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# Negative role of trihydrophobin 1 in breast cancer growth and migration

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**Trihydrophobin 1 (TH1) is a member of the negative elongation factor complex, which is involved in transcriptional pausing. Although the negative elongation factor complex attenuates the estrogen receptor α-mediated transcription, little is known about the relationship between TH1 and tumor progression. Here, we report that the protein level of TH1 was negatively correlated with the aggressiveness of human breast cancer. Immunohistochemical analysis revealed that TH1 expression in clinical stage III–IV primary breast cancer tissues was statistically significantly lower than that in stage I–II breast tissues ($P < 0.01$), and especially inversely associated with lymph node metastasis ($P < 0.001$). Furthermore, we showed that overexpression of TH1 in MDA-MB-231 breast cancer cells inhibited, and knockdown of TH1 in MCF-7 cells enhanced, cell proliferation and migratory ability. Moreover, upregulation of TH1 in MDA-MB-231 cells resulted in the decrease of cyclin D1, β-catenin, and ERK activity, and the increase of p21. In contrast, knockdown of TH1 in MCF-7 cells enhanced the expression of cyclin D1 and β-catenin, increased the activity of ERK, and downregulated the expression of p21. Additionally, overexpression of TH1 in MDA-MB-231 cells prevented. However, knockdown of TH1 in MCF-7 cells induced a number of molecular and cellular alterations associated with epithelial–mesenchymal transition. Taken together, our results suggest that TH1 might play an important role in regulation of proliferation and invasion in human breast cancer, and could be a potential target for human breast cancer treatment. (*Cancer Sci* 2010; 101: 2156–2162)**

Human trihydrophobin 1 (TH1) was originally identified during the positional cloning of mei-41 and was characterized further by the D.T. Bonthron group in 2000.[1,2] The *th1* gene, which is located in chromosome 20q13, was highly conserved and widely expressed. An independent study indicates that TH1 is identical to NELF-C/D, an integral subunit of the human negative elongation factor (NELF) complex.[3] The NELF complex is a four-subunit transcription factor, designated NELF-A, -B, -C/D, and -E in humans, that is physically associated with RNP polymerase II to induce transcriptional pausing.[4] The cofactor of BRCA1, COBRA1, which was isolated as a BRCA1 interacting protein and showed similar chromatin reorganizing activity to BRCA1, was found to be identical to NELF-B.[3,5] Previous studies have shown that COBRA1 and the rest of the NELF complex represses estrogen receptor (ER) α-mediated transcription and the growth of breast cancer cells in an estrogen-dependent manner.[6] Recently, we reported that TH1 is also linked to testicular development and targets the androgen receptor for degradation to suppress the transactivation of the androgen receptor.[7,8] Furthermore, we have shown that TH1 is a negative regulator of both A-Raf and PAK1 to regulate MEK/ERK signaling.[9–11] Although the understanding of its function has been advanced, little is known about the relationship between TH1 and breast tumor progression.

In this study, we report that the expression of TH1 was negatively correlated with the aggressiveness of human breast cancer cells and breast tumor tissues. Overexpression of TH1 in MDA-MB-231 cells inhibited the proliferation and migratory ability of cells and inhibited a number of molecular and cellular alterations associated with epithelial–mesenchymal transition (EMT). Results from MCF-7 cells with knockdown of TH1 further confirm that TH1 has a negative role in breast cancer cells. The expression of TH1 in breast cancer cells could regulate the expression of cyclin D1, β-catenin, and p21 as well as ERK activity, which might be involved in the biologic behavior of breast cancer cells. Taken together, these results suggest that a lack of TH1 expression in breast carcinoma might promote tumor progression and might serve as a useful target in breast cancer treatment.

## Materials and Methods

**Plasmids and cell lines.** Breast cancer cell lines, including MDA-MB-231, MCF-7, MDA-MB-435, T47D, MDA-MB-453, and SK-BR-3, were obtained from the Institute of Cell Biology of the Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM (Sigma, St. Louis, MO, USA) supplemented with 10% FBS (Gibco, Los Angeles, CA, USA). We constructed TH1 expression plasmids as described previously.[10,11] MDA-MB-231 cells were transfected with pcDNA3.1-Myc-TH1 or pcDNA3.1-Myc empty vector and MCF-7 cells were transfected with non-targeting shRNA construct or TH1 shRNA construct with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Cells were 1:10 passaged at 48 h and selected in medium containing G418 (600 μg/mL) for approximately 30 days. A single surviving clone was isolated by ring cloning and expanded into a stable cell line. Stable transfectants were identified by Western blot analysis and cultured with medium containing G418 (200 μg/mL).

**Tissue specimens.** All cases were retrieved from the Department of Pathology, Huashan Hospital, Shanghai, China. The patients had not previously received chemotherapy or radiation therapy. All cases were diagnosed by two experienced pathologists without discrepancy. The study was approved by the institutional review board at Huashan Hospital and all patients provided consent.

**Western blot assay.** Western blotting was carried out as described previously, using anti-TH1 antibody or indicated antibodies.[8,10] Anti-GAPDH, anti-cyclin D1, anti-β-catenin, anti-p53, anti-p-ERK (E-4), anti-ERK1/2, anti-p-MEK1(Ser298), anti-MEK1, anti-E-cadherin, anti-N-cadherin, anti-vimentin, and anti-twist antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-p21, anti-p27,

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anti-p-Akt (ser473), and anti-Akt antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA).

**Immunohistochemical staining.** Paraffin-embedded tissues were subjected to antigen retrieval by heating the slides in an autoclave at 120°C for 5 min in 0.1 M citric acid buffer (pH 6.0), then incubated with the rabbit anti-TH1 antibody (1:250) at 4°C overnight. After incubation in anti-rabbit detection reagent for 30 min at room temperature, the sections were developed in 0.05% diaminobenzidine containing 0.01% hydrogen peroxidase. For negative controls, the rabbit anti-TH1 antibody was replaced with normal goat serum by co-incubation at 4°C overnight preceding the immunohistochemical staining procedure.

**MTT assay.** Cells were seeded on 96-well plates at an initial density of $5 \times 10^3$/well. At each time point, cells were stained with 100 μL sterile MTT dye (0.5 mg/mL) for 4 h at 37°C, followed by removal of the culture medium and addition of 150 μL DMSO. The absorbance was measured using a Synergy 2 multi-mode microplate reader (BioTek Instruments, Winooski, VT, USA) at 490 nm. All experiments were carried out in triplicate.

**Wound-healing assay and Boyden chamber assay.** Wound-healing assay and Boyden chamber assay were carried out as described previously.[11] MDA-MB-231 cells plated on 6-well plates were grown to form a confluent monolayer, and wounds were made with sterile pipette tips. Photographs were taken at indicated time points after wounds were made. Cell migration assay was carried out using Boyden chambers (tissue culture treated, 6.5-mm diameter, 8-μm pores, Transwell; Costar, Cambridge, MA, USA) containing polycarbonate membranes. Serum starved cells were trypsinized and counted. Then 100 μL of $1 \times 10^6$ cells in serum-free medium was added to the upper chamber and 600 μL of appropriate medium with 10% FBS was added to the lower chamber. The Transwell was incubated for 12 h in MDA-MB-231 or 24 h in MCF-7 at 37°C. The migratory cells on the under-surface of the membrane were fixed and stained with 0.1% crystal violet for 20 min at room temperature. Photographs of three random regions were taken and the number of cells counted to calculate the average number of cells that had transmigrated.

**FACS cell analysis.** Cells at 70–80% confluence were incubated for 24 h in DMEM containing 0.5% FBS, then cultured back into DMEM with 10% FBS for 12 h. Cells were harvested and fixed in citric acid buffer for 30 min. After washing, the cell pellets were re-suspended in PBS and treated with 100 mg/L RNase and 10 mg/L propidium iodide for 20 min. Apoptotic cell fraction and cell cycle distribution were analyzed by FACScan cytometry (Becton-Dickinson, San Jose, CA, USA).

**Statistical analysis.** All values are expressed as the mean ± SD. The Student's *t*- test and chi-squared test were used to evaluate the experimental data. $P < 0.05$ means statistically significant.

## Results

**Expression of TH1 negatively correlated with aggressiveness of breast cancer.** To investigate the role of TH1 in breast cancer progression, we examined TH1 expression in six breast cancer cell lines, including four non-aggressive breast cancer cell lines (MCF-7, T47D, MDA-MB-453, and SKBR3) and two aggressive breast cancer cell lines (MDA-MB-435 and MDA-MB-231). Compared with the metastatic cell lines, non-metastatic cell lines had a higher expression level of TH1 (Fig. 1A). Furthermore, Western blot analysis showed that two out of four random primary breast cancer tissues had a low expression of TH1 compared with their matched adjacent non-cancerous tissues (Fig. 1B). These results imply that TH1 might have a negative role in breast tumor.

To determine whether the expression level of TH1 was associated with malignancy of breast tumors, we carried out immunohistochemical assays on human breast cancer tissues with an anti-TH1 antibody. Tissues were scored on the basis of the intensity of TH1 labeling and percentage of TH1-positive tumor cells (Fig. 2). Tissue was scored as: 0, negative TH1 staining for all tumor cells; 1, positive TH1 staining other than score 2; or 2, moderate TH1 staining for >50% tumor cells or strong staining for >5% tumor cells. Trihydrophobin 1 protein was detected both in the nuclei and cytoplasm of breast cancer cells. Positive TH1 staining was observed in tumor cells in 89 of 138 (64.5%) cases. Associations between TH1 expression and disease parameters in 138 breast carcinomas are summarized in Table 1. Statistical analysis revealed there was a significant inverse correlation of TH1 expression with tumor TNM stage ($\chi^2$-test, $P = 0.005$) and nodal stage ($\chi^2$-test, $P < 0.001$). This analysis indicated a significant negative correlation between TH1 protein levels and tumor aggressiveness. Taken together, these results imply that TH1 plays a negative role in breast tumor progression.

As TH1 is a negative regulator of ERK signaling and a subunit of NELF, which suppresses ERα transactivation,[6,10,11] we analyzed the association between TH1 expression and clinicopathological parameters. A negative relationship between TH1 expression and Ki67 or ERα positivity was expected. As shown in Table 2, Ki67 > 30% tumors tend to have low TH1 expression. However, we did not observe a significant association between TH1 immunoreactivity and Ki67 ($P = 0.093$), nor with other clinical prognosis markers, including ERα, progesterone receptor, HER2, or p53 ($\chi^2$-test, Table 2).

**Trihydrophobin 1 suppresses proliferation and migration of breast cancer cells.** To determine whether TH1 is a negative regulator of breast cancer growth and migration, we used two different breast cancer cell lines, MDA-MB-231 cell line, with low expression of TH1, and MCF-7 cell line, with high expression of TH1. We generated two MDA-MB-231 clones that overexpressed Myc-TH1 (MT1 and MT2) and one MDA-MB-231

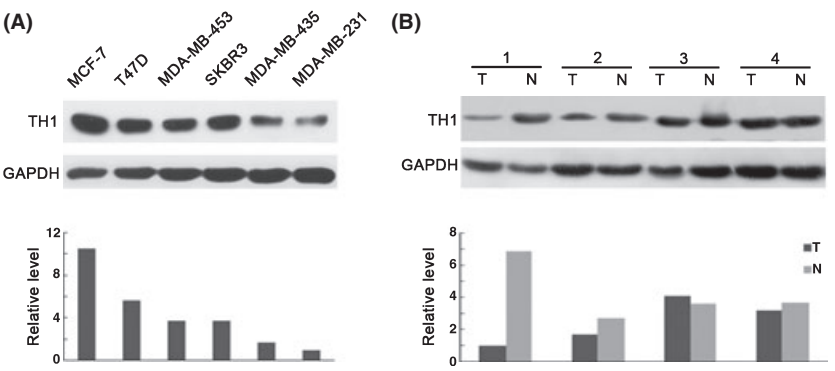


**Fig. 1.** Expression of trihydrophobin 1 (TH1) in breast cancer cell lines and tissues. (A) Expression of TH1 protein was detected in breast cancer cell lines. Extracts from four non-aggressive breast cancer cell lines (MCF-7, T47D, MDA-MB-453, and SKBR3) and two aggressive breast cancer cell lines (MDA-MB-435 and MDA-MB-231) were subjected to Western blot analysis. Expression levels were normalized for GAPDH. (B) Western blot analysis of TH1 protein from human primary breast cancer (T) and paired tumor-adjacent non-cancerous breast tissues (N), with each pair taken from the same patient. Expression levels were normalized for GAPDH.

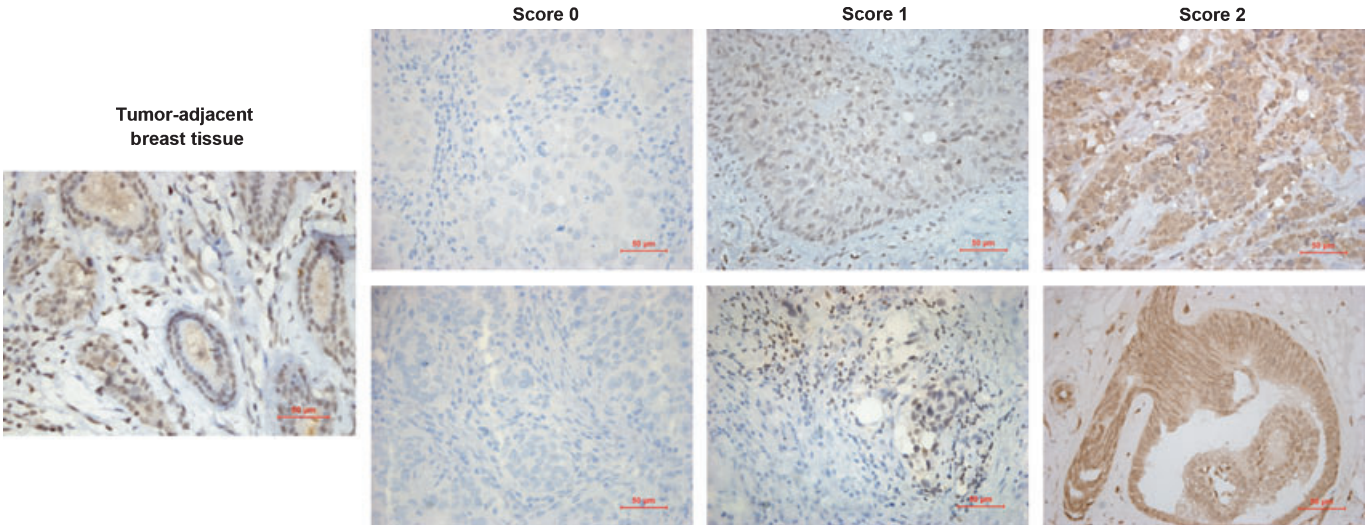


**Fig. 2.** Immunohistochemistry for trihydrophobin 1 (TH1) protein in breast cancer tissues. Immunostaining with anti-TH1 antibody was carried out on human breast cancer tissues. Tumor-adjacent non-cancerous breast tissues stained positively for TH1. Representative photographs are shown of negative and positive TH1 immunostaining in breast cancer cells. Tissue was scored as: 0, for negative TH1 staining for all tumor cells; 1, for positive TH1 staining other than score 2; or 2, for moderate TH1 staining for >50% tumor cells or strong staining for >5% tumor cells. Bar = 50 μm.

**Table 1. Association between trihydrophobin 1 (TH1) expression and disease parameters in 138 cases of breast carcinoma**

| | Total | TH1 | | | *P*-value |
|---|---|---|---|---|---|
| | | 0 (–) *n* = 49 | 1 (+) *n* = 80 | 2 (++) *n* = 9 | |
| TNM stage | | | | | |
| I and II | 100 | 28 | 63 | 9 | 0.005* |
| III and IV | 38 | 21 | 17 | 0 | |
| Tumor size | | | | | |
| ≤2 cm | 72 | 22 | 47 | 3 | 0.274 |
| 2 cm < T ≤5 cm | 58 | 24 | 28 | 6 | |
| >5 cm | 8 | 3 | 5 | 0 | |
| Lymph node metastasis | | | | | |
| $N_0$ | 68 | 13 | 50 | 5 | <0.001* |
| $N_1$ | 38 | 15 | 19 | 4 | |
| $N_2N_3$ | 32 | 21 | 11 | 0 | |
| BRE grade | | | | | |
| G1 | 35 | 12 | 19 | 4 | 0.486 |
| G2 | 86 | 29 | 52 | 5 | |
| G3 | 17 | 8 | 9 | 0 | |

*$P$ < 0.01. BRE, Bloom, Richardson, Elston-Ellis grading; 0, negative TH1 staining for all tumor cells; 1, positive TH1 staining other than score 2; 2, moderate TH1 staining for >50% tumor cells or strong staining for >5% tumor cells.

**Table 2. Association between trihydrophobin 1 (TH1) expression and clinicopathological parameters in 138 cases of breast carcinomas**

| | Total | TH1 | | | *P*-value |
|---|---|---|---|---|---|
| | | 0 (–) *n* = 49 | 1 (+) *n* = 80 | 2 (++) *n* = 9 | |
| Ki67 | | | | | |
| ≤30% | 92 | 27 | 59 | 6 | 0.093 |
| >30% | 46 | 22 | 21 | 3 | |
| ERα | | | | | |
| Negative | 48 | 17 | 26 | 5 | 0.388 |
| Positive | 90 | 32 | 54 | 4 | |
| PR | | | | | |
| Negative | 76 | 27 | 44 | 5 | 0.993 |
| Positive | 62 | 22 | 36 | 4 | |
| HER2 | | | | | |
| Negative | 110 | 38 | 65 | 7 | 0.870 |
| Positive | 28 | 11 | 15 | 2 | |
| p53 | | | | | |
| Negative | 69 | 26 | 38 | 5 | 0.781 |
| Positive | 69 | 23 | 42 | 4 | |

ER, estrogen receptor; PR, progesterone receptor; 0, negative TH1 staining for all tumor cells; 1, positive TH1 staining other than score 2; 2, moderate TH1 staining for >50% tumor cells or strong staining for >5% tumor cells.

clone with pcDNA 3.1-Myc (Control) (Fig. 3A). We also knocked down endogenous TH1 continuously by specific shRNA in MCF-7 cells (shTH1-1 and shTH1-2) and MCF-7 cells with scramble sequences shRNA (Scramble) as the control (Fig. 3B). In the MTT assay, we observed that overexpression of TH1 in MDA-MB-231 cells slowed down cell proliferation (Fig. 3C). Knockdown of TH1 in MCF-7 cells increased cell growth (Fig. 3C). Furthermore, rescue experiments showed that the inhibition of proliferation in MCF-7 by knockdown of TH1 can be resumed by re-expression of TH1 (Fig. S1). These results indicate that TH1 has an inhibitory role in proliferation of breast cancer cells.

To examine the migratory potential of these cells, we carried out a wound-healing assay. As shown in Figure 3(D), overexpression of TH1 markedly inhibited the migration of MDA-MB-231 cells. To further confirm the effect of TH1 on migration of breast cancer cells, we carried out a migration assay using a Boyden chamber with both MDA-MB-231 cells and MCF-7 cells. Overexpression of TH1 in MDA-MB-231 cells repressed, whereas knockdown of TH1 in MCF-7 cells elevated, the ability of cells to migrate (Fig. 3E). The results from these assays indicate that the migratory potential of cancer cells was significantly suppressed by TH1 overexpression and enhanced by TH1 knockdown. Taken together, these data indicate that TH1


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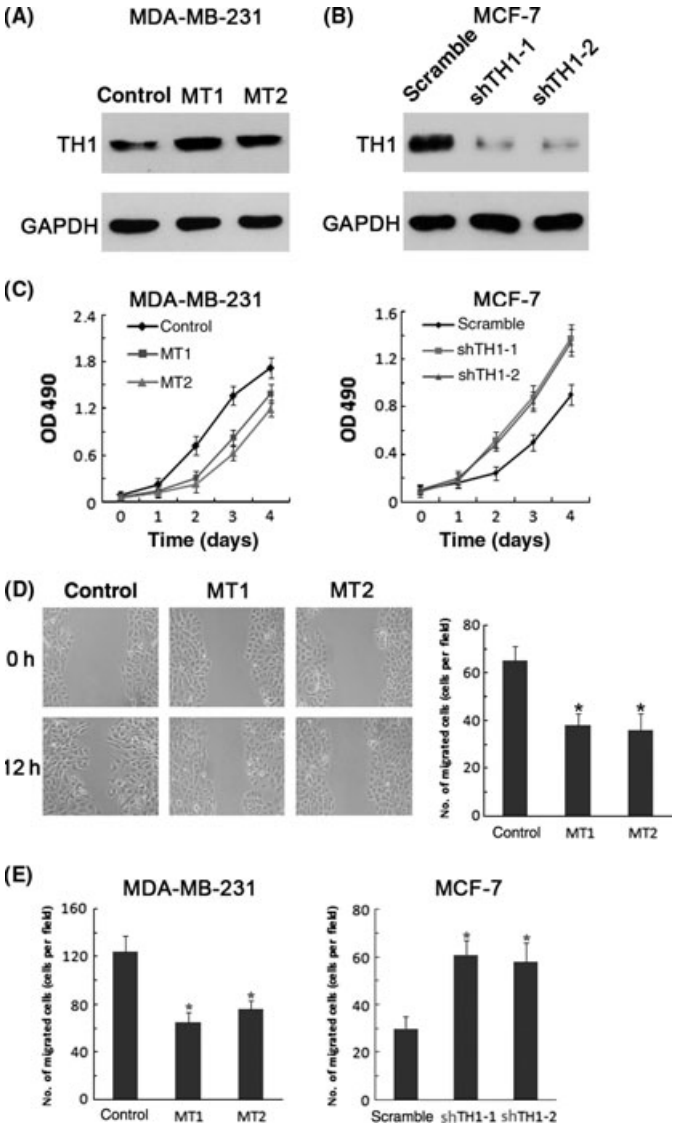


**Fig. 3.** Trihydrophobin 1 (TH1) suppresses proliferation and migration of breast cancer cells. (A) Western blot analysis of TH1 in MDA-MB-231 with pcDNA3.1-Myc (Control) and pcDMA3.1-Myc-TH1 (MT1 and MT2) stably transfected cells. (B) Western blot analysis for TH1 in MCF-7 cells transfected with scramble shRNA (Scramble) and TH1 shRNA (shTH1-1 and shTH1-2). (C) Detection of cell proliferation by MTT assay in the control and TH1-overexpressing MDA-MB-231 cells or in the scramble and TH1-knockdown MCF-7 cells. Data represent the mean ± SD from triplicate samples and are representative of three independent experiments. (D) Photographs taken at 0 h and 12 h after wounds were made in control and TH1-overexpressing MDA-MB-231 cells as results of a wound-healing assay. The mean ± SD of migrated cell numbers obtained from three parallel experiments. $*P < 0.05$. (E) Effects of TH1 on the cell migration in MDA-MB-231 cells and MCF-7 cells by Boyden chamber assay. The mean ± SD of migrated cell numbers obtained from three parallel experiments. $*P < 0.05$.

expression levels were inversely correlated with the aggressive phenotype of breast cancer cells, including increased cell growth and migratory potential.

**Effects of TH1 on associated regulators of cell cycle in breast cancer cells.** To investigate the potential molecular mechanism by which TH1 regulates breast cancer cell proliferation, we measured the cell cycle distribution and apoptotic cell fraction in TH1-overexpressing MDA-MB-231 cells. In flow cell cytometry analysis, overexpression of TH1 in MDA-MB-231 cells increased $G_0$–$G_1$ phase populations (Fig. 4A,B). Cells in S phase and $G_2$/M phase were diminished compared with control. However, no differences in the apoptotic cell fraction amounts were observed in MDA-MB-231 cells (Fig. 4A). These data indicate that TH1 might suppress breast cancer cell growth through blocking the $G_0$/$G_1$ to S phase transition in the cell cycle.

To understand the mechanism underlying the cell cycle arrest mediated by TH1, we examined the expressions of cyclins, cyclin-dependent kinases (CDKs), CDK inhibitors, and related signal factors. Western blot analysis indicated that overexpression of TH1 in MDA-MB-231 cells clearly downregulated cyclin D1 and upregulated p21 (Fig. 4C). In contrast, no obvious difference was detected for the expression of p27 and p53. Furthermore, the phosphorylation of MEK and ERK were decreased in TH1-overexpressing MDA-MB-231 cells (Fig. 4C), which is consistent with our previous reports.[10,11] Overexpression of TH1 also reduced the expression level of β-catenin, which can upregulate the transcription of cyclin D1.[12] In contrast, overexpression of TH1 had no obvious effect on the phosphrylation of Akt, which can activate the Wnt/β-catenin pathway (Fig. 4C).[13]

Subsequently, we explored a similar set of investigations in MCF-7 cells. Knockdown of TH1 in MCF-7 cells decreased $G_0$–$G_1$ phase cell populations and increased S phase populations, compared with the Scramble cells (Fig. 4D). Knockdown of TH1 in MCF-7 cells also increased ERK activity and enhanced the expression of β-catenin and cyclin D1. In contrast, knockdown of TH1 in MCF-7 cells decreased the amount of p21 protein. Taken together, these results suggest that cyclin D1, p21, β-catenin, and the ERK pathway are involved in the process of TH1-induced cell cycle arrest.

**Trihydrophobin 1 regulates expression of EMT-associated proteins.** Tumor progression towards the malignant phenotype requires the loss of the epithelial phenotype and the acquisition of a fibroblastic or mesenchymal one, known as EMT.[14] The morphological changes during EMT are driven by a number of molecular and cellular alterations, including decrease or loss of epithelial cell markers (e.g. E-cadherin) and de novo expression of mesenchymal markers (e.g. N-cadherin, vimentin).[15] We observed that TH1 overexpression lead to an obvious alteration in the phenotype of MDA-MB-231 cells (Fig. 3D), so we examined the associated markers and transcriptional factors of EMT. As expected, the accumulation of E-cadherin and decrease of vimentin was observed in the TH1-overexpressing MDA-MB-231 cells (Fig. 5A). Overexpression of TH1 also reduced the expression levels of Twist, which is an important transcription repressor of E-cadherin (Fig. 5A).[16] Moreover, knockdown of TH1 in MCF-7 cells could increase the expression of Twist and decrease the levels of E-cadherin (Fig. 5B). These results indicate that overexpression of TH1 could inhibit, and knockdown of TH1 could lead to, the EMT process. These findings support the theory that TH1 expression might decrease the aggressive potential in breast tumor cells.

## Discussion

We have described how TH1 expression plays a critical role in the progression of breast cancer, supported by both clinical and experimental data. These results showed that TH1 expression is decreased in aggressive breast cancer cell lines, compared to non-aggressive breast cancer cells. Immunohistochemical and statistical analyses indicate that TH1 expression is negatively correlated to tumor stage, and especially lymph node metastasis. Although we did not observe a significant association between TH1 expression and Ki67 ($P = 0.093$), Ki67 > 30% tumors tend to have low TH1 expression and TH1 has a negative role in the proliferation of breast cancer cells. An expansion of sample

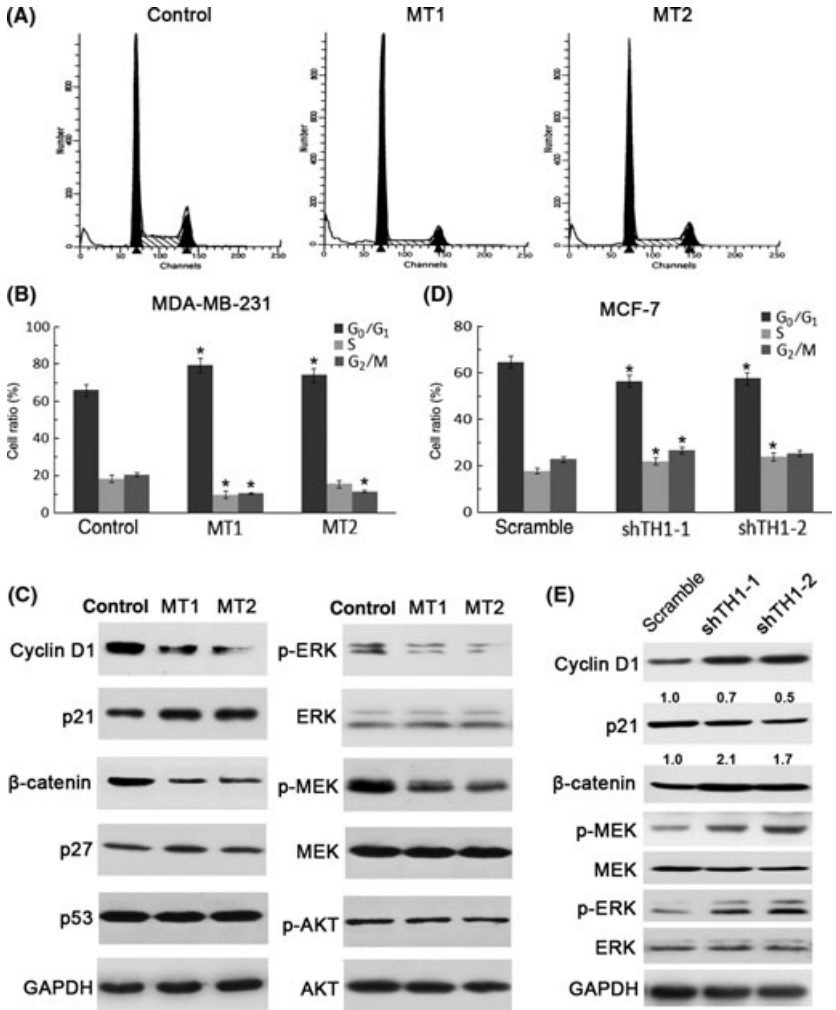


**Fig. 4.** Effects of trihydrophobin 1 (TH1) on associated regulators of the cell cycle in breast cancer cells. (A) Representative FACS analyses of the cell cycle distribution of control and TH1-overexpressing MDA-MB-231 cells (MT1 and MT2). (B,D) Percentages of cells in the $G_0/G_1$, S, and $G_2/M$ phases were counted in MDA-MB-231 cells or in MCF-7 cells. The mean ± SD of cell ratios were obtained from three parallel experiments. $^*P < 0.05$. (C,E) Western blot analyses of expression of cyclin D1, p21, β-catenin, p27, p53, p-ERK, total ERK, p-MEK, total MEK, p-Akt (ser473), and total Akt in control and TH1-overexpressing MDA-MB-231 cells or in MCF-7 cells transfected with scramble shRNA (Scramble) or TH1 shRNA (shTH1-1 and shTH1-2). GAPDH was used as the loading control.

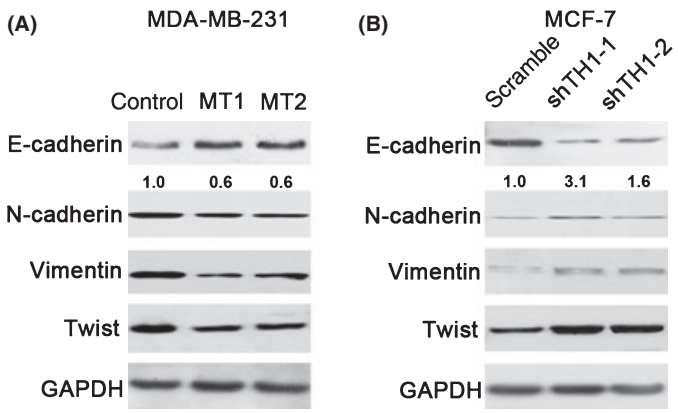


**Fig. 5.** Trihydrophobin 1 (TH1) regulates the expression of epithelial–mesenchymal transition-associated proteins in breast cancer cells. (A) Western blot analysis for expression of E-cadherin, N-cadherin, vimentin, and Twist in MDA-MB-231 cells. (B) Western blot analysis for expression of E-cadherin, N-cadherin, vimentin, and Twist in MCF-7 cells. GAPDH was used as the loading control.

scale might be required to further elucidate the association between TH1 and Ki67.

Gene expression is tightly controlled and regulated and deregulation of gene expression has been widely demonstrated in tumors. Previous studies have revealed that the abundance of COBRA1 and the other NELF subunits were mutually influenced in a tightly coordinated fashion.[17] Recently, we reported that TH1 is a specific substrate that is targeted for degradation through E6AP-catalyzed polyubiquitination.[18] Furthermore, analysis of the promoter of *th1* gene shows that a CpG island is associated with the TH1 promoter and transcriptional start site, which might have a role in the regulation of TH1 expression. In this study a decrease of TH1 expression was observed in breast cancer and had a negative role in breast cancer growth and invasion. However, the regulatory mechanism of TH1 expression in breast cancer cells remains elusive.

In our current study, we found that upregulation of TH1 slowed down the growth of human breast cancer cells by MTT assay and accumulation of TH1 suppressed the migration ability of cells, as indicated by wound-healing assay and Boyden chamber assay (Fig. 3). We also provided evidence that TH1 overexpression could block $G_1/S$ phase transition (Fig. 4). Many components of the cyclins, CDKs, and CDKIs are involved in mediating $G_1/S$ phase transition.[19,20] Decreased $G_1/S$ phase transition might be facilitated by TH1-mediated cyclin D1 downregulation and p21 upregulation (Fig. 4). It is well known that p21 is a tumor suppressor protein and a negative regulator in the $G_1/S$ transition.[21] It can interact with CDK4/6 complexes to inhibit their activity and cell proliferation.[22] Loss of p21 expression has been reported in various human cancers, and is associated with loss of control of cell growth.[23] Previous studies have indicated that cyclin D1 and p21 contribute to


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the alteration of cell cycle progression.[22] It has been shown that p21 can be upregulated in a p53-dependent manner or in a p53-independent manner.[24] In our present study, TH1 could upregulate p21 in a p53-independent manner.

Our results presented the attenuation of MEK/ERK signaling in breast cancer cells with overexpression of TH1. A previous study has shown that ERK signaling can enhance cell proliferation and promote $G_1$/S progression, leading to increased cyclin D1 and reduction of p21.[25] These results imply that the downregulation of cyclin D1 and upregulation of p21 in TH1-overexpressing MDA-MB-231 cells might be through inhibition of MEK/ERK signaling. However, β-catenin was found to be decreased in TH1-overexpressing cells. Cyclin D1, as a downstream target of the Wnt pathway, can be downregulated by the reduction of β-catenin.[26]

Migration plays an important role in the process through which cancer cells escape from the primary site to achieve tumor spreading.[27,32] Migration requires cell motility, which is polarized rearrangements of the actin/myosin cytoskeleton to move directionally.[28,29] The inhibition of the MAPK pathway impairs the motility of cells through repressing the phosphorylation of Rac1 and RhoA, which is involved in the assembly and turnover of focal adhesions.[30,31] Our previous studies have shown that ERK activity is important for cell migration.[11] Negative regulation of the MAPK pathway by TH1 might be one of the mechanisms that defines the role of TH1 in the motility and migration of breast cancer cell.

In breast cancer, lymph node metastasis is an early event in cancer cell spreading and is an important mark for breast cancer staging.[32] Epithelial-mesenchymal transition, migration, and invasion are critical events for cancer cell dissemination.[33] A process by which tumor-associated epithelial cells obtain mesenchymal features, EMT results in reduced cell–cell contact and increased motility.[14,34] Invasion mediated by EMT has been largely attributed to the loss of E-cadherin, a tumor invasion suppressor.[35] A key mechanism of E-cadherin loss is that the transcriptional repressors of E-cadherin, such as Twist, Snail, and Slug, is transactived by a series of signal pathway in breast cancer cells.[16] Transforming growth factor-β signaling was first revealed to induce EMT in normal mammary epithelial cells.[36] Moreover, integrin, RTK, Notch, and Wnt signal pathways are related to EMT.[37–39] As expected, upregulation of TH1 restored the expression of E-cadherin and decreased the expression of Twist. The reduction of Twist in TH1-overexpressing cells might be targeted by inhibition of ERK signaling and the Wnt/β-catenin pathway.

Taken together, these results indicate that TH1 could be a negative regulator of breast cancer growth and invasion. The expression of TH1 in breast cancer cells regulates the expression of cyclin D1, β-catenin, and p21 as well as ERK activity, which might be involved in the biologic behavior of breast cancer cells. Therefore, TH1 could be useful as a therapeutic target for metastatic breast disease.

## Acknowledgments

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Re-expression of trihydrophobin 1 (TH1) rescues the TH1 RNAi-induced inhibition of proliferation in MCF-7 cells. (A) Detection of cell proliferation by MTT assay in Scramble, shTH1-1 transfected with pcDNA3.1-Myc (shTH1-1 3.1 vector), and pcDMA3.1-Myc-TH1 (shTH1-1 3.1 MT) MCF-7 cells. (B) Detection of cell proliferation by MTT assay in Scramble, shTH1-2 transfected with pcDNA3.1-Myc (shTH1-2 3.1 vector), and pcDMA3.1-Myc-TH1 (shTH1-2 3.1 MT) MCF-7 cells. Western blot analysis for expression of TH1 and GAPDH in these cells is also shown. Data represents the mean ± SD from triplicate samples and are representative of three independent experiments.

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