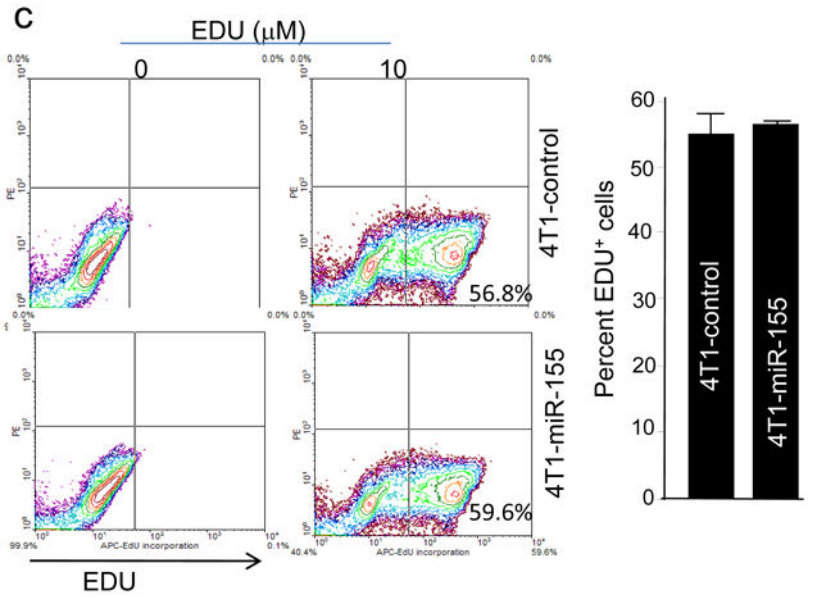
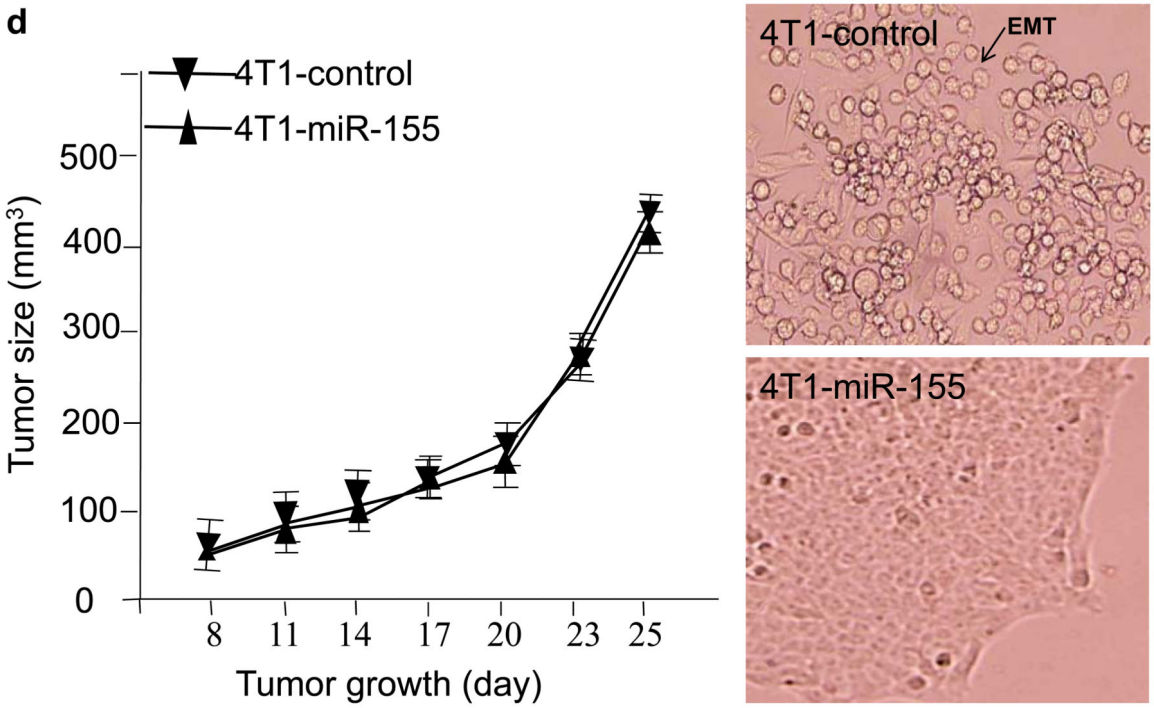


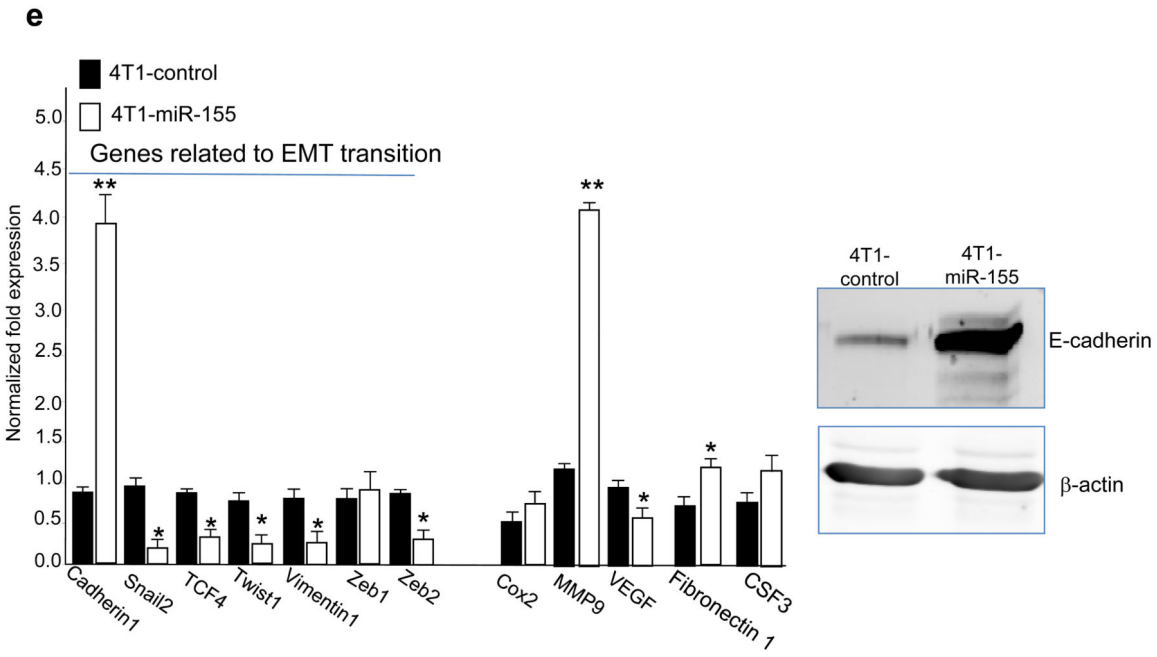
Figure 1. 4T1 tumor cells undergo EMT *in vivo*

(a) Changes in cell morphology of 4T1 cells. 2×10^5 4T1 cells (left panel) were injected into the mammary fat pads, and 4T1 cells were recovered from lung (right upper panel) and tumor (right lower panel) at 25 days post-injection. Cell morphology was analyzed by light microscopy. Representative optical fields are shown. (b) Changes in mRNA abundance were analyzed by quantitative RT–PCR. Relative mRNA levels were determined by the $\Delta\Delta C_t$ method using GAPDH and β-actin for internal cross-normalization and the indicated primers (Supplementary table 5). Values are mean ± standard error of mean (S.E.M.) of at least three independent experiments (n=5).









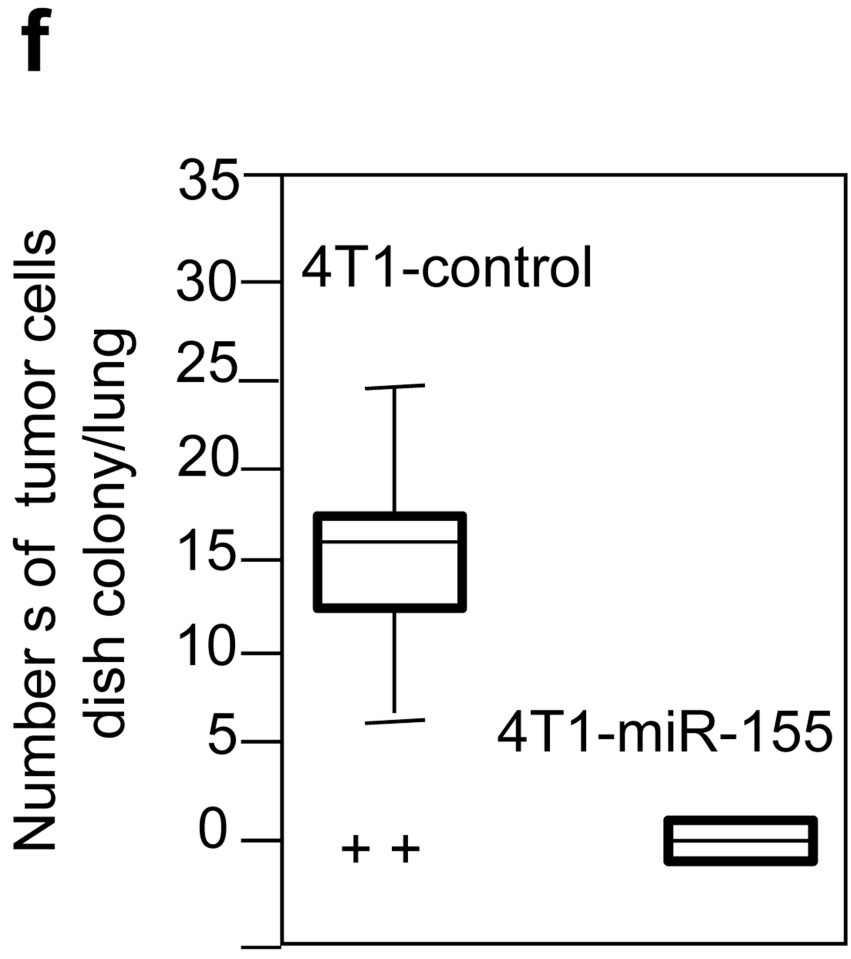


Figure 2. miR-155 prevents 4T1 tumor cells from undergoing EMT *in vivo*

(a) Quantitative real-time PCR expression analyses of microRNAs (left panel) or mature miR-155 (right panel) were carried out in 4T1 cells recovered from tumor tissue and lung. Results are represented as the mean ± S.E.M. of at least three independent experiments. (b) RT-PCR analyses of expression of mature (left panel) and primary miR-155 and genes as listed in supplemental table 5 were carried out in 4T1-control and 4T1-miR-155 cells. Representative gel images are shown (right panel). (c) miR-155 had no effect on *in vitro* 4T1 cell proliferation. 4T1-control or 4T1-miR-155 cells (0.5×10^6) were cultured overnight and then continued in culture in the presence or absence of EDU conjugated with Alexa florescent dye (10 μM) for 2 h. Representative FACS images are shown (Left panel). The percentage of EDU positive cells were analyzed using flow cytometry and results are expressed as the percent mean ± S.E.M. of three independent experiments (right panel) (n = 5). (d) 6-week-old female BALB/c mice were injected with 4T1-control or 4T1-miR-155 cells (1.0×10^5 cells/mammary fat pad in 50 μl PBS for each mouse; 10 mice per group). Tumor volume was measured every 3 days. Each point represents the mean volume ± S.E.M. of 10 mice from each group (three independent experiments). After mice were sacrificed, tumor cells were recovered from tumor tissue. Cultured cell morphology was analyzed by light microscopy. Representative optical fields are shown. (e) Quantitative RT-PCR expression analyses of genes as listed in the figure 2e were carried out in 4T1-control and 4T1-miR-155 cells recovered from tumor tissue. Results are expressed as the mean ± S.E.M. of at least two independent experiments. E-Cadherin protein in recovered 4T1-control and 4T1-miR-155 cells were analyzed by western blotting. (f) miR-155 prevents tumor dissemination. Lung tissues from the same groups of mice as described in figure 2d were used for recovering 4T1 tumor cells. Numbers of tumor cell colonies were quantified. Results are expressed as the box plot for the 4T1-control group and the 4T1-miR-155 group for at least three independent experiments (n = 10 mice/group). A potential outlier is marked with a plus sign in the plot.

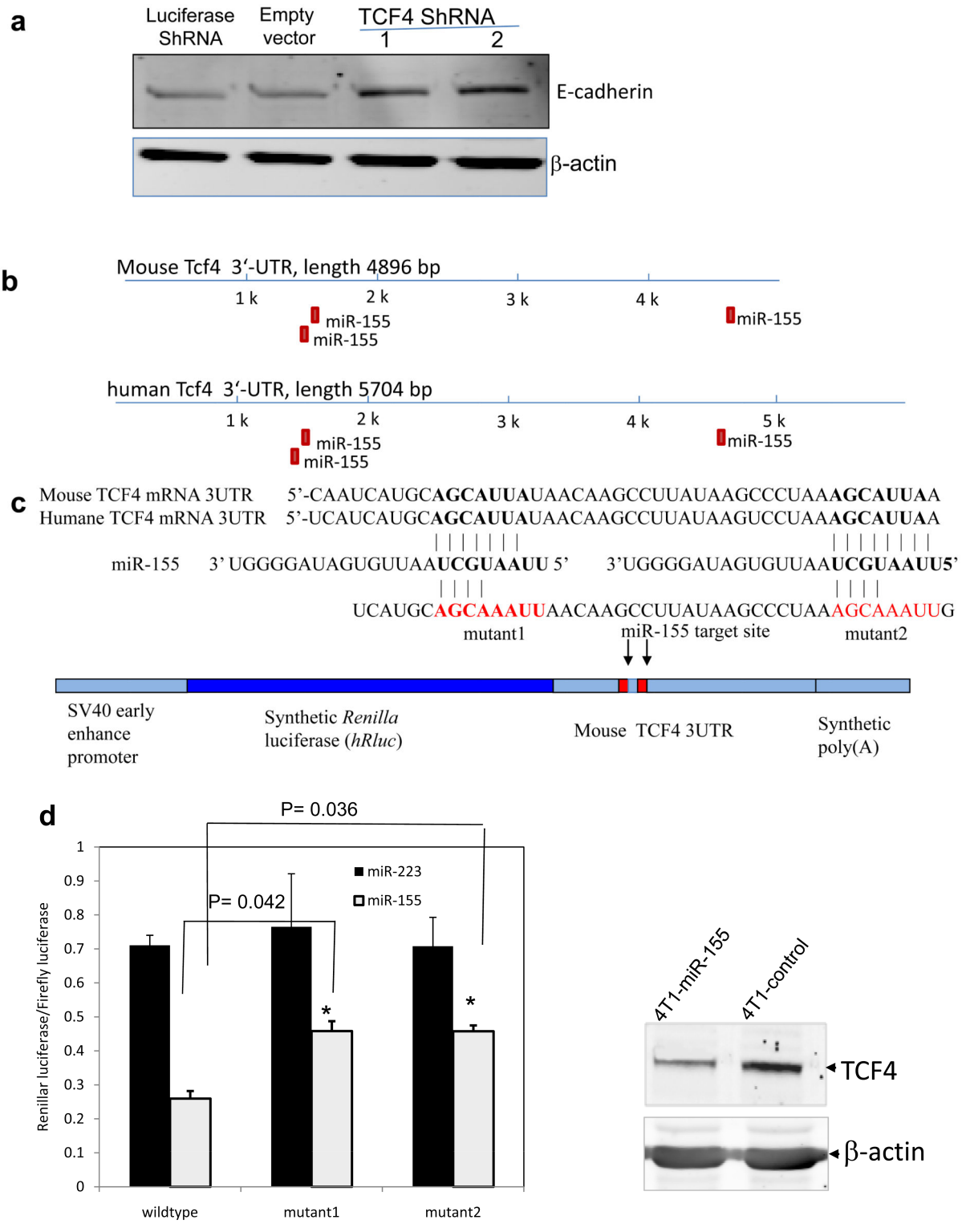
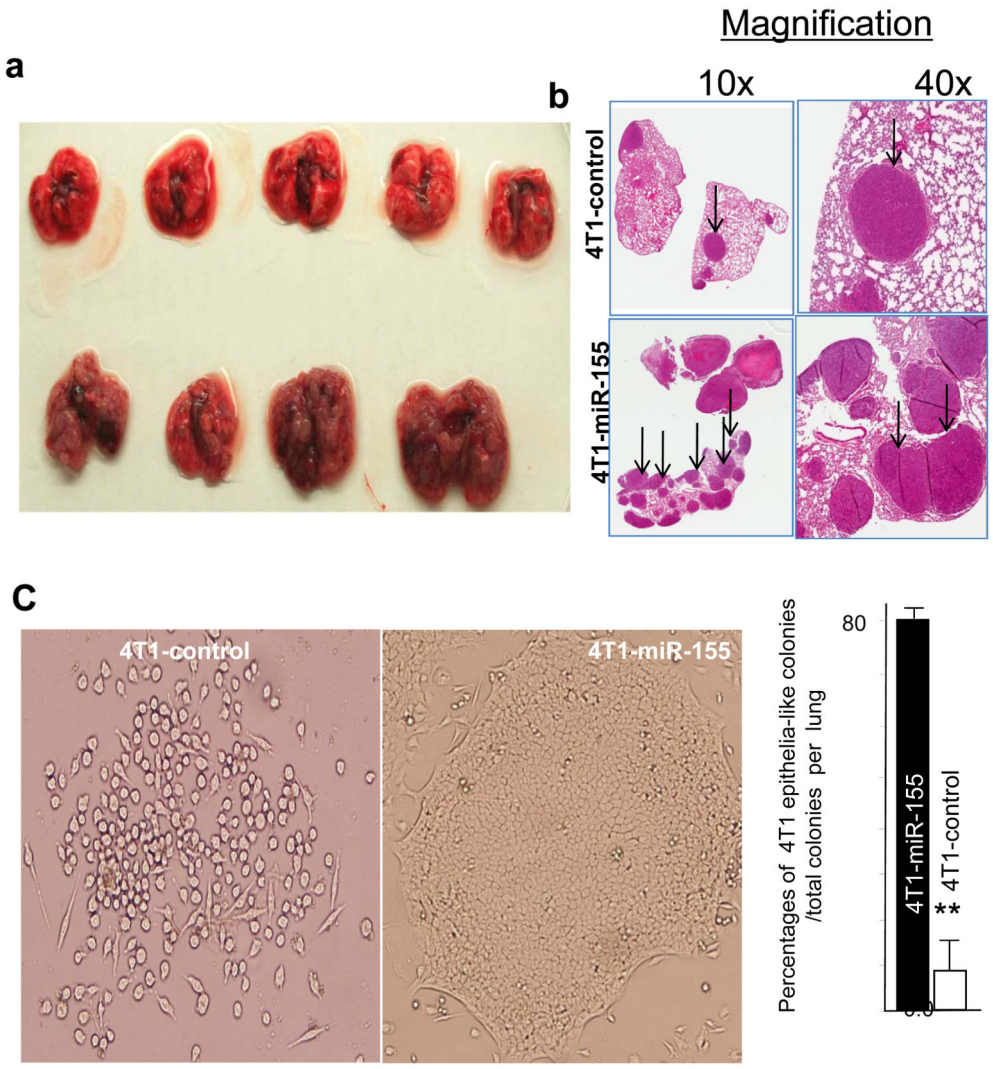


Figure 3. TCF4 is a direct target of miR-155

(a) 4T1 cells were transfected with a MDH1-PGK-GFP retroviral vector expressing different shRNA for TCF4 (numbers 2, 3), a ShRNA binding to luciferase (lane 1) or MDH1-PGK-GFP retroviral empty vector (lane 2) as controls. Three days after retrovirus infection, GFP^{+}

cells were sorted by FACS. 50 μg of total protein were used for western blotting analysis of E-cadherin, or β-actin was used as a control. (b) Conserved miR-155 target sites are located at the 3′ -UTR of TCF4 mRNA and are shown as alignment of mouse and human sequences in the 3′-UTR of TCF4 mRNA. The representation in 3b is limited to the region around the miR-155 complementary site. In bold, the “seed” region with a conserved anchoring adenosine (complementary to the first nucleotide of miR-155) matches to the eighth nucleotide of the miRNA. (c) Schematic representation of the construct used in the luciferase reporter assay. (d) Luciferase reporter assays were performed by transiently transfecting 293T cells with a vector over-expressing miR-155 or miR-223 with the indicated 3′-TCF4-UTR luciferase reporter plasmids. Renilla luciferase values were normalized to Firefly luciferase. Results represent the ratio of Renilla luciferase to Firefly luciferase (left panel). An asterisk (*) indicates a p-value of less than 0.05 in a Student's t-test. TCF4 protein levels in 4T1-control and 4T1-miR-155 cells recovered from primary tumor were determined by western blotting (right panel).



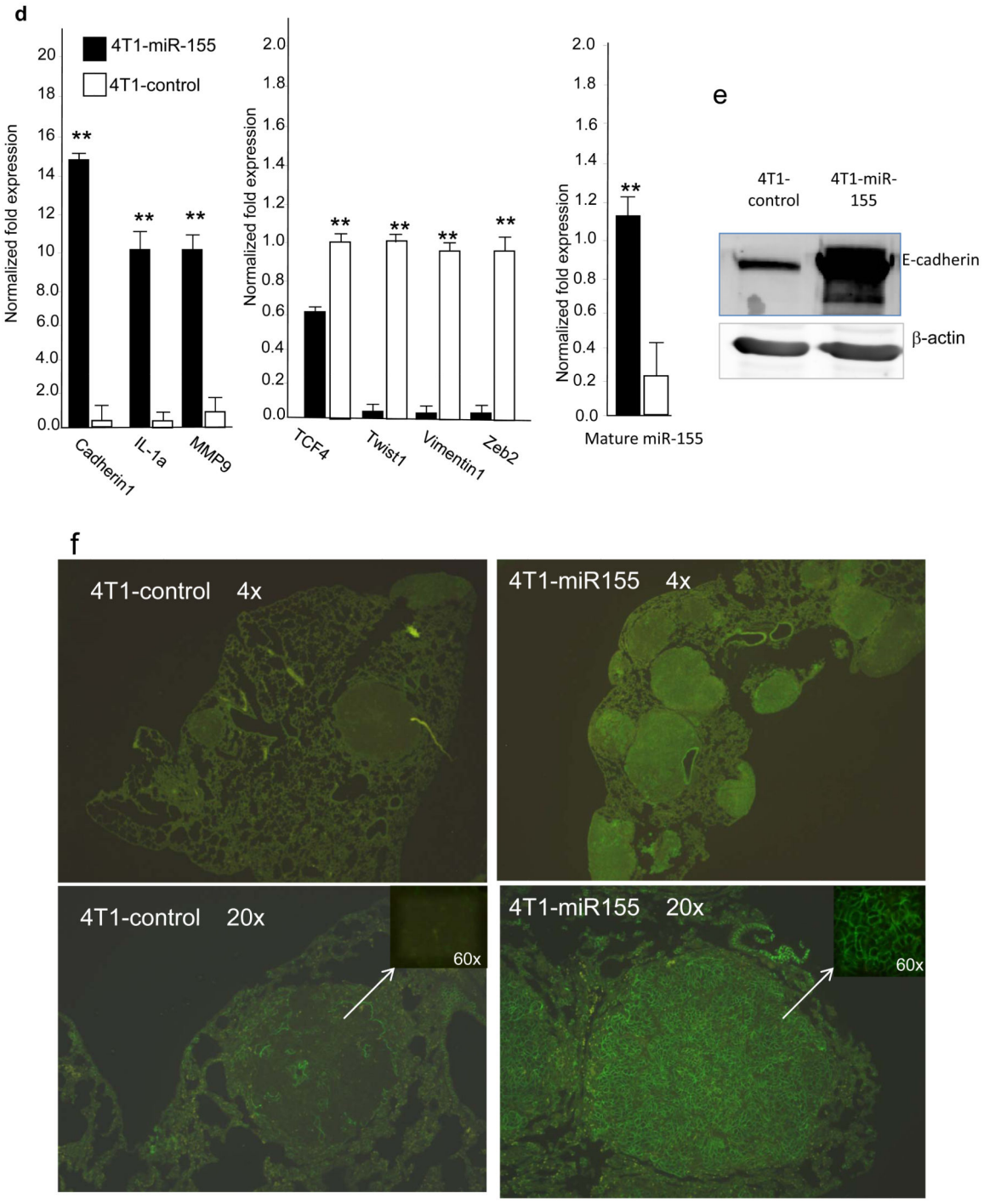


Figure 4. miR-155 promotes macroscopic tumor formation in the lung

6-week-old female BALB/c mice were injected intravenously with 4T1-control or 4T1-miR-155 cells (0.5×10^5 cells in 50 μl PBS for each mouse with 5 mice per group). Twenty-five days post-injection, the lungs from each mouse were removed and observed for tumor nodules. Mice injected with 4T1-miR-155 cells had numerous tumor nodules. Photographs represent one of five independent experiments (a). Lung sections of the same groups of mice

as described in figure 4a were H&E stained. Arrows indicate microscopic tumor. Representative images at low (10×) and high (40×) magnification are shown (b). After mice were sacrificed, recovered tumor cells from each of lung were visualized using light microscopy. Representative optical fields are shown (c, left panel), and numbers of epithelia-like colonies from cells isolated from each lung were counted under a light microscope. Results are represented as the percentages of mean ± S.E.M. of numbers of epithelia-like colonies/total cell colonies per lung (c, right panel) (n=5) with three independent experiments. Quantitative RT-PCR expression analyses of genes as listed (left panel) or mature miR-155 (right panel) were carried out in 4T1-control and 4T1-miR-155 cells recovered from the lung. Results are represented as the mean ± S.E.M. of four independent experiments (d). E-cadherin protein in recovered 4T1-control and 4T1-miR-155 cells from lung were carried out by western blotting analysis (e). Fixed lung sections were immunofluorescent stained with an anti-E-Cadherin antibody (f). Each photograph is representative of three different independent experiments. Representative images at 4×, 20× and 60× magnification are shown.

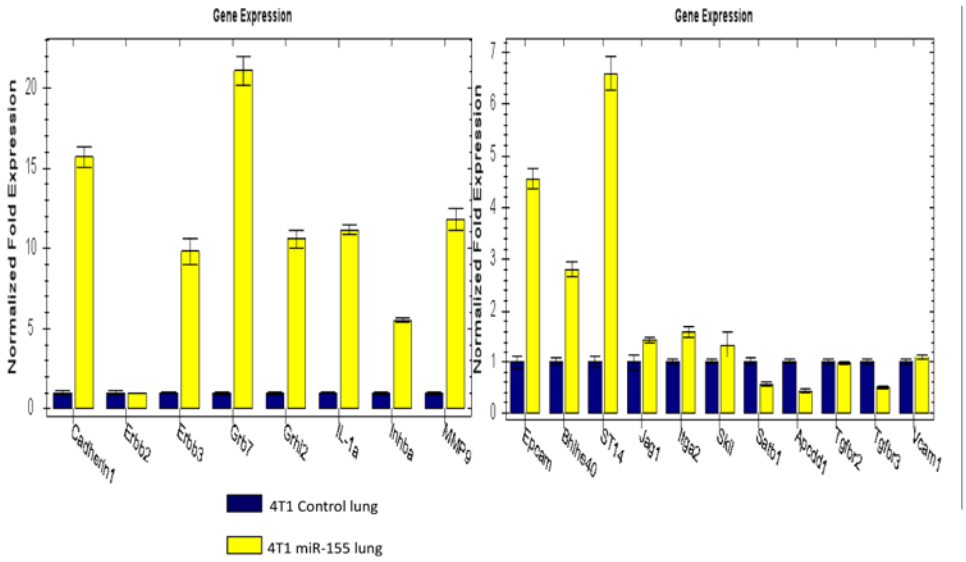


Figure 5. Genes differentially expressed between 4T1-miR-155 and 4T1-control recovered from lung and identified by cDNA microarray were verified by Real-time PCR

Quantitative real-time PCR expression analyses of genes as listed were carried out in 4T1-control and 4T1-miR-155 cells recovered from control lung and miR-155 lung. Results represent the mean ± S.E.M. of four independent experiments.

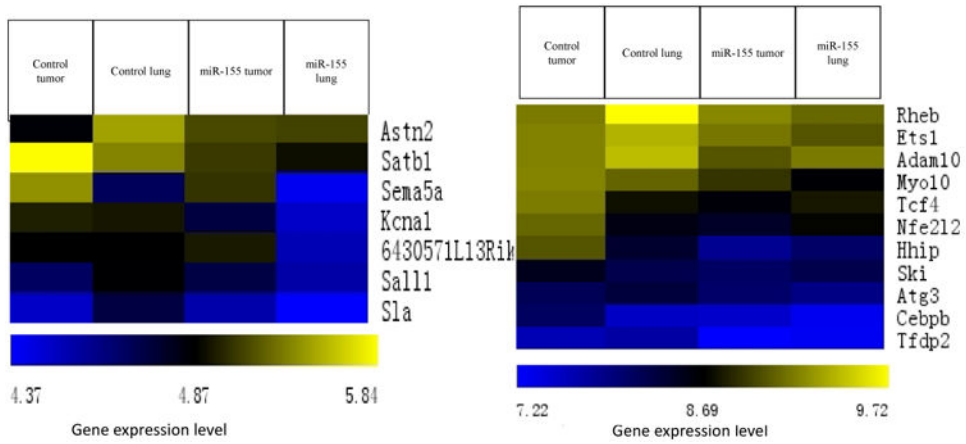


Figure 6. Heat map of genes containing at least one conserved miR-155 target site at 3′-UTR and differentially expressed in 4T1-miR-155 and 4T1-control

Hierarchical clustering of genes are differentially expressed in 4T1 cells recovered from tumor (miR-155 tumor) and lung (miR-155 lung) in comparison with 4T1-control recovered from tumor (control tumor) and lung (control lung). An expression ratio (log 2) scale is shown. The blue-yellow color-coded score indicates the significance level.

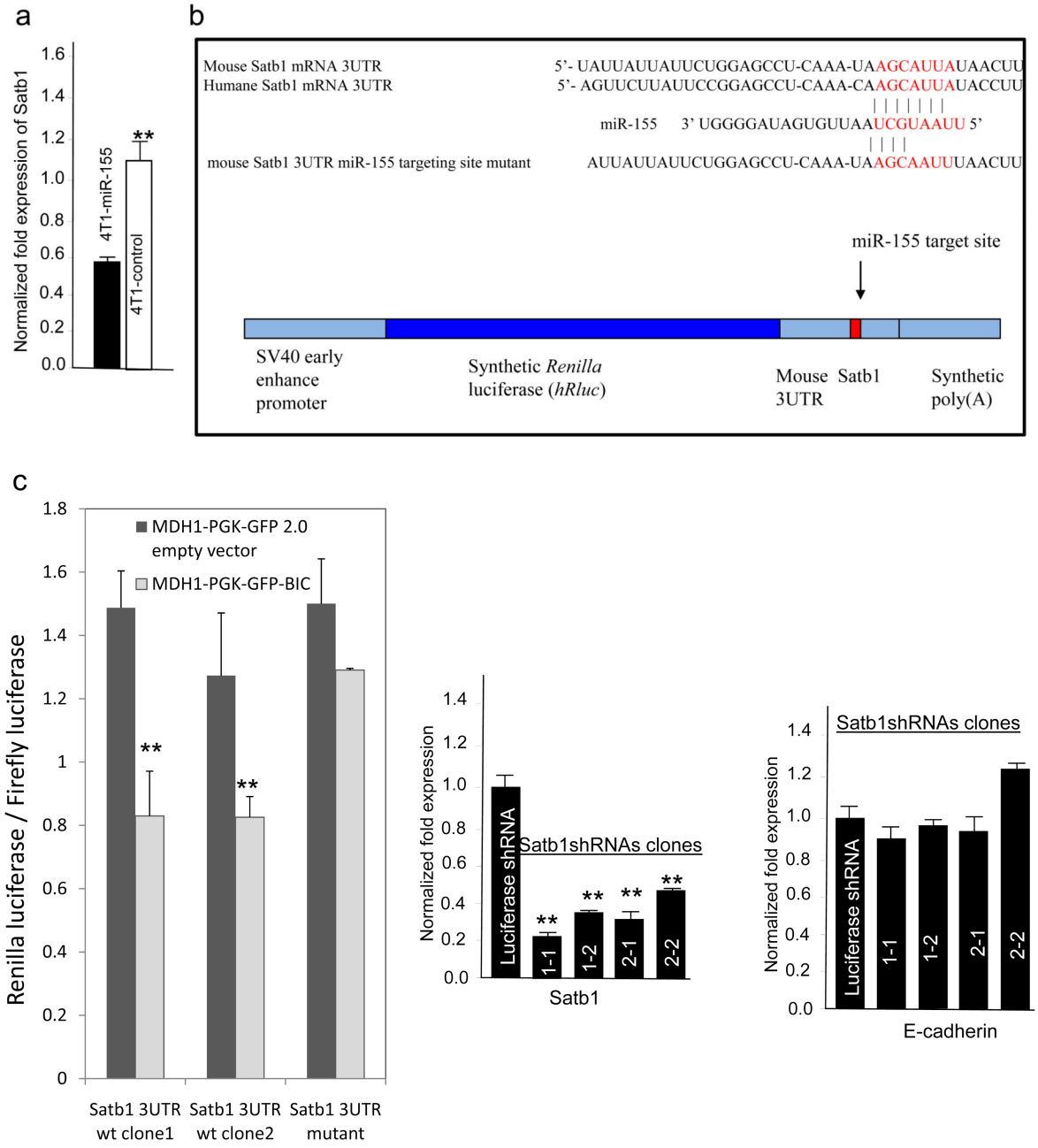


Figure 7. Expression of Satb1 is suppressed by stable-expression of miR-155

(a) Quantitative real-time PCR expression analyses of Satb1 were carried out in 4T1-control and 4T1-miR-155 cells recovered from the lung. Results represent the mean ± S.E.M. of three independent experiments. (b) Conserved miR-155 target site is located at the 3′-UTR of Satb1 mRNA and are shown as alignment of mouse and human sequences in the 3′-UTR of Satb1 mRNA (b, top panel). The representation in 7b is limited to the region around the miR-155 complementary site. In bold, the "seed" region with a conserved anchoring adenosine (complementary to the first nucleotide of miR-155) matches to the eighth nucleotide of the miRNA. Schematic representation of the construct used in the luciferase reporter assay (b, bottom panel). (c) Luciferase reporter assays were performed by

transiently transfecting 293T cells with a vector over-expressing miR-155 or a control pMDH1-PGK-GFP vector with the indicated 3′-Satb1-UTR luciferase reporter plasmids. Renilla luciferase values were normalized to Firefly luciferase. Results represent the ratio of Renilla luciferase to Firefly luciferase (left panel). An asterisk (*) indicates a p-value of less than 0.05 in a Student's t-test. 4T1 cells were transinfected with a retrovirus over-expressing shRNA targeting mouse Satb1 at two different sites with a duplicate clone for each site or luciferase gene as a control. 72 h after the transfection, GFP^+ cells were sorted out. The expression of Sabt1 (c, middle panel) and E-cadherin (c, right panel) in sorted cells was analyzed by real-time PCR. Results represent the mean ± S.E.M. of three independent experiments.