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# TGF-β-Inducible Gene TMEPAI Converts TGF-β from a Tumor Suppressor to a Tumor Promoter in Breast Cancer

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## Abstract

TMEPAI is a TGF-β-induced transmembrane protein that is overexpressed in several cancers. How TMEPAI expression relates to malignancy is unknown. Here we report high expression of TMEPAI in ER/PR-negative and HER2-negative breast cancer cell lines and primary breast cancers that was further increased by TGF-β treatment. Basal and TGF-β-induced expression of TMEPAI was inhibited by the TGF-β receptor antagonist SB431542 and overexpression of Smad7 or a dominant negative mutant of Alk-5. TMEPAI knockdown attenuated TGF-β-induced growth and motility in breast cancer cells, suggesting a role for TMEPAI in growth promotion and invasiveness. Further, TMEPAI knockdown decreased breast tumor mass in a mouse xenograft model in a manner associated with increased expression of PTEN and diminished phosphorylation of Akt. Consistent with effects via the PI3K pathway, tumors with TMEPAI knockdown exhibited elevated levels of the cell cycle inhibitor p27kip1 and attenuated levels of DNA replication and expression of HIF-1α and VEGF. Together, these results suggest that TMEPAI functions in breast cancer as a molecular switch that converts TGF-β from a tumor suppressive to a tumor promoting role.

## Introduction

Both single-copy gains and high level regional amplification of chromosomal arm 20q (~ 5- to 10-fold) occur in human breast cancer (1,2) and other tumors (2). This suggests a role for genes on 20q in tumor pathogenesis. 20q amplification is associated with immortalization and avoidance of cell senescence (3). TGF-β overactivity induces replicative senescence in untransformed cells and in oncogene transduced primary epithelial cultures (4) but is paradoxically oncogenic in established cancer, including breast cancer (5,6). Genes involved in bypassing senescence checkpoints could be the “missing links” that connect TGF-β to oncogenesis. *TMEPAI*, a TGF-β inducible gene (7) mapped to 20q13.3 (8), encodes a NEDD4 E3 ubiquitin ligase binding protein (9) and is overexpressed in cancers (7,10–13) including breast cancer (10,13). We speculated whether *TMEPAI* plays a role in breast cancer by favoring growth and invasion and/or antagonizing the tumor suppressive functions of TGF-β. We investigated the consequences of *TMEPAI* expression and knockdown using in vitro culture models and in vivo murine xenografts. TMEPAI profoundly affected the growth, motility and invasiveness of cultured breast cancer cells, growth of tumor xenografts, and expression of PTEN, $p27^{kip1}$, Hif-1α and VEGF. In view of our data showing *TMEPAI* gene amplification in breast cancer (13), we suggest that overexpression and/or increased or altered function of TMEPAI may be a “molecular switch” that converts TGF-β from tumor suppressor to tumor promoter. A recent report that TMEPAI sequesters Smad proteins to decrease TGF-β signaling (14) and our unpublished data are consistent

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with this premise. However, our findings suggest that the effects of TMEPAI may be even more pervasive and critically relevant to cancer progression than its Smad sequestering function would suggest.

## Materials and Methods

### Cell culture and Cell proliferation

All previously authenticated breast cell lines obtained from ATCC before 2009 were used. All of them tested positive for human origin and absence or presence of estrogen receptor α and HER2. Breast cancer cells [MDA-MB-231, BT-20, and MDA-MB-468(DMEM); HCC-1937 and T-47D (RPMI); CAMA-1 and MCF-7 (Minimal Essential Medium)] were grown in their respective medium with 10% fetal bovine serum. hTERT-HME1 cells were grown in Mammary Epithelial Basal Medium with required additives. All cells were maintained at 37°C in 5% $CO_2$. MDA-MB-231 cells, after receipt, were grown initially in L-15 medium without $CO_2$ and later shifted to DMEM. Cell proliferation was measured by either counting cells in a haemocytometer or quantitation of total cell DNA by Hoechst 33258. Since all isoforms of TGF-β behaved similarly in TMEPAI induction, all experiments described here were carried out with TGF-β1 at 2ng/ml concentration.

### Quantitative real-time-PCR

Total RNA was used for qPCR with TMEPAI specific primers and SYBR green PCR master mix in an Applied Biosystems 7500 Real-Time PCR System. The nucleotide sequences for PCR primers were: TMEPAI:5′-GCACAGTGTCAGGCAACGG -3′(forward)and 5′-AGATGGTGGGTGGCAGGTC-3′ (reverse); 18S rRNA:5′-GAGAAACGGCTACCACATCC-3′ (forward) and 5′-CACCAGACTTGCCCTCCA-3′ (reverse).

### TMEPAI knockdown and Immunoblotting

pLKO.1-based lentiviral vectors were packaged in 293T cells. shRNAs of human TMEPAI (shRNA1:5′-GAGCAAAGAGAAGGATAAACA-3′ and shRNA2:5′-GTCCCTATGAATTGTACGTTT-3′) cloned in lentiviral vectors were from Open Biosystems. MDA-MB-231 cells were infected with viral supernatants for 24 h at 37°C with 8 μg/ml Polybrene (Sigma) and selected with puromycin to obtain stable lines with TMEPAI knockdown. Total cell-lysates were immunoblotted as described(15).

### Tumorigenesis and Migration Assays

MDA-MB-231 cells ($2 \times 10^6$) expressing control or TMEPAI shRNA were implanted subcutaneously in 5- to 6-week-old female nude mice (6 animals/each group). Tumor volumes were measured with a caliper weekly. After 6 weeks, mice were sacrificed. Tumors were removed and processed for immunoblotting and immunohistochemistry (IHC). Migration and invasion assay was performed using Transwell Matrigel Invasion chamber (16) and wound induced migration as described (17,18).

## Results and Discussion

### TMEPAI gene amplification and expression in invasive breast ductal cancers and TGF-β regulation of TMEPAI expression

We reported in abstract form that *TMEPAI* gene is commonly amplified in breast cancers, particularly in ductal carcinomas, including a majority of triple negative tumors (13). We used array comparative genomic hybridization (aCGH) to detect genomic imbalances in cancers from 97 patients. While 45/85(53%) invasive ductal carcinomas and 2/11 (18%)

invasive lobular carcinomas showed gain (26/97, 26.8%) or high copy gain (21/97, 21.6%) of TMEPAI, 18 of 31(58.1%) triple negative cancers showed gene amplification. Most tumors with gene amplification were grade 3 tumors (34/47, 72.3%) (13). While these studies are being prepared for publication in article form, we felt that *TMEPAI* amplification may be a factor that increases cancer aggressiveness. In silico analysis of the Oncomine data base using published methods(19)suggested that *TMEPAI* expression is higher ($P < 0.001$) in invasive breast cancer compared to normal breast (supplement Fig. S1). Given TMEPAI amplification in 58.1% of triple negative breast cancers, we tested for TMEPAI protein expression in 4 triple negative breast cancers and corresponding normal/benign tissues by western blotting. Each of four matched normal/benign tissues did not express TMEPAI, whereas all four cancers exhibited varied levels of expression(Fig. 1A).

TMEPAI expression was assessed in 7 breast cancer cell lines. Three of four triple negative or phenotypically basal-like lines expressed more TMEPAI protein (MDA-MB-231, BT-20, HCC1937) than three estrogen receptor (ER) positive non invasive lines (MCF-7, T47D and CAMA-1) (Fig. 1B). MDA-MB-231 cells are devoid of ER and HER2 receptors and highly sensitive to TGF-β (20). Treatment of MDA-MB-231 cells with TGF-β for 6h resulted in ~ 40-fold induction of TMEPAI mRNA (Fig. 1C, top panel)and ~9 fold increase of protein (Fig. 1C, bottom panel). Induction was blocked by SB431542, a TGF-β receptor I (Alk5) kinase inhibitor (Fig. 1C). Induction by TGF-β was minimal or nil for TMEPAI mRNA (Fig. 1C, top panel) or protein (supplement Fig. S2) in benign human mammary epithelial cells immortalized with telomerase (hTERT-HME1). Smad7 and dominant negative TGF-β receptor I (DN Alk5) blocked basal as well as TGF-β induced TMEPAI suggesting a requirement for TGF-β receptor and Smad dependent TGF-β signaling for induction (Fig. 1D, top panel). MCF-7 cells do not express or induce TMEPAI in response to TGF-β; however, they did respond when Alk5 was overexpressed (Fig. 1D, bottom panel) suggesting defective TGF-β receptor I in these cells. Thus, induction of TMEPAI may be a key hallmark of invasive breast cancer cells with intact TGF-β signaling.

### Effects of TMEPAI knockdown on TGF-β dependent growth and migration

We used lentiviruses expressing 2 different TMEPAI shRNAs to assess their effects on growth, motility and invasive behavior of MDA-MB-231 cells. Both shRNAs ablated TMEPAI protein expression (Fig. 2A). TMEPAI was not expressed even in the presence of TGF-β. TMEPAI knockdown by either shRNA resulted in decreased cell growth, measured as increase of total DNA (Fig. 2B), or as cell number (not shown). Although TGF-β caused early growth inhibition of wild type and control shRNA expressing cells, there was a remarkable growth spurt after 72 hours of treatment; consequently, TGF-β treated cells outnumbered those without the cytokine by 96 hours (Fig. 2B). This effect was also observed in complete absence of serum (not shown). Importantly, TMEPAI shRNA inhibited proliferation regardless of exposure to TGF-β, at all time points (Fig. 2B). TMEPAI knockdown altered the morphological phenotype of MDA-MB-231 cells. By 72–96 hours of growth, cells with control shRNA displayed elongated and spindly morphology; without TGF-β, occasional cells showed loss of contact inhibition and growth of cells one on top of the other; with TGF-β, loss of contact inhibition was pronounced (supplement Fig. S3). In contrast, cells with TMEPAI shRNA displayed a cobblestone type epithelial morphology regardless of TGF-β treatment (supplement Fig. S3). We found a time-dependent increase of TMEPAI in TGF-β treated MDA-MB-231 cells that correlated with proliferation induced by the cytokine, including the late growth spurt (Fig. 2C). These data suggest that a critical concentration of TMEPAI may need to accumulate before the TGF-β induced growth spurt occurs.

Transwell invasion assays revealed extensive migration of MDA-MB-231 cells expressing control shRNA across matrigel in presence of TGF-β (Fig. 2D). Migration across the

membrane, and therefore, invasion through matrigel, was impaired in cells expressing TMEPAI shRNA regardless of TGF-β treatment (Fig. 2D). We reported that wound induced migration of epithelial monolayers is associated with increased autocrine TGF-β signaling (17,18). Therefore, we tested whether TMEPAI responds to wounding of MDA-MB-231 confluent monolayers. Wounding caused increased TMEPAI transcript and protein that was blocked by TGF-β receptor inhibitor SB431542 (Fig. 3A and 3B). Moreover, SB431542 inhibited the migration of wounded MDA-MB-231 cells (Fig. 3C), an effect mimicked by TMEPAI shRNA but not control shRNA (Fig. 3D). Because TMEPAI knockdown increases TGF-β signaling (14); and our unpublished data), these results show that TMEPAI affects cancer cell motility downstream of Smads. Whether TMEPAI knockdown attenuates cancer cell growth by releasing sequestered Smads, i.e., by increasing TGF-β signaling, or, as seems likely, by other effects as well, needs investigation. As indicated before, TGF-β inhibited MDA-MB-231 cell proliferation early, but promoted growth later (Fig. 2B). We are currently investigating how TMEPAI relates to this duality of TGF-β action that mirrors its paradoxical promotion of aggressiveness in established cancers (5).

### Inhibition of TMEPAI expression decreases tumor xenograft growth

Tumor volumes of MDA-MB-231 xenografts from cells with TMEPAI shRNA were less compared to those with control shRNA after 28 days (Fig. 4A, $p<0.05$). In agreement, the weights of TMEPAI knockdown tumors were correspondingly less (supplement Fig. S4). Expression of proliferation marker Ki67 was decreased by >70% in TMEPAI shRNA tumors relative to shRNA controls (Fig. 4B; and supplement Fig. S5) without any significant increase in apoptotic index by TUNEL staining (not shown) and expression of angiogenic factor VEGF was dramatically reduced in xenografts expressing TMEPAI shRNA (Fig. 4B and 4C). Both cultures and tumors derived from TMEPAI knockdown cells expressed lower levels of Hif-1α protein (Fig. 4C). Furthermore, TMEPAI knockdown resulted in reduced Akt phosphorylation, an event that promotes growth, and this was associated with increased expression of growth suppressors PTEN and $p27^{kip1}$ (Fig. 4D).

Our results show that profound effects of TMEPAI knockdown on the biology of a triple negative breast cancer cell line are accompanied by important alterations of several gene products that control cancer progression. While we have not investigated how these diverse effects come about, we suggest that they are related to the potent actions of TMEPAI expression or knockdown. The multiplicity of effects on tumor promoters (HIF-1α, VEGF) and tumor suppressors (PTEN, TGF-β, $p27^{kip1}$) congruently converging along a potentially beneficial direction is unusual for an intervention involving a single gene product. Therefore, we hypothesize that TMEPAI is a “master regulator’ of cancer progression. Our report provides detailed studies on only one cell line MDA-MB-231. Nevertheless, this cell line is a well studied and common model for aggressive breast cancers with demonstrated relevance in published work. While we are currently studying a more diverse variety of breast cancer cells, the ancillary data we provide offer support to our belief in the importance of TMEPAI and its relevance to cancer biology. Therefore these findings merit early attention by investigators in the field.

The supportive data include identification of the region amplified on 20q in breast cancers as the TMEPAI gene, provide evidence for local copy number variations and suggest that the majority of triple negative and invasive phenotypes are associated with copy gain (13). Furthermore, elevation of TMEPAI protein expression was observed not only in primary tumors but also in several breast cancer cell lines (Fig. 1). TMEPAI expression was detected mainly in invasive phenotypes of breast cancer cell lines.

Importantly, the data indicate that constitutive aberrant expression of *TMEPAI* not only promotes growth, migration, and invasion but also overcomes growth suppression by TGF-

β. For these reasons it seems possible that molecular events affecting TMEPAI and/or associated proteins may constitute a "molecular switch" that converts TGF-β, normally a tumor suppressor, to a tumor promoter role in breast cancer.

Triple negative tumors remain a major cause of breast cancer mortality because of their invasiveness and metastatic potential and lack of suitable molecular targets for treatment. This first report of a role for TMEPAI in tumor growth and invasiveness and its relationship to TGF-β should spur interest in further investigation of its role in cancer cell signaling. Such studies could lead to the development of tumor biomarkers and treatment targets.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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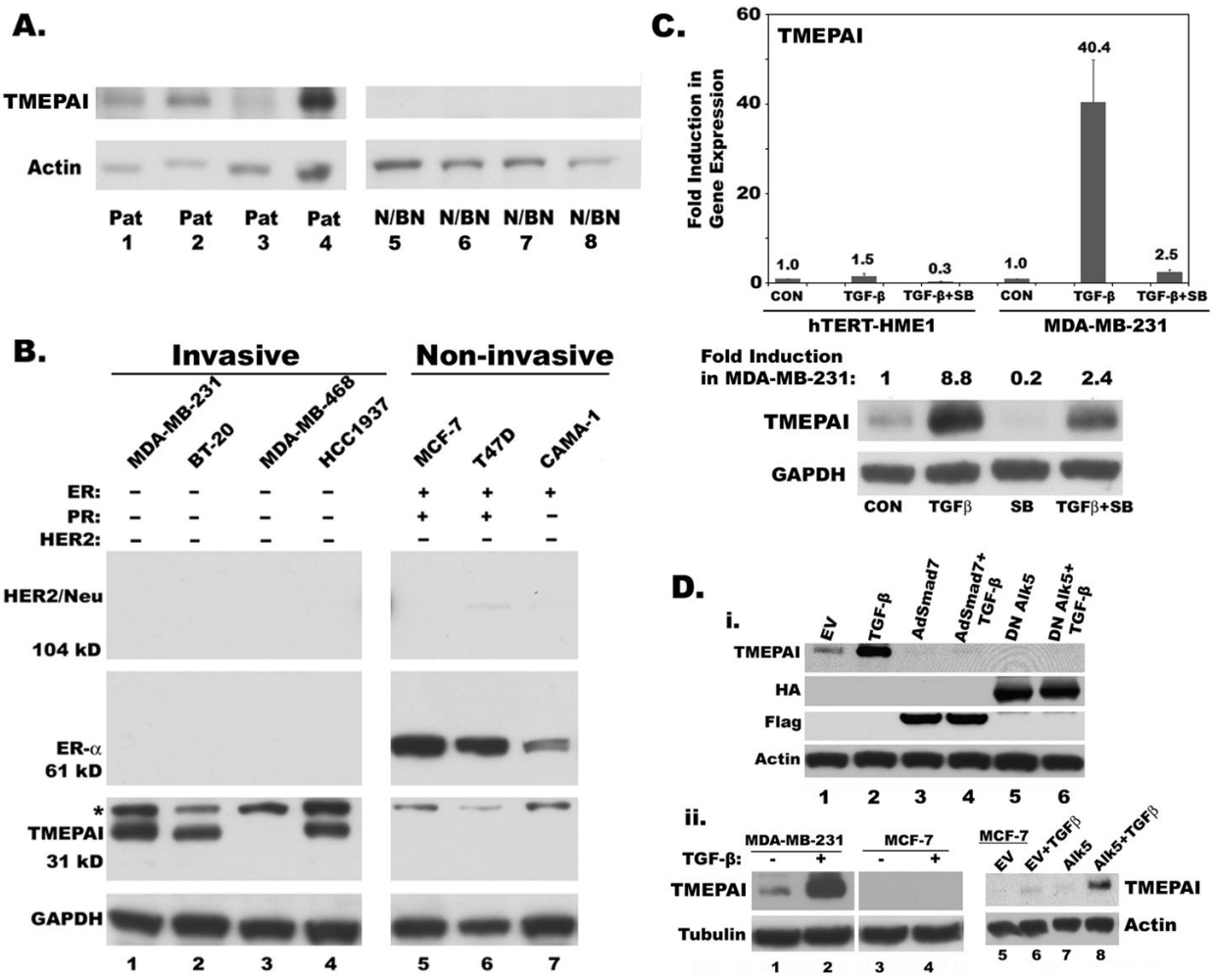


**Figure 1. TMEPAI expression in invasive breast ductal cancers and its regulation by TGF-β signaling**

A. Western blot of TMEPAI in primary human patient (Pat1-4) breast tumor samples and corresponding normal/benign (N/BN1-4) samples; B. Western blot analysis for TMEPAI in invasive and non-invasive breast cancer cell lines. Asterisk (*) indicates non-specific band interacting with TMEPAI antibody. C. Effect of TGF-β and inhibition by SB431542 (SB) on TMEPAI mRNA by Q-PCR in hTERT-HME1 breast epithelial cells and MDA-MB-231 breast cancer cells and TMEPAI protein levels in MDA-MB-231 cells. D. Inhibition of Basal and TGF-β induced TMEPAI protein expression by Smad7 and DN ALk5 (i). Exogenous expression of Alk5 in MCF-7 cells restores induction of TMEPAI by TGF-β in MCF-7 cells (ii).

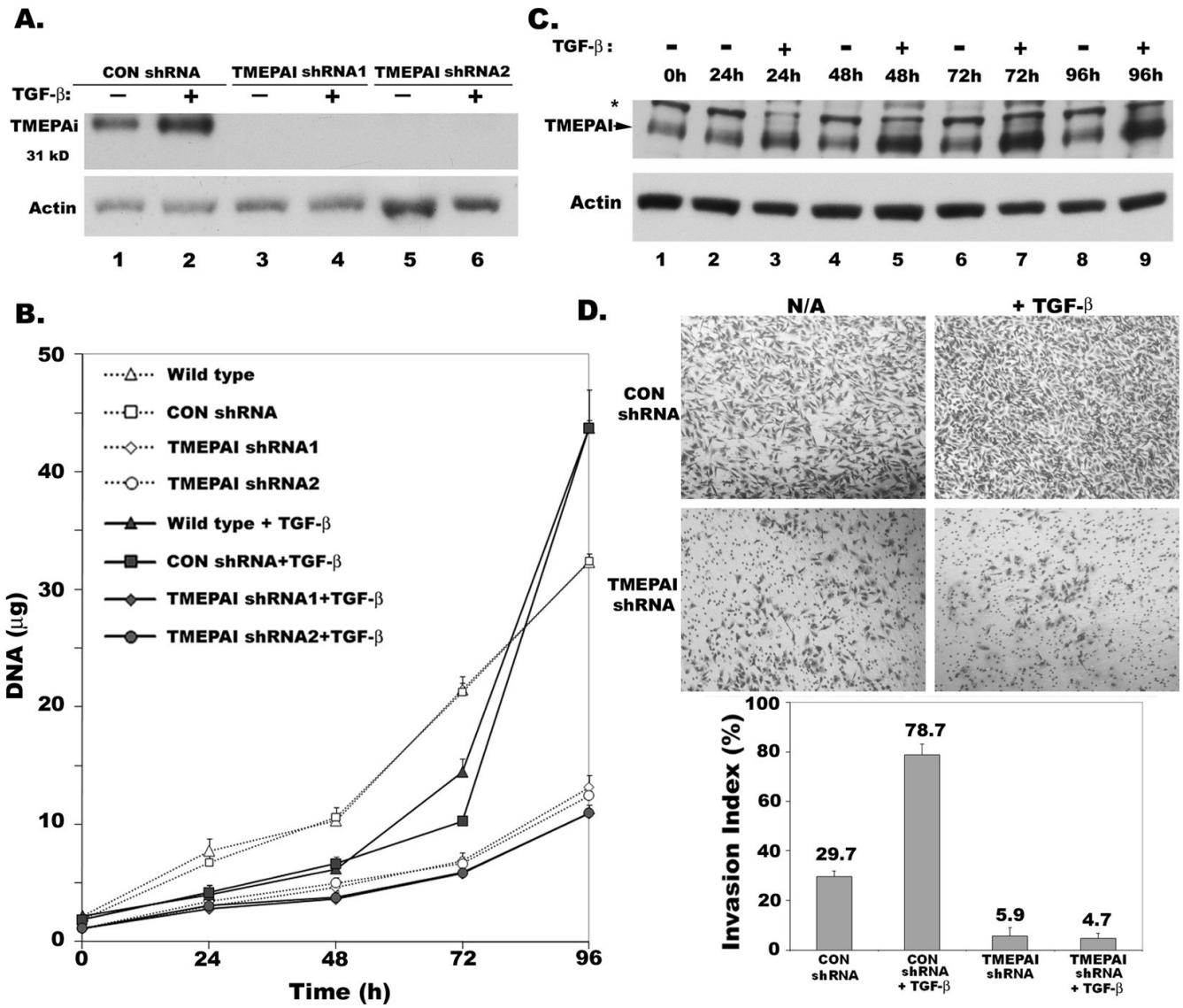


**Figure 2. TMEPAI knockdown blocks TGF-β stimulated growth, migration and invasion**

A. Lack of TMEPAI protein expression in MDA-MB-231 cells stably expressing 2 different TMEPAI shRNAs. B. Growth curves of wild type MDA-MB-231 cells and cells expressing control shRNA and TMEPAI shRNA 1 and 2 with or without TGF-β. Cell proliferation was measured by quantitating total DNA. C. Time course of TMEPAI expression in MDA-MB-231 cells with exposure to TGF-β. Asterisk (*) indicates non-specific antibody reactive band. D. Effect of TGF-β and TMEPAI shRNA on relative invasion of MDA-MB-231 breast cancer cells in Transwell Matrigel invasion assay. Invasion index measured as relative migration of cells across the Matrigelin all groups was calculated.

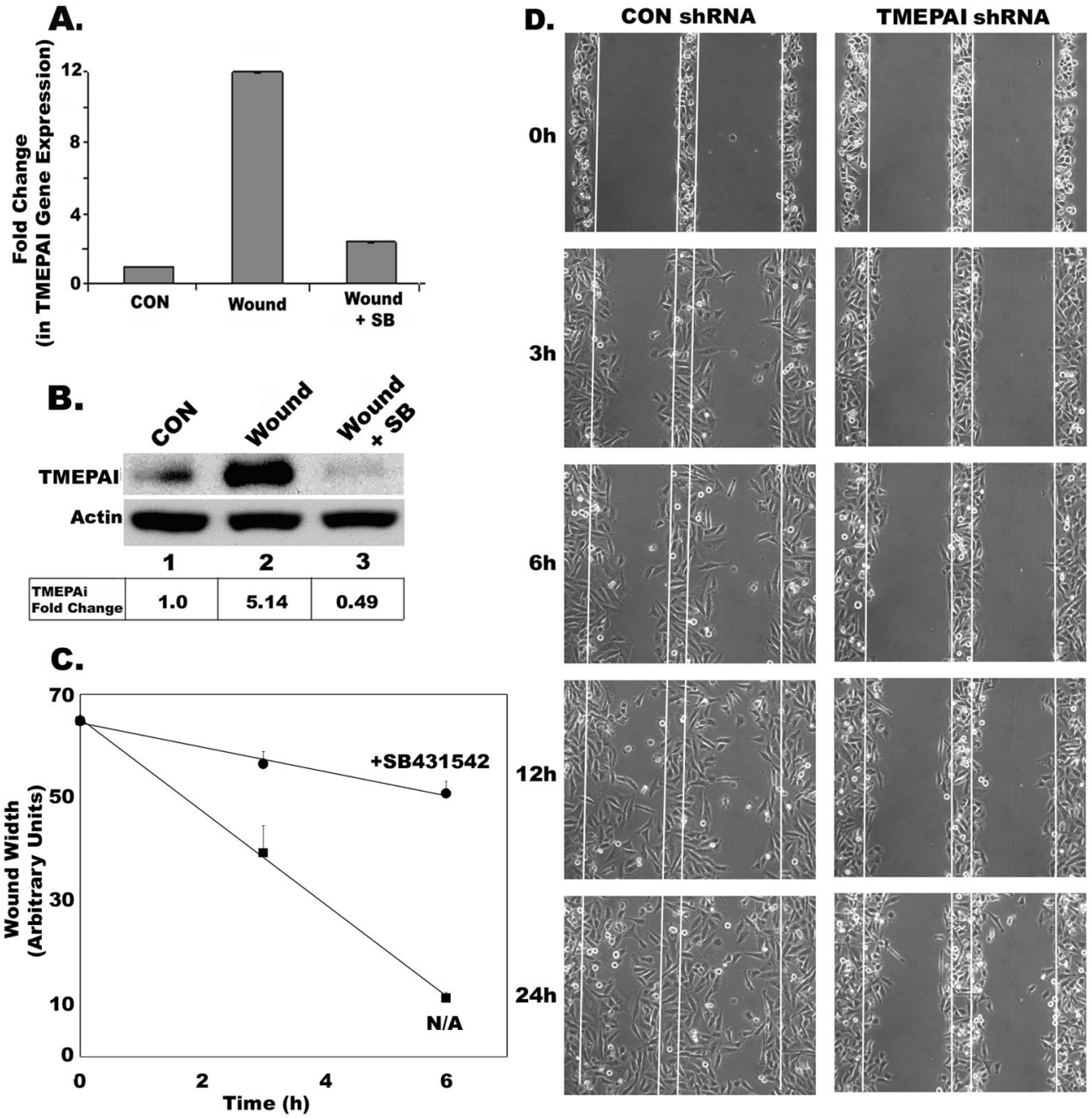


**Figure 3. Wound induced TMEPAI expression and cell migration**

Wound induced TMEPAI mRNA (A) and protein expression (B) in MDA-MB-231 cells is blocked by SB431542 (SB). (C) Wound induced migration of MDA-MB-231 cells, which is inhibited by SB431542, was measured as wound width and plotted against time in hours (h). (D) Inhibition of wound stimulated migration of MDA-MB-231 by TMEPAI shRNA.

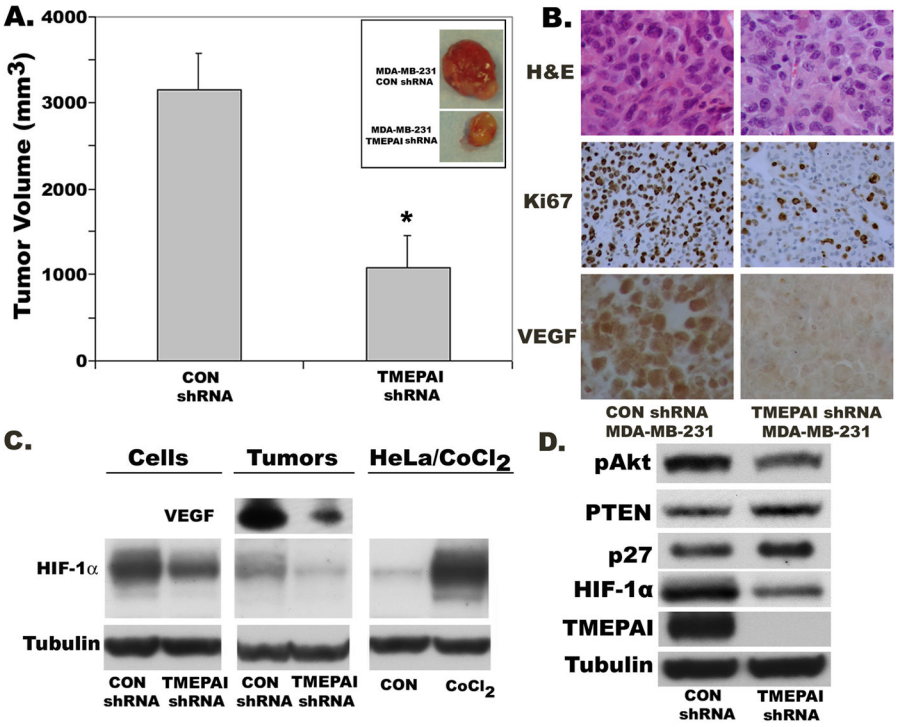


**Figure 4. Inhibition of TMEPAI expression decreases human breast tumor growth in nude mice**

A. Reduced breast tumorigenic potential *in vivo* of TMEPAI knockdown cells as measured by tumor volume (*P< 0.05). Inset shows representative tumors. B. Reduced expression of VEGF and Ki67 in tumors formed by TMEPAI knockdown cells compared to cells expressing control shRNA. C. Relative expression of HIF-1α in control and TMEPAI knockdown cells and xenograft breast tumors. HeLa cells treated with cobalt chloride were used as positive control for HIF-1α expression. D. Expression of pAkt, PTEN, $p27^{kip1}$ and HIF-1α and TMEPAI in cells expressing control shRNA and TMEPAI shRNA.