

ORIGINAL ARTICLE

Re-expression of microRNA-375 reverses both tamoxifen resistance and accompanying EMT-like properties in breast cancer

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Epithelial–mesenchymal transition (EMT) is an initiating event in tumor cell invasion and metastasis. It has been shown to occur in resistance to a range of cancer therapies, including tamoxifen. MicroRNAs (miRNAs) have been associated with EMT as well as resistance to standard therapies. To investigate the role of miRNAs in the development of resistance to tamoxifen as well as accompanying EMT-like properties, we established a tamoxifen-resistant (TamR) model by continually exposing MCF-7 breast cancer cells to tamoxifen. In addition to the molecular changes known to be involved in acquired tamoxifen resistance, TamR cells displayed mesenchymal features and had increased invasiveness. Genome-wide miRNA microarray analysis revealed that miRNA-375 was among the top downregulated miRNAs in resistant cells. Re-expression of miR-375 was sufficient to sensitize TamR cells to tamoxifen and partly reversed EMT. A combination of mRNA profiling, bioinformatics analysis and experimental validation identified metadherin (MTDH) as a direct target of miR-375. Knockdown of MTDH partially phenocopied the effects of miR-375 on the sensitivity to tamoxifen and the reversal of EMT. We observed an inverse correlation between the expression of miR-375 and its target MTDH in primary breast cancer samples, implying the pathological relevance of targeting. Finally, tamoxifen-treated patients with higher expression of MTDH had a shorter disease-free survival and higher risk of relapse. As most cancer-related deaths occur because of resistance to standard therapies and metastasis, re-expression of miR-375 or targeting MTDH might serve as potential therapeutic approaches for the treatment of TamR breast cancer.

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INTRODUCTION

Up to 70% of all breast cancer patients overexpress the nuclear receptor estrogen receptor- α (ER- α),¹ making it an excellent candidate for endocrine therapy. Tamoxifen is an ER antagonist that competitively inhibits the interaction of estrogen with ER, thus repressing ER activity,^{2,3} and is commonly administered in adjuvant first-line treatment of ER- α + patients. However, up to 50% of patients with metastatic disease do not respond to first-line treatment with tamoxifen; moreover, many initial responders experience relapse.⁴ Emerging evidence suggests a strong link between resistance to therapies and the induction of epithelial–mesenchymal transition (EMT) in cancer.⁵ Morphologically, EMT is characterized by the loss of tight cell–cell junctions and accompanied by re-organization of the actin cytoskeleton, resulting in spindle shaped mesenchymal-like cells, which are capable of migrating and invading other tissues.⁶ The EMT process is mimicked by cancer cells, which detach from the primary tumor, invade through the extracellular matrix, and migrate to form distant metastases upon certain cues in the tumor microenvironment (for example, EGF, TGF- β , Wnt and so on). Therefore, determining the mechanisms that connect EMT and the development of drug resistance could be a key approach for the development of novel therapeutic strategies to overcome both drug resistance and potentially prevent metastasis initiation.

MicroRNAs (miRNAs) are 20–22 nucleotide noncoding RNAs, which mostly anneal in the 3'-UTR of protein coding mRNAs at sequences that have imperfect complementary, leading to post-transcriptional silencing of the respective genes. The involvement of miRNAs in drug resistance and EMT has been described. A recent study by Miller *et al.*⁷ investigated the role of miRNAs in tamoxifen resistance in breast cancer where differentially expressed miRNA signatures representing tamoxifen resistant (TamR) and sensitive cell lines were identified. They showed that two upregulated miRNAs, miR-221/222, alone were able to confer resistance to tamoxifen, potentially owing to their large gene-targeting spectrum. Moreover, miRNAs have also been described to regulate the EMT process. One example is the miRNA-200 family, which targets the transcriptional repressors (for example, ZEB1 and ZEB2) of E-cadherin and blocks the invasion of cancer cells.⁸ However, to the best of our knowledge, miRNAs that regulate both the resistance to tamoxifen as well as accompanying EMT-like properties, resulting in higher invasive capacity in breast cancer, have not been reported until now.

In the current study, we developed and characterized an *in vitro* model of tamoxifen resistance to investigate miRNAs that are involved in the acquisition of tamoxifen resistance in breast cancer. We identified several miRNAs that are downregulated in TamR compared with wild-type (WT) MCF-7 cells. One of the most significantly downregulated miRNAs was miR-375. Re-expression

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of miR-375 sensitized TamR cells to tamoxifen and inhibited the invasive capacity of these cells. We then identified a target of miRNA-375, metadherin (MTDH), which was upregulated in TamR cells. RNAi-mediated silencing of MTDH partly mimicked the effects that we observed upon re-expression of miRNA-375 with respect to sensitization to tamoxifen and reversal of EMT and invasive capacity. Finally, we found that elevated MTDH levels were inversely correlated with miR-375 expression and positively correlated with poorer disease-free survival in tamoxifen-treated patients.

RESULTS

Characterization of TamR MCF-7 breast cancer cell line

In order to develop an *in vitro* model of acquired tamoxifen resistance the epithelial breast cancer cell line MCF-7, expressing ER- α (parental cell line or WT), was continuously exposed to 5 μ M tamoxifen for 1 year until cells had acquired resistance to tamoxifen. Resistance of the cells to tamoxifen was verified by cell cycle and cell viability assays (Figures 1a and b). Tamoxifen induced a G1 arrest of the cell cycle exclusively in the WT cells, whereas TamR cells were resistant to the cytostatic effects of the

drug. However, exposure to 10 μ M tamoxifen was sufficient to induce G1 arrest in both WT and TamR cells (Supplementary Figure S1) demonstrating the preserved sensitivity of TamR cells to tamoxifen of a higher concentration.

Next, we wanted to see if the results from the cell cycle assay were reflected in differences in the viability of cells. Although the viability of WT cells was significantly reduced by 25% after 5 μ M tamoxifen treatment ($P < 0.001$), that of the TamR cells were not affected under these conditions (Figure 1b). Furthermore, dose-response curves showed a significant difference in EC₅₀ values for WT and TamR cells (7.627 and 7.083 μ M, respectively, $P = 0.0078$ as determined by the F-test, Supplementary Table S1) validating our *in vitro* model of tamoxifen resistance. We then investigated whether there was a difference in the proliferation rate between WT and TamR cells under normal growth conditions. WT cells had a higher proliferation rate as measured by direct cell counting ($P < 0.05$, Figure 1c, left panel) and a luciferase-based viability assay ($P < 0.001$, Figure 1c, right panel) after 48 and 72 h compared with TamR cells.

As TamR cells are no longer responsive to the effects of tamoxifen at 5 μ M concentration, and as it was proposed that

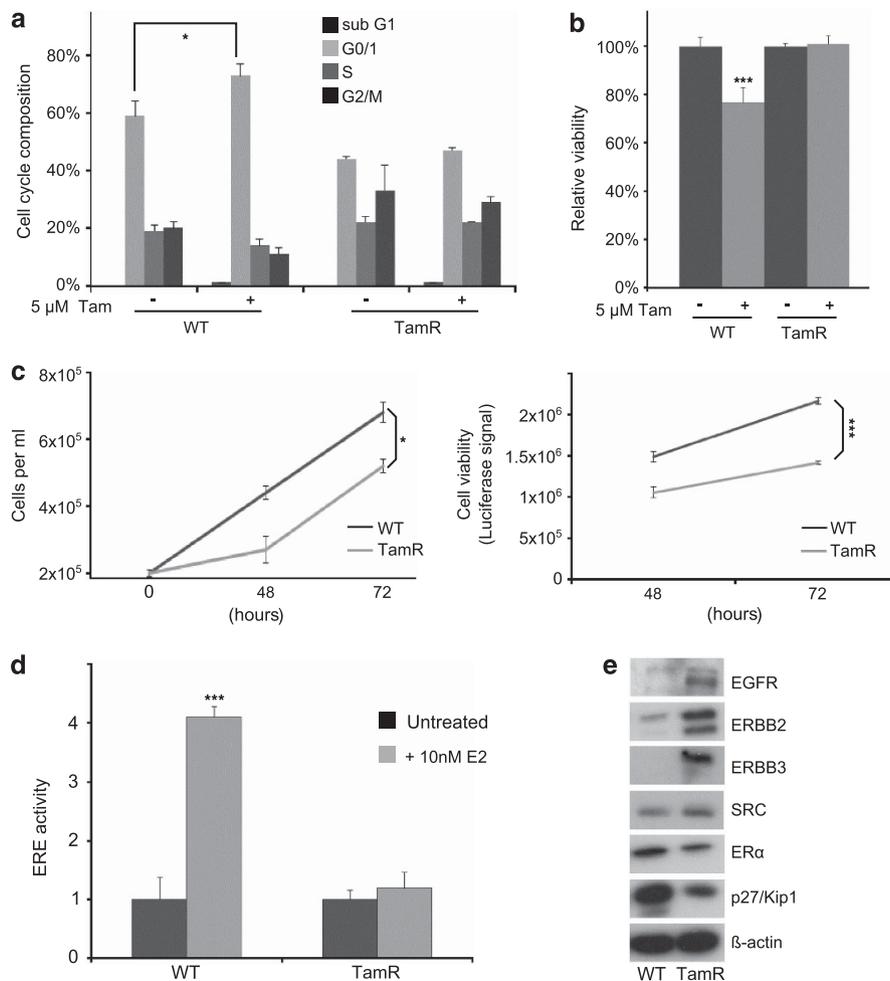


Figure 1. MCF7 cells have acquired resistance to tamoxifen. **(a)** The effects of tamoxifen on the cell cycle progression. MCF7-WT and MCF7-TamR cells were seeded in the presence of 5 μ M 4-hydroxytamoxifen (shown as 5 μ M Tam) or ethanol vehicle, and total DNA content was determined by 7-AAD staining after 72 h. Results are shown as the average and s.d. of three biological and two technical replicates each. $*P < 0.05$, $**P < 0.001$, $***P < 0.001$, determined by a two-sided *t*-test, hereafter. **(b)** The effects of tamoxifen on cell viability. MCF7-WT and MCF7-TamR were untreated or treated with tamoxifen, and viable cells were counted by a luciferase-based viability assay after 72 h. **(c)** Proliferation rate of WT and TamR MCF-7 cells. Proliferation rates were measured after 48 and 72 h by direct counting (left) and with a luciferase-based viability assay (right). **(d)** ERE activity was measured after stimulating cells with E2. The results are shown as average of three biological and four technical replicates each. **(e)** Western blot validation of molecular markers of tamoxifen resistance, with β -actin as the loading control.

long-term tamoxifen treatment may induce sensitivity to Estradiol⁹, we next examined if TamR cells might be sensitive to estrogen. To this end, we transfected WT and TamR cells with a luciferase-based reporter construct driven by an estrogen response element (ERE). After stimulation with E2 (estrogen), luciferase activity significantly increased in WT cells, although it remained at basal levels in TamR cells demonstrating that our TamR model is also resistant to Estradiol ($P < 0.001$) (Figure 1d). Finally, we examined the expression of molecular markers, for example, ErbB family receptors,¹⁰ Src kinase,¹¹ p27/Kip and ER- α ,¹² that all had previously been suggested to be involved in the development of tamoxifen resistance. In line with the previous findings, protein levels of EGFR, ErbB2 and ErbB3 as well as Src were upregulated, and those of p27/Kip1 and ER- α were downregulated in TamR cells compared with WT cells (Figure 1e). Overall, these results demonstrate that TamR MCF-7 cells had indeed acquired tamoxifen resistance and that these had obtained previously reported properties at the molecular and phenotypical levels.

TamR cells display mesenchymal-like markers and gain invasive properties

We next investigated whether the acquisition of the TamR phenotype was accompanied by morphological changes of the cells. WT MCF-7 cells grew in tightly packed cobblestone-like clusters, which is a typical characteristic of epithelial cells. In contrast, TamR MCF-7 cells appeared to have lost their tight cell-cell contacts and grew as loosely packed spindle-like cells and began spreading as individual cells (Figure 2a). This suggested an

EMT had taken place resulting in the acquisition of mesenchymal properties. We investigated the expression and localization of a key epithelial marker, E-cadherin, and examined actin cytoskeletal changes in the TamR cells compared with WT MCF-7 cells. E-cadherin predominantly localized at cell-cell contacts in WT cells, while the staining intensity was reduced in TamR cells (Figure 2b). Phalloidin staining revealed that actin filaments, which regulate cell migration, were re-organized in TamR cells. Furthermore, E-cadherin expression was strongly reduced at the mRNA level in TamR MCF-7 cells as compared with the WT cells (CDH1 in Figure 2c). Next, we examined the expression of other EMT marker genes by performing gene expression profiling in both WT and TamR MCF-7 cells. Intriguingly a range of "epithelial" marker genes were downregulated, whereas many "mesenchymal" marker genes were upregulated in TamR cells (Supplementary Table S2). Quantitative RT-PCR analysis verified the array results (not shown) and additionally showed that a number of 'EMT' genes, which had not been detected on the array, such as fibronectin (*FN1*) and *ZEB1*, were also upregulated in TamR cells (Figure 2c).

As TamR MCF-7 cells had gained a distinctive morphology of mesenchymal-like cells and as they also expressed increased levels of mesenchymal markers, we hypothesized that these cells should have gained increased invasive properties. Using Boyden chamber-based invasion assays we observed that, upon EGF stimulation, TamR MCF-7 cells had significantly higher invasion capacity as compared with their WT MCF-7 parental cells ($P = 0.026$, Figure 2d). Furthermore, tumorigenicity of the TamR MCF-7 cells was analyzed by growing cells on PolyHEMA, which is a test for anchorage-independent growth.¹³ While WT cells grew

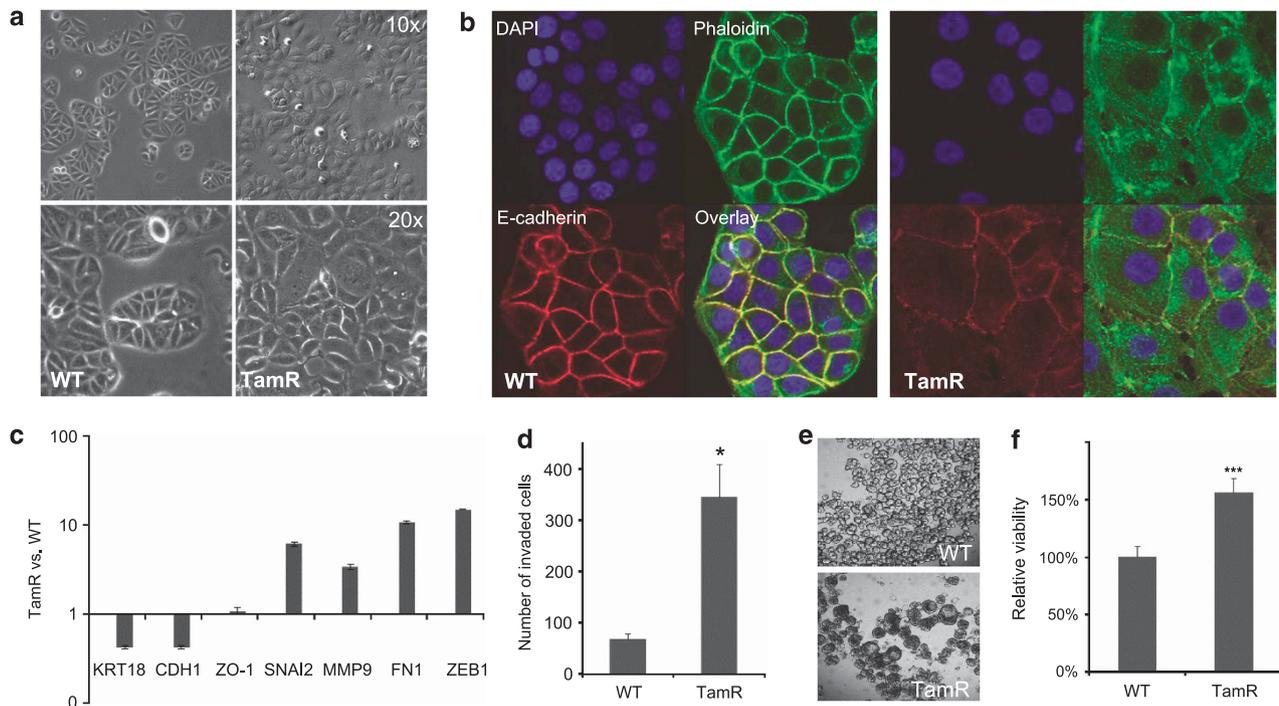


Figure 2. TamR cells are morphologically distinct, more invasive and tumorigenic than their WT parental cells. **(a)** Morphological differences between WT and TamR MCF-7 cells. Bright field images were taken at $\times 10$ and $\times 20$ magnification, respectively, when cells were 70% confluent. **(b)** Immunofluorescence staining of cell-cell junction protein E-Cadherin and the actin cytoskeleton. WT and TamR MCF-7 cells were stained with E-cadherin (red), Phalloidin (green) as well as DAPI and pictures were taken at $\times 40$ magnification. The results from A and B are shown as representative images from three biological replicate experiments. **(c)** Quantification of mRNA levels of epithelial and mesenchymal markers by qRT-PCR. Shown are the fold changes of expression in TamR compared with WT cells, normalized to *ACTB* and *HPRT*, and are presented as average of two biological as well as three technical replicates of each. **(d)** Comparison of EGF-driven invasion capacity of WT and TamR MCF-7 cells using a Boyden chamber-based invasion assay. The results are presented as average of two biological and three technical replicates each. **(e)** Anchorage-independent growth capacity of cells was determined by seeding cells in PolyHEMA-coated plates for 96 h. Pictures were taken at $\times 5$ magnification. **(f)** Proliferation of cells on PolyHEMA plates was measured with WST-1 reagent. The results are shown as averages of two biological and six technical replicates each. * $P < 0.05$, ** $P < 0.001$, *** $P < 0.001$, determined by a two-sided *t*-test.

Table 1. List of differentially expressed microRNAs in TamR MCF7 compared with WT MCF7 cells

miRNA	Fold change (log2)
<i>Top downregulated (LogFC > -2)</i>	
hsa-miR-135a	-3.66
hsa-miR-489	-3.45
hsa-miR-375	-3.11
hsa-miR-653	-3.08
hsa-miR-135b	-2.89
hsa-miR-556-3p	-2.50
hsa-miR-190b	-2.28
hsa-miR-556-5p	-2.19
hsa-miR-561	-2.19
hsa-miR-548 h	-2.08
<i>Top upregulated (LogFC > 2)</i>	
hsa-miR-551b	3.78
hsa-miR-519a	3.01
hsa-miR-376a*	2.90
hsa-miR-31	2.82
hsa-miR-224	2.45
hsa-miR-521	2.39
hsa-miR-31*	2.30
hsa-miR-655	2.26
hsa-miR-205	2.17
hsa-miR-518 f	2.05
hsa-miR-520 h	2.02
hsa-miR-455-3p	2.02

Abbreviation: miR, microRNA.

distributed over the PolyHEMA plates in small clusters, TamR cells grew in large tightly packed colonies (Figure 2e). Quantification of the growth of cells without substratum on PolyHEMA showed that TamR cells were significantly more capable of anchorage-independent growth than their WT counterparts ($P < 0.001$) (Figure 2f). All together, these data indicate that TamR cells had acquired EMT-like properties and become more invasive and tumorigenic compared with WT MCF-7 cells.

miRNA-375 is downregulated in TamR cells and its re-expression sensitizes cells to tamoxifen and reverts EMT-like properties

In order to identify miRNAs that are potentially involved in the underlying mechanisms of tamoxifen resistance and induction of EMT-like properties, we employed a miRNA microarray, and determined the miRNAs, which were differentially expressed between WT and TamR MCF-7 cells. Microarray analysis showed a significant downregulation of 10 miRNAs and upregulation of 12 miRNAs in TamR MCF-7 compared with WT cells (absolute log fold-change $|\log FC| > 2$, Benjamini-Hochberg adjusted P -value < 0.05 , Table 1).

miRNA-375 was among the top downregulated hits and its downregulation was validated by qRT-PCR (Figure 3a). This miRNA has been shown to be positively correlated with ER- α expression in breast cell lines¹⁴ and its ectopic expression has recently been reported to inhibit invasion in melanoma.¹⁵ Therefore, we next investigated if the downregulation of miRNA-375 could be involved in acquiring resistance to tamoxifen and gaining EMT-like properties in the TamR cells. For this purpose, TamR MCF-7 cells were transfected with either control precursor miRNAs or

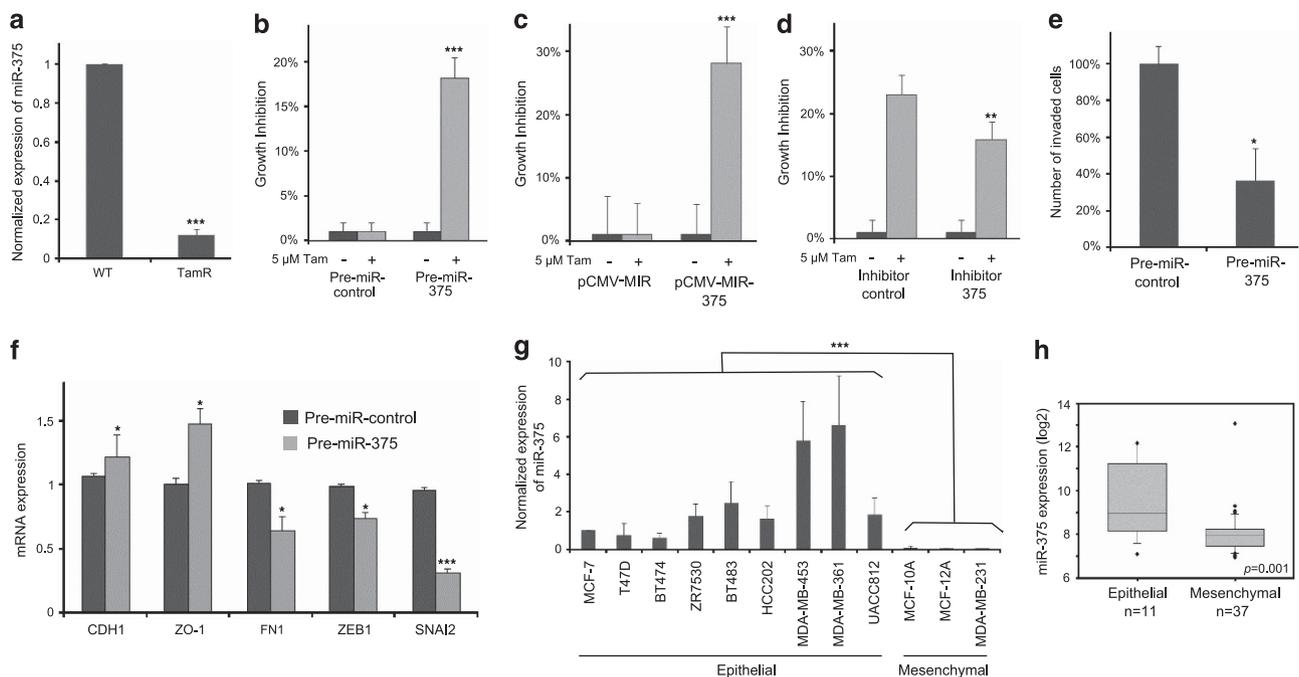


Figure 3. Re-expression of miRNA-375 in TamR cells sensitizes cells to tamoxifen, inhibits invasion and reverses EMT-like properties while knockdown of miRNA-375 promotes resistance to tamoxifen in WT cells. (a) qRT-PCR data validation of the downregulation of miRNA-375 in TamR cells compared with WT cells, normalized to *RNU44* and *RNU48*, which was obtained from miRNA microarrays. The results are shown as an average of four biological and three technical replicates. (b) Sensitizer effect of miRNA-375, using precursor molecules, on the TamR phenotype of cells as measured by Cell Titer Glo Luminescent Cell Viability assay analysis. (c) Sensitizer effect of miRNA-375, using an overexpression vector, on the TamR phenotype of cells. The results are shown as an average of three biological and four technical replicates. (d) Transfection with miRNA-375 inhibitor promotes resistance to tamoxifen. (e) miRNA-375 re-expression reduces cell invasion capacity of TamR MCF-7 cells in a Matrigel invasion chamber assay. The results are shown as an average of three biological and three technical replicates. (f) qRT-PCR data show that re-expression of miRNA-375 modulates the expression of EMT markers. The results are normalized to *ACTB* and *HPRT* and are shown as an average of four biological and three technical replicates. (g) Expression of miRNA-375 is reduced in a group of mesenchymal breast cell lines compared with epithelial ones. The results are normalized to *RNU44* and *RNU48* and are shown as an average of two biological and three technical replicates. * $P < 0.05$, ** $P < 0.001$, *** $P < 0.001$, determined by a two-sided t -test. (h) Boxplot depicting the expression level of miRNA-375 in a group of 11 epithelial cell lines compared with 37 mesenchymal cell lines from the NCI60 panel.

miRNA-375 precursor, and treated with or without tamoxifen. In the case of the precursor control, there was no effect on cell viability with or without tamoxifen treatment in TamR MCF-7 cells. However, in the case of miRNA-375 precursor, cell viability was reduced by 20% (Figure 3b) only in the presence of tamoxifen ($P < 0.001$), showing that recovery of miRNA-375 expression was capable of sensitizing TamR cells to tamoxifen. Considering the expression level achieved by transfecting precursor miRNAs is unphysiologically high (Supplementary Figure S2), we employed a miRNA overexpressing vector system to recapitulate our results. This vector induced around a 30-fold increase in the expression of miRNA-375 compared with the empty vector (Supplementary Figure S3). In the same experiment as described above, pCMV-MIR-375 was also able to sensitize cells to tamoxifen (Figure 3c). Additionally, we performed a loss-of-function experiment in WT MCF7 cells using an inhibitor against miRNA-375 (Supplementary Figure S4). In line with the above data, although the viability of cells transfected with the control was reduced by around 25%, viability of cells transfected with of miRNA-375 inhibitor was reduced by only 15%, (Figure 3d) indicating that loss of miR-375 promotes resistance to tamoxifen.

Next, we asked if miRNA-375 re-expression could also modulate the invasive capacity of the TamR MCF-7 cells. After transfection of cells with miRNA-375 precursor, cell invasion was significantly reduced compared with control precursor transfected cells (Figure 3e). This reduction in invasion was accompanied by decreased expression of mesenchymal genes (*fibronectin*, *ZEB1* and *SNAIL2/slug*) and increased expression of epithelial markers, E-Cadherin and *ZO-1*, upon re-expression of miRNA-375 (Figure 3f). Additionally, we analyzed miRNA-375 expression in a panel of 12-breast-cell lines including normal epithelial and cancer cells, which had previously been classified as either epithelial or mesenchymal.^{16,17} Interestingly, miRNA-375 expression was significantly reduced in the mesenchymal group of cell lines ($P < 0.001$), similar to the TamR MCF-7 cells, compared with epithelial cell lines (Figure 3g).

To examine a potential general role of miR-375 in cancer invasion, we analyzed miRNA-375 expression in the NCI-60 cell line collection representing 9 different tumor types (<http://dtp.nci.nih.gov/index.html>; Experiment ID: 372685) where cells had been defined as either epithelial or mesenchymal by Park *et al.*¹⁸ based on their respective ratio of E-cadherin to vimentin expression. In line with the data obtained in breast cancer cell lines, miRNA-375 expression was significantly reduced in the mesenchymal group of cell lines (Figure 3h). Furthermore, we were able to demonstrate the effects of miRNA-375 overexpression on EMT-like properties in a number of breast, lung and ovarian cell lines having mesenchymal phenotypes (Supplementary Figure S5). Overall, these results indicate that restoration of miRNA-375 sensitizes TamR cells to tamoxifen, knockdown of miRNA-375 induces resistance of WT cells to tamoxifen and miRNA-375 reduces invasion capacity of TamR cells, potentially rendering an epithelial phenotype. Overall, the link between miRNA-375 and EMT appears to be not restricted to breast cancer but to be a rather widespread mechanism in cancer.

Finally, to investigate the relevance of miRNA-375 in breast cancer patients, we analyzed the association between miRNA-375 expression and a number of clinicopathological features of breast cancer in two public data sets (Table 2). In both data sets, we observed that miRNA-375 levels were significantly higher in tumors of patients that were positive for ER ($P < 0.001$). In GSE19783, miRNA-375 was also significantly higher expressed in patients with WT p53, supporting the tumor suppressive role of this miRNA. Furthermore, in GSE22220, miRNA-375 was significantly reduced in the higher grade 3 tumors ($P = 0.033$) compared with lower grades. Overall, we could show that there was an association between miRNA-375 expression and clinicopathological characteristics of breast cancer patients

in two independent data sets thus supporting our *in vitro* findings.

MTDH is a direct target of miRNA-375

Having identified miRNA-375 as a regulator of both tamoxifen resistance and EMT-like properties, we were then interested in identifying which genes this miRNA modulates in TamR MCF-7 cells. As the miRNA-375 level was strongly reduced in TamR cells, we hypothesized that its potential targets should be upregulated. Therefore, we merged the list of upregulated genes in TamR cells (logFC > 0.5 difference in expression compared with WT cells) with potential target genes of miRNA-375, obtained from three publicly available prediction algorithms (TargetScan, PITA and DIANA-mT). This generated a list of 114 overlapping hits (Figure 4a, Supplementary Table S3).

Among those, we found MTDH (also known as AEG-1/Lyric) to be of special interest because of its established relevance in drug resistance and breast cancer metastasis.¹⁹ We hypothesized that upregulation of MTDH in response to reduced levels of miR-375 might be involved in both resistance to tamoxifen and accompanying EMT-like properties that we had observed in the TamR cells. To test this hypothesis, we first verified that MTDH was upregulated in TamR cells at mRNA and protein levels (Figure 4b). To investigate whether MTDH is regulated by miRNA-375, we next transfected cells with miRNA-375 precursors and examined MTDH expression. Both mRNA and protein levels were strongly decreased (Figure 4c). We then analyzed the 3'-UTR of MTDH and identified one potential target site for miRNA-375 located at position 1453–1461 (Figure 4d).

To determine whether the reduced expression of MTDH was due to direct targeting of miRNA-375, we cloned reporter constructs containing the 3'-UTR (both long and short versions as described in methods) of MTDH downstream of a luciferase open reading frame. For the long version, transfection with miRNA-375 precursor significantly reduced relative luciferase activity in both TamR ($P < 0.01$) and WT cells ($P < 0.001$), suggesting MTDH as a target of miRNA-375 (Figure 4e). Furthermore, mutations in the predicted miR-375 target sites abrogated inhibition by miR-375 precursor (Figure 4f), confirming the functionality of these target sites. We could further validate these results in an unrelated nontumor cell line, HEK-293FT, showing that the observed effects are not restricted to our model system. Finally, inhibition of miR-375 in MCF-7 cells resulted in an increase in relative luciferase activity (Figure 4g). In conclusion, the results

Table 2. Association of miRNA-375 expression and clinicopathological features of breast cancer^a

Feature	Number of patients	Average expression of miR-375	P-value
<i>GSE19783</i>			
ER status			
Negative	61	6.893	
Positive	38	8.417	$P < 0.001$
TP53 mutation			
WT	64	8.252	
MUT	37	7.144	$P = 0.033$
<i>GSE22220</i>			
ER status			
0	82	11.035	
1	128	11.923	$P < 0.001$
Tumor grade			
G1-G2	123	11.7	
G3	63	11.195	$P = 0.033$

Abbreviations: ER, estrogen receptor; miR, microRNA; WT, wild-type. ^aNot all features were available for all patients.

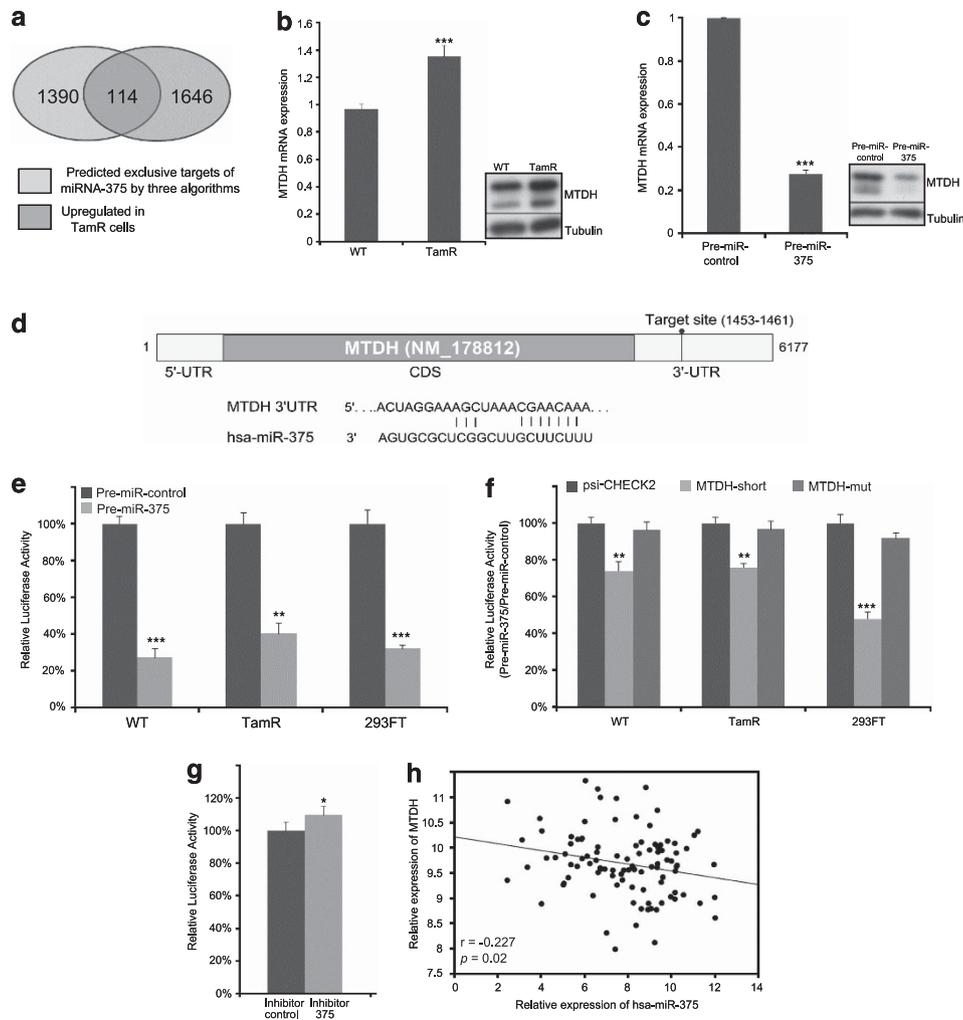


Figure 4. MTDH is a direct target of miRNA-375. **(a)** Intersection of genes which are upregulated in TamR cells with $\log_{2}FC > 0.5$ and genes predicted to be targets of miR-375 identified 114 genes, as candidate direct targets of miR-375, one of which is Metadherin (MTDH). **(b)** qRT-PCR and western blotting shows that MTDH/Metadherin is upregulated in TamR cells at mRNA and protein level, respectively. Tubulin was used as a loading control. **(c)** Re-expression of miRNA-375 downregulates MTDH 48 h after transfection both at mRNA and protein levels. *ACTB* and *HPRT* were used to normalize qRT-PCR data. **(d)** Gene structure of *MTDH* showing the predicted target site of miRNA-375 in its 3'-UTR. **(e)** Validation of direct targeting of *MTDH* (long version) by miRNA-375 with luciferase reporter assay. **(f)** Mutagenesis of target sites in the 3'-UTR of *MTDH* gene (short version) abrogates direct targeting of miRNA-375. **(g)** In WT MCF7, transfection with miRNA-375 inhibitor increases luciferase activity. Results are presented as an average of four biological and three technical replicates. * $P < 0.05$, ** $P < 0.001$, *** $P < 0.001$, determined by a two-sided *t*-test. **(h)** Correlation analysis of miR-375 and its target MTDH in breast cancer patient samples presented as a scatter plot. MTDH expression is negatively correlated with miRNA-375 expression (Pearson's correlation $r = -0.227$, $P = 0.02$).

of the luciferase assay confirmed that MTDH is a direct target of miR-375.

Finally, in order to examine the pathological relevance of this interaction, we analyzed a breast cancer patient data set (GSE19783) where complementary miRNA and mRNA expression data were available (sample size $n = 101$). As shown in Figure 4h, MTDH expression was negatively correlated with miRNA-375 expression in primary breast cancer samples (Pearson's correlation $r = -0.227$, $P = 0.02$). In summary, MTDH, a known factor in cancer metastasis and chemoresistance, is upregulated in TamR cells and a direct target of miRNA-375, and their expression is negatively correlated in breast cancer patients.

Loss of MTDH restores sensitivity to tamoxifen, reduces the invasive capacity of TamR cells, and is correlated with better disease-free survival in tamoxifen-treated patients

We have shown that restoration of miRNA-375 expression sensitized TamR cells to tamoxifen and inhibited invasion and

an EMT-like cell state. The direct targeting of MTDH by miRNA-375 led us to hypothesize that downregulation of MTDH by miRNA-375 in TamR cells could be involved in resistance and/or EMT. For this purpose, we silenced MTDH in TamR cells using RNA interference (Figure 5a). Knockdown of MTDH could indeed partially phenocopy the effects observed on sensitization to tamoxifen and EGF-driven invasion upon overexpression of miRNA-375 (Figures 5b and 5d), suggesting that it is involved in both tamoxifen resistance and EMT.

To exclude that these effects are potentially due to off-target effects of the small interfering RNA (siRNA) pools used for gene silencing, we tested the knockdown efficiency of four individual siRNAs directed against MTDH. All individual siRNAs strongly reduced MTDH expression (Supplementary Figure S6). We selected two individual siRNAs, #2 and #3, and tested their effects on sensitization and invasion. In both experiments the individual siRNAs were capable of inducing sensitization to tamoxifen and reducing cell invasion to a similar degree as the siRNA pool (Figures 5c and e, respectively) supporting the specificity of the

observed effects. Furthermore, we examined the expression of those EMT marker genes that we had analyzed upon over-expression of miRNA-375 (Figure 3d) and also after knockdown of MTDH (Figure 5f). siMTDH significantly increased E-Cadherin and ZO-1 expression and decreased the mesenchymal markers ZEB1 and SNAI2 showing that the involvement of miRNA-375 in EMT could, at least in part, be via targeting MTDH.

Finally, we asked if the expression of MTDH has a prognostic potential in breast cancer patients treated with tamoxifen. Therefore, we analyzed a publicly available patient data set (GSE1378) where hormone receptor-positive patients ($n = 60$) had been treated with adjuvant tamoxifen monotherapy and a minimum of 5-year follow-up data were available. Higher expression of MTDH significantly correlated with poorer disease-free survival and a higher risk of relapse ($P = 0.0233$, Figure 6a). In an additional independent data set, GSE1456, comprised of data from a population-based registry of patients who had been treated with tamoxifen ($n = 90$), elevated MTDH expression significantly associated with poorer relapse-free survival in the

tamoxifen-treated subgroup ($P = 0.009$, Figure 6b). All together, data from two independent studies suggest that the expression level of MTDH is correlated with survival of patients treated with tamoxifen.

DISCUSSION

In this study, we identified that miRNA-375 expression was reduced in TamR cells compared with their WT parental cells, and that its re-expression was capable not only of sensitizing cells to tamoxifen but also of reversing EMT-like properties and reducing invasion. Our findings indicate that tamoxifen-sensitive cells (WT MCF-7) expressing miRNA-375 at high levels directly represses MTDH expression, and that this regulation confers the cells with a tamoxifen sensitive and epithelial phenotype. In contrast, after developing resistance to tamoxifen, miRNA-375 expression is lost leading to a higher expression of MTDH and other targets, and the cells become resistant and more mesenchymal-like as well as invasive. Our results are supported by clinical data where we

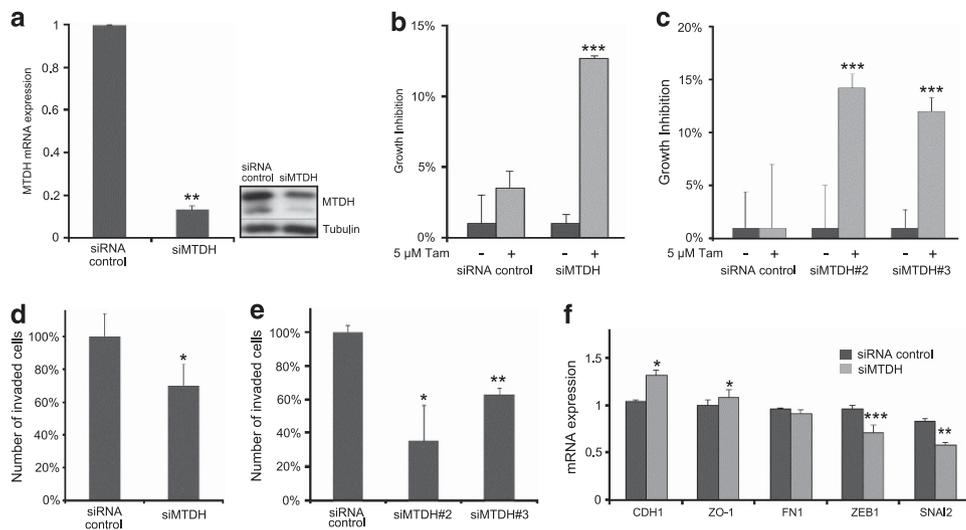


Figure 5. Silencing of MTDH phenocopies the effects of miR-375 re-expression on sensitization of TamR cells to tamoxifen, inhibition of invasion and reversion of EMT-like properties. **(a)** qRT-PCR and western blotting validate the downregulation of MTDH after siRNA knockdown in TamR cells. *ACTB* and *HPRT* were used to normalize qRT-PCR data. **(b)** Sensitizer effect of silencing MTDH using siRNA pool on the TamR phenotype of cells. **(c)** Sensitizer effect of silencing MTDH with individual siRNAs #2 and #3 on the TamR phenotype of cells. The results are shown as an average of three biological and four technical replicates. **(d)** MTDH knockdown reduces the invasion capacity of TamR MCF-7 cells. EGF-driven invasion was analyzed in cells 48 h after transfection with control siRNA or MTDH siRNA or **(e)** with individual siRNAs. The results are shown as an average of three biological and three technical replicates. **(f)** qRT-PCR data show that silencing of MTDH can modulate the expression of EMT markers, normalized to *ACTB* and *HPRT*. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, determined by a two-sided *t*-test.

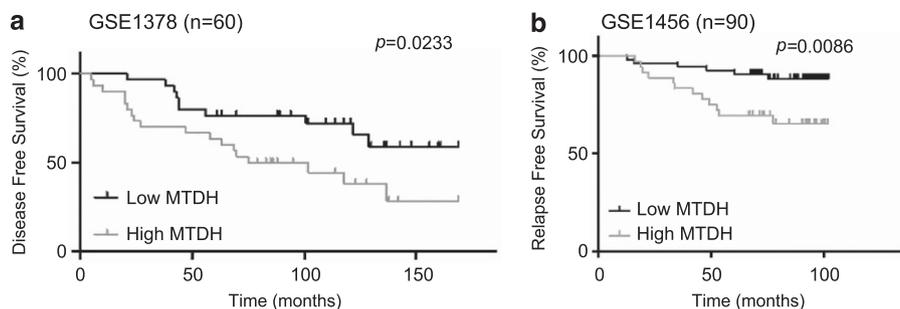


Figure 6. Higher expression of MTDH is significantly correlated with poorer disease-free survival and higher risk of relapse in tamoxifen-treated patients. **(a)** Analysis of 60 patients and **(b)** 90 patients who were treated with adjuvant tamoxifen monotherapy reveals that those having high expression of MTDH have a significantly poorer probability of survival compared with patients having low expression of MTDH. The low group was defined by having MTDH expression lower than the median of all patients in one study, and the rest patients were of the high group.

found a statistically significant inverse correlation between the expression of miRNA-375 and its target MTDH in primary breast cancer samples. Importantly, higher expression of MTDH was correlated with poorer disease-free survival in tamoxifen-treated patients supporting the role of MTDH in TamR breast cancer.

It is becoming increasingly evident that miRNAs are key modulators of drug resistance and EMT in many types of cancers. Few reports have been published so far revealing roles of miRNAs in tamoxifen resistance. Miller *et al.*⁷ reported that miR-221/222 were upregulated in TamR cells and in high ErbB2/HER2-expressing patients and were capable of conferring resistance to tamoxifen. More recently, it was shown that downregulation of miRNA-451 was responsible for resistance to tamoxifen by directly targeting the oncogene 14-3-3 zeta.²⁰ In line with our study, other groups have also characterized EMT in a similar *in vitro* model of tamoxifen resistance. Kim *et al.*²¹ observed a downregulation of E-cadherin expression that was accompanied by the upregulation of N-Cadherin, Vimentin and an EMT transcription factor, Snail, in TamR cells compared with WT cells. These findings match our own observations. Furthermore, we observed a strong induction of ZEB1 expression, which is a transcriptional repressor, binding and repressing E-Cadherin expression.²² Another group proposed that EMT induction in TamR cells was owing to increased levels of phosphorylated β -catenin, which is associated with EGFR, leading to increased transcription of EMT regulators.²³ However, none of these studies has addressed the potential co-regulation of EMT and tamoxifen resistance by miRNAs, as had been shown in the case of other drug resistance models.^{24,25} This led us to explore the potential of miRNAs linking tamoxifen resistance and EMT.

Whole-genome miRNA profiling comparing WT and TamR MCF-7 cells identified several miRNAs, which might be involved in the observed resistance and/or EMT-like properties of TamR cells. One of these downregulated miRNAs, miRNA-375, has already been reported to be downregulated in a range of cancers, including gastric cancer,²⁶ hepatocellular carcinoma²⁷ and more recently, melanoma,¹⁵ potentially suggesting a tumor-suppressor role for this miRNA. A recent study by Kong *et al.*²⁸ also described the tumor suppressive effects of miRNA-375 in esophageal squamous cell carcinoma where they showed that loss of miRNA-375 led to increased proliferation, invasion and metastatic potential of the cell system and poorer survival, similar to the results we have shown in breast cancer. Several studies have described miRNA-375 to be epigenetically regulated.^{14,15} DNA hypermethylation of the promoter of miRNA-375 might also explain the downregulation of miRNA-375 in our TamR model, but further studies are required to investigate whether epigenetic regulation of the miRNA is indeed also relevant in this cell system.

In search for the potential targets of miRNA-375, which might be involved in the observed resistance to tamoxifen and EMT-like phenotype, we identified *MTDH* as one of the candidate genes. *MTDH* has been shown in a number of studies to be involved in breast cancer metastasis. In line with our findings, a recent study by Li *et al.*,²⁹ could show that *MTDH* overexpression was capable of inducing EMT and modulating invasion as well as metastasis in breast cancer. It was also shown that overexpression of *MTDH* increased mesenchymal markers while downregulating E-cadherin expression, matching the increased E-cadherin expression upon *MTDH* knockdown we observed in the TamR cells. In another study, it has been shown that *MTDH* overexpression is associated with poor prognosis and increased risk of metastasis in breast cancer.¹⁹ In that study, a mouse model was employed to show that *MTDH* overexpression alone could mediate seeding of metastatic cells in the lungs as well as enhancing resistance to a range of chemotherapeutic drugs. This study also suggested that, although *MTDH* activation in breast cancer can be mostly attributed to a genomic gain of chromosome 8q22, there should be additional regulatory mechanisms. Indeed, in our work we

could show that *MTDH* expression can also be elevated upon gaining of resistance and EMT-like properties by downregulation of its targeting miRNA, miRNA-375.

In summary, we identified that loss of miRNA-375 in the TamR breast cancer model can be a potential modulator of both acquired tamoxifen resistance and EMT-like properties. Moreover, it can modulate invasive potential by the direct regulation of the *MTDH* oncogene. Furthermore, our study defined a novel role for *MTDH* in the context of tamoxifen resistance where our *in vitro* findings are supported by the clinical data that *MTDH* expression is correlated with disease-free survival of patients treated in an adjuvant tamoxifen monotherapy setting. As the majority of cancer-relevant deaths arise from resistance to therapy and metastasis, re-expression of miRNA-375 or inhibition of *MTDH* might be potential therapeutic approaches for the treatment of TamR breast cancer in future.

MATERIALS AND METHODS

Cell culture and development of TamR MCF-7 cell line

The human breast cancer cell lines, MCF-7 and MDA-MB-231, and lung cancer cell lines, H1703 and H1299 were obtained from ATCC (Manassas, VA, USA). HEK-293FT cells were obtained from Invitrogen (Carlsbad, CA, USA). The human ovarian cancer cell line, Ovar-5 was a kind gift from Dr Yasser Riazalhosseini. MCF-7 cells were cultured in MEM without phenol red supplemented with 10% FBS, 1% Pen/Strep, 1% L-glutamine and 1% sodium pyruvate (Invitrogen). Resistant cells (TamR) were developed by culturing parental cells (WT) in the presence of 5 μ M 4-hydroxytamoxifen (Sigma-Aldrich, St Louis, MO, USA) for ~1 year. Parental cells (WT) were cultured in parallel to resistant ones without addition of tamoxifen. HEK-293FT cells were grown in D-MEM high glucose medium containing 10% FBS, 1% pen/strep and 500 μ g/ml geneticin. Ovar-5, H1299 and H1703 were cultured in RPMI supplemented with 10% FBS and 1% Pen/Strep. MDA-MB-231 cells were cultured as previously described.³⁰ Medium without antibiotics was used for transfection. Multiplex human cell authentication as well as contamination tests of the cell lines were performed at the DKFZ Core Facility.

ERE reporter assay

For the measurement of ERE activity, luciferase reporter assays were performed using a reporter vector, 17-ERE-bGlob-Luc+, which drives the transcription of the *Firefly* luciferase reporter gene under control of an ERE. Both WT and TamR MCF-7 cells were seeded in 96-well plates and cotransfected with *Firefly* and normalizer *Renilla* (pRL-TK (Promega, Madison, WI, USA)) luciferase reporters. Twenty-four hours after transfection, cells were starved for 18 h and stimulated with 10 nM E2 for 24 h before cell lysis. ERE reporter activities were measured using the Dual Luciferase Reporter Assay System (Promega) according to manufacturer's protocol. *Firefly* luciferase values were normalized to *Renilla* luciferase activity.

Direct cell counting

WT and TamR MCF-7 cells were seeded in six-well plates. After 24 and 48 h, cells were harvested in 1 ml of medium and counted in triplicates using a Neubauer-counting chamber and a bright field microscope.

Whole genome miRNA/mRNA expression profiling

Total RNA was isolated according to the manufacturer's protocol using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Sentrix HumanWG-6 arrays (Illumina, San Diego, CA, USA) were used for mRNA profiling. Illumina Human miRNA microarrays were used for miRNA profiling. Quality control of total RNA (Agilent Bioanalyzer, Santa Clara, CA, USA) as well as labeling and array hybridization was performed at the DKFZ microarray Core Facility. Expression profiling data was normalized with the variance stabilization transformation algorithm (Bioconductor vsn package). Differential expression was determined with linear models, established with the limma package in Bioconductor.

Transfections with miRNA precursors, inhibitors, miRNA-expression vectors and siRNAs

Cells were seeded in full growth medium. After 24 h, medium was replaced with medium without antibiotics. Precursor miRNAs (Ambion, Austin, TX, USA), miRCURRY LNA miRNA inhibitors (Exiqon, Vedbaek, Denmark), miRNA-overexpression vectors pCMV-MIRs (Origene, Oxon, UK) and siRNAs (Dharmacon, Lafayette, CO, USA) were transfected to a final concentration of 50 nM, 100 nM, 25 ng/ml and 20 nM, respectively, using Lipofectamine 2000 transfection reagent (Invitrogen). For silencing of MTDH, either pools of four siRNAs or individual siRNAs were used (for sequences, see Supplementary Table S4).

Cell lysis and western blotting

Preparation of protein lysates and western blotting was previously described.³¹ Briefly, 10 µg of protein was separated by SDS-PAGE and exposed to primary antibodies as listed in Supplementary Table S5. HRP-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Quantitative RT-PCR for miRNAs and protein-coding genes

qRT-PCR for mRNA and miRNAs was carried out as previously described.³⁰ Sequences of primers and the respective UPL probe numbers are given in Supplementary Table S6. *ACTB* and *HPRT* were used as mRNA housekeeping genes while small RNAs *RNU44* and *RNU48* were used as miRNA housekeeping genes.

Cell viability assay and cell cycle analysis

Cells were seeded in the presence of 4-hydroxytamoxifen or ethanol solvent control. After 72 h, 7-AAD cell cycle assay was done according to the manufacturer's protocol (BD Pharmingen, San Diego, CA, USA). Stained cells were measured by flow cytometry (FACSCalibur, BD Bioscience, Heidelberg, Germany) using Cell Quest Pro software (BD Bioscience). Cell viability was measured using Cell Titer Glo Luminescent Cell Viability assay (Promega) following the manufacturer's instructions. Transfections were carried out in 96-well plates (5×10^3 cells per well) in five replicates.

Immunofluorescence staining and microscopy

For analysis with fluorescence microscopy, cells were seeded on square cover slips in six-well plates. Subsequently, cells were fixed with 2% paraformaldehyde for 15 min. For permeabilization, 0.2% Triton X-100 in PBS was applied for 5 min. Specimens were blocked for 30 min with 3% BSA/PBS. For detection of E-Cadherin, specimens were incubated with an anti-E-Cadherin antibody (Santa Cruz, CA, USA) for 1 h at room temperature. Alexa Fluor 647-labeled secondary antibody (Invitrogen) was applied for 1 h at room temperature. For staining of filamentous actin, cells were incubated with Alexa Fluor 488 phalloidin (Invitrogen) for 30 min at room temperature. For nuclear staining, cells were treated with 1 µg/µl 4',6-Diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) for 10 min. All antibodies and staining reagents were diluted in 3% BSA/PBS, and cells were washed three times with PBS after each step of the staining procedure. Cover slips were mounted with ProLong Gold antifade reagent. Fluorescence images were obtained using a Zeiss LSM 510 Meta microscope (Zeiss, Jena, Germany).

PolyHEMA assay

For PolyHEMA assays, 5×10^3 cells were seeded per well of PolyHEMA plates. 96 h after seeding, pictures were taken at $\times 5$ magnification with an Axiovert 25 light microscope (Hund, Wetzlar, Germany). In order to quantify the proliferation of cells on PolyHEMA, 10 µl WST-1 reagent (Roche, Penzberg, Germany) was added to each well and absorbance was measured at 450 nm after 2 h (Tecan, Männedorf, Switzerland).

Invasion Assay

For invasion assays, 1.5×10^5 cells were seeded in Matrigel invasion chambers (BD Pharmingen) in the presence of 25 ng/ml EGF (Sigma-Aldrich, Saint-Louis, MO, USA). 10% FBS was used as a chemoattractant. Invaded cells were trypsinized after 48 h and counted with flow cytometry (FACS Calibur) using Cell Quest Pro software (both from BD Bioscience).

Cloning 3'-UTR constructs and luciferase reporter assay

Long (2270 bp; NM_178812.3: 2008–4277) and short (101 bp; NM_178812.3: 3469–3569) 3'-UTR sequences of MTDH were amplified by PCR using human genomic DNA of the MCF-7 TamR cell line and cloned downstream of the *luciferase* ORF using the *XhoI* and *NotI* sites in the psiCHECK2 vector. To mutate the predicted binding site of miR-375 in the shorter 3'-UTR-containing vector, the QuickChange site-directed mutagenesis kit (Promega) was used. Sequences of primers used for cloning both constructs as well as those used in mutagenesis are listed in the Supplementary Table S7. For the luciferase reporter assay, cells were co-transfected with 50 nM of precursor miRNAs or 100 nM miRNA inhibitors and 15 ng (WT, TamR cell lines) or 5 ng (HEK-293FT cell line) of the luciferase reporter vector. *Firefly* and *Renilla* luciferase activities were measured after 48 h with a Dual-Luciferase Reporter Assay System (Promega) using a luminometer (Tecan), according to the manufacturer's instructions. Relative luciferase activity was determined by the ratio of *Renilla* luciferase signal intensity to that of *Firefly* for normalization. The average and s.d. of the ratio were estimated by the Delta-method (Bioconductor ratioAssay).

miRNA target prediction

The miRWalk database was used to identify predicted targets of miRNA-375.³² 3'-UTRs with a seed match of at least 7 bases and a *P*-value <0.05 were searched for using three database algorithms: TargetScan, PITA and DIANA-mT. The 114 overlapping genes that were upregulated in TamR cells and predicted to be targets of miRNA-375 are listed in Supplementary Table S3.

Analysis of patient data

Two datasets which included mRNA and miRNA expression data for human primary breast tumors were obtained from the NCBI GEO database (GEO Accession GSE19783³³ and GSE22220³⁴). mRNA expression data from 60 patients and 90 patients who had received tamoxifen monotherapy was obtained from the NCBI GEO database (GEO Accession GSE1378³⁵ and GSE1456,³⁶ respectively). Correlations and statistical analyses were carried out with SigmaPlot (Systat Software Inc, Chicago, IL, USA). Unless otherwise stated, all *P*-values were calculated by means of a two-sided *t*-test where *P*-values <0.05 were considered as significant. Kaplan–Meier survival curves and EC₅₀ dose-response analysis were carried out in GraphPad software (GraphPad software Inc., La Jolla, CA, USA).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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