Cellular Signalling 59 (2019) 24–33

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

TMEPAI/PMEPA1 inhibits Wnt signaling by regulating β-catenin stability and nuclear accumulation in triple negative breast cancer cells

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ARTICLE INFO

Keywords:
TMEPAI
Wnt signaling
β-catenin

ABSTRACT

Transmembrane prostate androgen-induced protein (TMEPAI) is a type I transmembrane protein induced by several intracellular signaling pathways such as androgen, TGF-β, EGF, and Wnt signaling. It has been reported that TMEPAI functions to suppress TGF-β and androgen signaling but here, we report a novel function of TMEPAI in Wnt signaling suppression. First, we show that TMEPAI significantly inhibits TCF/LEF transcriptional activity stimulated by Wnt3A, LiCl, and β-catenin. Mechanistically, TMEPAI overexpression prevented β-catenin accumulation in the nucleus and TMEPAI knockout in triple negative breast cancer cell lines promoted β-catenin stability and nuclear accumulation together with increased mRNA levels of Wnt target genes *AXIN2* and *c-MYC*. The presence of TGF-β type I receptor kinase inhibitor did not affect the enhanced mRNA expression of *AXIN2* in TMEPAI knockout cells. These data suggest that TMEPAI suppresses Wnt signaling by interfering with β-catenin stability and nuclear translocation in a TGF-β signaling-independent manner.

1. Introduction

Transmembrane prostate androgen induced protein (TMEPAI), also known as prostate transmembrane protein, androgen induced 1 (PMEPA1), or solid tumor associated gene 1 (STAG1), was originally identified as a prostatic protein induced by testosterone or its derivatives [1,2]. It is a type 1b transmembrane protein consisting of a short extracellular domain, a single-pass transmembrane domain (TM), and intracellular domain, including two PPxY (PY) motifs and a Smad interaction motif (SIM) [3,4].

During tumor progression, cancer cells undergo a number of alterations in cellular signaling pathways which control cell proliferation, survival, motility, and metabolism. Many proteins in these signaling pathways are currently under investigation as cancer therapeutic targets [5,6]. TMEPAI is constitutively and highly expressed in many types of cancers including triple negative breast cancer (TNBC), an aggressive subtype of breast cancer compared with other types of breast cancers, and associated with poor prognoses in TNBC [7,8]. In consistent with these findings, we previously demonstrated that knockdown of TMEPAI in human lung cancer cells reduces tumorigenic activities (such as xenograft tumor formation and sphere formation) [9], TMEPAI is thought to be an oncogenic protein even though its role in tumorigenesis is still not fully understood.

TMEPAI itself is induced by TGF-β [4,7,8,10] and ERK signaling [2,11]. In addition, our previous study showed that TMEPAI is a downstream target of Wnt signaling in which the Wnt/β-catenin/TCF7L2 pathway preferentially activates *TMEPAI/PMEPA1* gene transcription together with TGF-β signaling [12]. Since the Wnt signaling pathway controls cell proliferation and stem cell maintenance [13], we investigated the role of TMEPAI in Wnt signaling with respect to mechanistic interaction with Wnt downstream targets. Here, we demonstrate that TMEPAI inhibits Wnt signaling and subsequent *AXIN2* and *c-MYC* transcription by regulating β-catenin protein stability and nuclear accumulation.

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https://doi.org/10.1016/j.cellsig.2019.03.016
Received 11 December 2018; Received in revised form 15 March 2019; Accepted 15 March 2019
Available online 16 March 2019

2. Materials and methods

2.1. Cell culture

MDA-MB-231, Hs578T, human embryonic kidney (HEK) 293, and HEK-293 T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma) containing 10% fetal bovine serum (FBS) (Life Technologies) and penicillin-streptomycin solution (Wako). Hs578T cells and Hs578T cells stably expressing TMEPAI isoform A were cultured in the above medium supplemented with 10 μg/ml insulin and 8 μg/ml blasticidin hydrochloride. BT-549 cells were cultured in RPMI-1640 medium (Sigma) supplemented with 10% FBS, 10 μg/ml insulin and penicillin-streptomycin solution. Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2. Plasmid transfections were performed with FuGENE 6 (Promega) or polyethylenimine (PEI) "MAX" (Polysciences) and siRNA transfection was performed with Lipofectamine3000 (Life Technologies), all according to manufacturer protocols. Wnt3A conditioned medium and control medium were prepared from L Wnt-3A cells (ATCC; CRL-2647) and L cells (ATCC; CRL-2648), respectively, according to the manufacturer's protocol (ATCC).

2.2. Plasmids

Human TMEPAI isoform A, B, D, and mutants were cloned and constructed in the pcDNA3.1/V5-HisA vector (thermo Fisher Scientific). A CSII-CMV-MCS-IRES2-Bsd vector was used to generate TMEPAI lentiviral expression vectors. All TMEPAI isoform A mutants were made with a QuickChange site-directed mutagenesis kit (Stratagene). In brief, TMEPAI isoform A PY1 mutant (Y161A), PY2 mutant (Y232A), double PY mutant (Y161A, Y232A) and SIM mutant (^{186}PPNR to AAAA) were constructed in the pcDNA3.1/V5-His-A vector. Our mouse β-catenin expression plasmid was constructed in the pCAG-GS plasmid [14]. The TOP-flash luciferase reporter and $(CAGA)_{12}$-luciferase reporter plasmids were described previously [4,15].

2.3. Knockout and knockdown of TMEPAI

TMEPAI knockout (KO) cell lines from MDA-MB-231, Hs578T, and BT-549 cells were established using CRISPR/Cas9 system. TMEPAI KO clones (MDA-MB-231 clone #2 and #4, Hs578T clone #5 and #18, here after referred to as KO plus clone number) were established using a guideRNA designed for targeting TMEPAI exon-2 (5′-CAGCCGGCACA GCCAGGGG-3′) and BT-549 TMEPAI knockout clone #22 (KO#22) was established using a pair of guideRNAs designed for targeting intron-3 (5′-CCAGAAGCGATCCTGAGAC-3′) and exon-4 3′-UTR (5′-AGAGAAAC TGTATGTGCGA-3′) to subtract whole exon-4 cording region. TMEPAI knockdown (KD) was performed using TMEPAI siRNA (ON-TARGET plus SMART pool human PMEPA1 #L-010501-00, Dharmacon) and non-targeting siRNA (ON-TARGET plus control pool #D-001810-10-20) was used as a control.

2.4. Lentiviral vectors

Lentiviral TMEPAI expression vector CSII-CMV-MCS-IRES2-Bsd-TMEPAI was transfected together with pMDLg/pRRE, pRSV-Rev, and pMD2.G into HEK-293T cells using FuGENE 6 according to the manufacturer's protocol. At 36, 48, 60, and 72 h after transfection, lentivirus solution was collected from the culture medium. Hs578T TMEPAI knockout #5 cells were then cultured with filtered viral solution for 24 h in the presence of 1 μg/ml polybrene (Sigma) and selected with 8 μg/ml blasticidin (Wako).

2.5. Luciferase assay

Cells were transfected with the TOP-flash *firefly* luciferase reporter (T-cell factor (TCF)/Lymphoid-enhancer binding factor (LEF) Reporter Plasmid), pRL-CMV *Renilla* luciferase control reporter, β-catenin expression plasmid, and TMEPAI expression plasmid using PEI "MAX". After transfection, cells were cultured for 24 h. Where indicated, the cells were stimulated with Wnt3A conditioned medium or 10 mM lithium chloride (LiCl) in the absence of FBS for 18 h. The cell lysates were mixed with Luciferase Assay Reagent (Promega) and luciferase activities were measured by a Berthold Micro Lumat*Plus* luminometer. The data was normalized to corresponding *Renilla* luciferase activity. Each transfection was carried out in triplicate.

2.6. Cytoplasmic and nuclear fractionation of experimental cells

Cells at 80–90% confluency were incubated with TGF-β for 7 h in total and Wnt3A conditioned medium was added for the last 3 h. Cytoplasmic fractionation of the cell lysate was obtained using hypotonic lysis buffer (20% glycerol, 20 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid [HEPES], 10 mM NaCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM PMSF, 20 mM β-glycerophosphate, 40 mM NaF, 100 units/ml aprotinin, 1 mM dithiothreitol) and the resultant nuclear fraction was lysed using TNE lysis buffer (10 mM Tris [pH 7.4], 150 mM NaCl, 10 mM EDTA, 1% NP40, 1 mM PMSF, 20 mM β-glycerophosphate, 40 mM NaF, 100 units/ml aprotinin, 5 μg/ml leupeptin).

2.7. SDS-PAGE and Western blotting

Cells were washed with PBS and suspended in Tris-HCl 62.5 mM (pH 8.0) or TNE lysis buffer, then mixed with an equal volume of 2× sample buffer (4% SDS, 1.44 M β-mercaptoethanol, 20% glycerol, 125 mM Tris [pH 7.4], 0.01% bromophenol blue). The total cell lysates were then subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), then electro-transferred to PVDF membrane (GE Healthcare) as previously described [4]. The membrane was incubated with antibodies. Bound antibodies were detected by chemiluminescence reaction using Immuno Star Zeta (Wako) and EZ capture MG (ATTO) according to the manufacturers' protocols. Antibodies used were: rabbit anti-TMEPAI [4], mouse anti-V5 (Thermo Fisher Scientific), mouse anti-FlagM5 (Sigma-Aldrich), mouse anti-α-tubulin (Wako), mouse anti-β-actin (Sigma), mouse anti-β-catenin (BD Transduction Laboratory), and rabbit anti-HDAC3 (Santa Cruz). Tubulin was used for a cytoplasm-specific loading control, HDAC3 was used for a nucleus-specific loading control, and β-actin was used as a general loading control, where appropriate.

2.8. RNA extraction and reverse transcription-PCR (RT-PCR)

Total RNA was isolated with ISOGEN II (Nippon Gene) according to the manufacturer's protocol. Reverse transcription reactions were performed using High Capacity RNA-to-cDNA Master Mix (Thermo Fisher Scientific) according to the manufacturer's protocol.

2.9. Real-time quantitative PCR

Real-time quantitative PCR (qRT-PCR) was performed with Gene Ace SYBR qPCR mix α Low ROX (Nippon Gene) by a 7500 Fast thermal cycler (Life Technologies, Applied Biosystems) using standard curve method according to the manufacturer's protocol. The primers used were: human *AXIN2* (forward 5′-TCCCAGACTCAGTGGGAAGA-3′, reverse 5′-GCCTGGTGTTGGAAGAGACA-3′), human *c-MYC* (forward 5′-CCTCGGATTCTCTGCTCTCC-3′, reverse 5′-TTTCTTCCTCATCTTCTT GTTCCTC-3′), and human *β-ACTIN* (forward 5′-CGTACAGGTCTTTGC GGATG-3′, reverse 5'-GCACTCTTCCAGCCTTCCTT-3′).

2.10. Co-immunoprecipitation assay

HEK-293T cells were transfected with Flag tagged β-catenin and V5

tagged TMEPAI isoform A wild type and its mutants, double PY motifs mutant and SIM mutant, expression plasmids and then incubated for 40 h. MG132 (10 mM) was added into media 6 h before harvest. Cells were lysed with TNE lysis buffer and cell lysates were incubated with anti-V5-antibody (Thermo Fisher Scientific) and protein G sepharose (GE healthcare). Precipitants were subjected to SDS-PAGE and western blotting.

2.11. Cell proliferation assay

MDA-MB-231 parental and TMEPAI KO cell lines (#2 and #4) were seeded on 12-well plates at a total of 5×10^4 cells per well and cultured for 2, 4, and 6 days. Cells were collected by trypsinization at the indicated time points and diluted in 0.2% trypan blue solution (Wako) before counting cell number using Neubauer cell counter chamber.

2.12. Analysis of RNA sequencing data in TCGA invasive breast cancer cases (BRCA)

We processed RNAseq data from 1093 TCGA invasive breast cancer cases (BRCA) to rank 1212 breast cancer samples in a descending order by RSEM-normalized RNA-seq values of *PMEPA1/TMEPAI*. We used the gene expression data of the top 220 samples (TMEPAI.High) and the bottom 220 samples (TMEPAI.Low) for gene set enrichment analysis (GSEA) to examine the enriched biological processes in PMEPA1.High samples using 4436 gene sets derived from the GO Biological Process Ontology (C5: BP: GO biological process) [16,17]. In GSEA, the enrichment statistic and metric for ranking genes were set to *Weighted* and to *Signal2Noise*, respectively. The statistical significance of the enrichment score was assessed against 1000 gene_set permutations using the analysis phenotype TMEPAI.High vs TMEPAI.Low. Gene sets with FDR q-value < 0.05 were considered of statistical significance.

2.13. Statistical analysis

The differences in luciferase reporter activities and relative mRNA expressions were evaluated using a standard two-tailed student *t*-test. Probability values of < 0.05 were considered significant.

3. Results

3.1. TMEPAI inhibits Wnt signal-induced TOP-flash-luciferase reporter activity

Human *TMEPAI* transcription results in four isoforms (A, B, C and D) (Sup. Fig. 1) and is coordinated by both TGF-β and Wnt signaling [4,12]. First, we confirmed our previous finding of the inhibitory role of TMEPAI in TGF-β/Smad signaling [4]. As expected, overexpression of TMEPAI isoforms A, B, and D in HEK-293 cells inhibited the $(CAGA)_{12}$-luciferase reporter, which is specifically activated by the TGF-β-induced Smad3 and Smad4 complex (Sup. Fig. 2). We then tested the role of TMEPAI in Wnt signaling by overexpressing TMEPAI isoforms A, B and D in HEK-293 cells. Overexpression of these isoforms remarkably inhibited the TOP-flash-luciferase reporter, which functions as an indicator of Wnt-induced β-catenin/TCF/LEF transcriptional activity, upon treatment with Wnt3A conditioned medium (Fig. 1A) suggesting an inhibitory role of TMEPAI in both Wnt and TGF-β signaling.

To assess the endogenous role of TMEPAI, we established TMEPAI knockout cells using a CRISPR/Cas9 system in triple negative breast cancer (TNBC) cell lines MDA-MB-231, Hs578T and BT-549. The TMEPAI knockout cells of MDA-MB-231 and BT-549 cells showed vanished expression of TMEPAI even after stimulation of TGF-β. Although the marginal expressions of TMEPAI were observed in monoallelic Hs578T KO clones after TGF-β stimulation, TMEPAI protein level was decreased to a 10% or less and these cell lines could be used to elucidate TMEPAI's function in a cell (Sup. Fig. 3). Since these cell lines highly express TMEPAI protein in the presence of TGF-β ligand, we performed TCF/LEF reporter assays under TGF-β stimulation. Compared with the corresponding parental cells, deletion of TMEPAI enhanced TOP-flash-luciferase reporter activities induced by treatment with Wnt3A conditioned medium in MDA-MB-231 (Fig. 1B). Furthermore, CRISPR/Cas9-induced TMEPAI knockout enhanced TOP-flash-luciferase reporter activities in Hs578T (Fig. 1C) and BT-549 (Fig. 1D) cells upon Wnt3A conditional medium treatment. These results further support the suppressive function of TMEPAI in Wnt signaling.

3.2. TMEPAI interferes with Wnt signaling at downstream of β-catenin stabilization

β-catenin is a key intracellular mediator of Wnt signaling. In the absence of Wnt ligand, β-catenin is phosphorylated by Casein Kinase 1 (CK1) and glycogen synthase kinase-3β (GSK3β) in a destruction complex that is further ubiquitinated by β-transducin repeat-containing protein (βTrCP) and subsequently degraded by proteasomes [13,18,19]. Upon Wnt ligand activation of Frizzled receptor and co-receptor, lipoprotein receptor-related protein (LRP) interacts with the destruction complex and interferes with its functions [18,19] resulting β-catenin stabilization and translocation into the nucleus to regulate transcription of Wnt target genes together with TCF/LEF transcriptional factor [19].

To investigate the molecular target(s) of TMEPAI in this cascade, we induced Wnt signaling by treatment of LiCl, a GSK3β inhibitor and direct overexpression of β-catenin as ligand-independent Wnt signal activators. Overexpression of TMEPAI inhibited TCF/LEF transcriptional activity induced by LiCl and β-catenin (Fig. 2A and B). Additionally, the knockout of endogenous TMEPAI in MDA-MB-231 cells also enhanced overexpressed β-catenin-induced TOP-flash reporter activity (Fig. 2C). These data suggest that TMEPAI suppresses Wnt signaling downstream of β-catenin accumulation.

3.3. Knockout of TMEPAI enhances β-catenin stability

To reconfirm how TMEPAI inhibits Wnt/β-catenin signaling, we examined β-catenin protein levels in the TMEPAI knockout and knockdown TNBC cell lines MDA-MB-231 and Hs578T. Because basal TMEPAI expression in both breast cancer parental cell lines is relatively low, we initially pre-treated all cells with TGF-β for 4 h to induce TMEPAI expression then co-stimulated with TGF-β and Wnt3A conditioned medium for 3 h as indicated in Fig. 3A. Total β-catenin levels were increased in both TMEPAI knockout (Fig. 3B and C) and knockdown (Fig. 3D and E) cells compared with the corresponding parental/control cells in both MDA-MB-231 and Hs578T lines. Although Hs578T TMEPAI knockout cells tend to show less increasement of β-catenin expression, it might be a result of monoallelic incomplete knockout in *TMEPAI* gene. This effect could be reversed by lentiviral TMEPAI overexpression in Hs578T TMEPAI knockout KO#5 cells (Fig. 3F). These data therefore indicate that TMEPAI suppresses Wnt signaling through regulation of β-catenin protein stability.

3.4. TMEPAI inhibits β-catenin nuclear accumulation

To further understand the effect of TMEPAI on β-catenin stabilization, we assessed β-catenin protein levels and localization in the cytoplasm and nuclei of TMEPAI knockout cells and their parental counterparts. Both nuclear and cytoplasmic β-catenin was increased in TMEPAI knockout MDA-MB-231 and Hs578T cells compared with their parental cells (Fig. 4A and B). In addition, overexpression of TMEPAI suppressed nuclear β-catenin levels in the presence of Wnt3A conditioned medium in HEK293 cells (Sup. Fig. 4). These data indicate that TMEPAI inhibits β-catenin stabilization and nuclear accumulation.

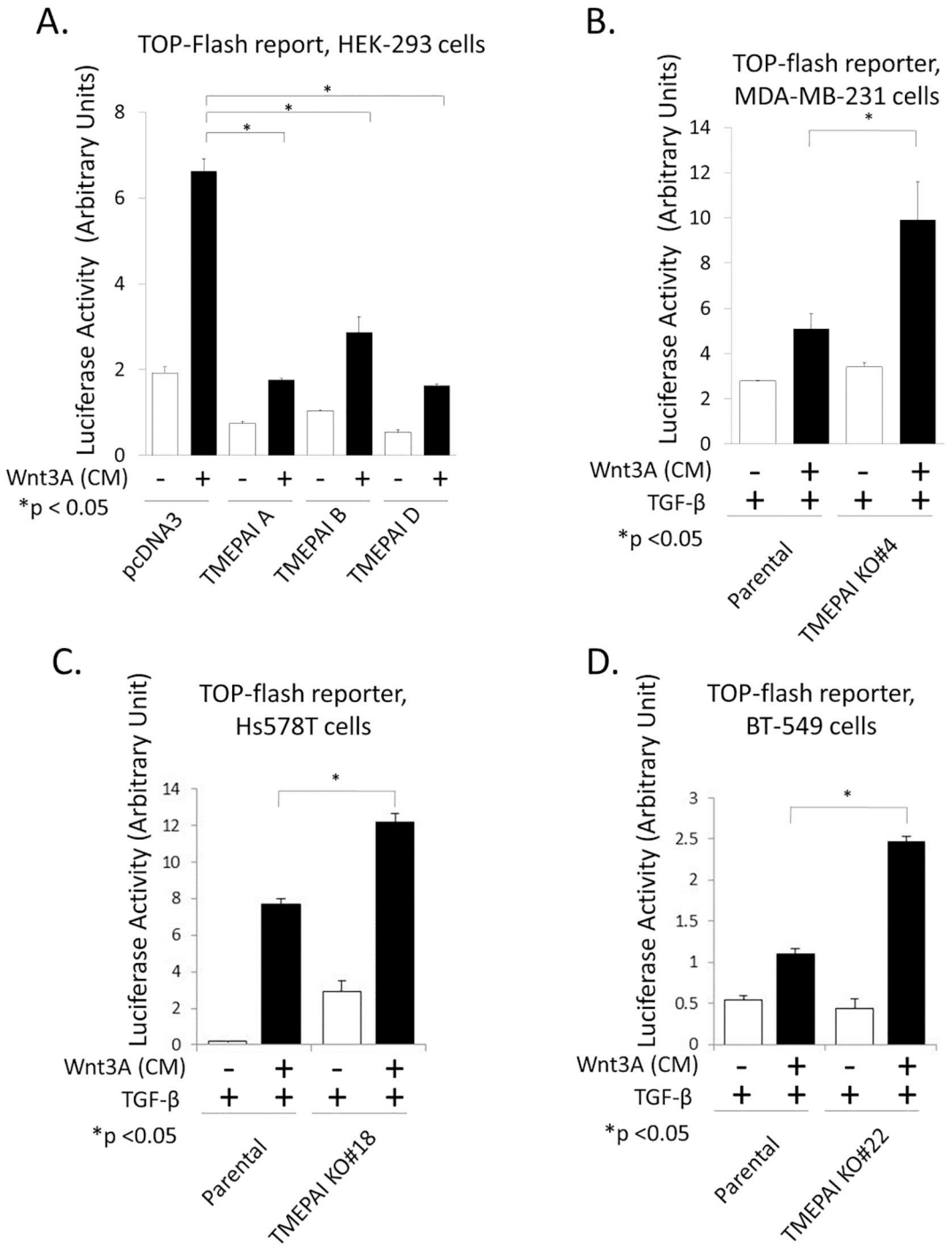

Fig. 1. TMEPAI inhibits TOP-flash reporter activity induced by Wnt3A. (A) TMEPAI isoform A, B, and D expression plasmids were transfected into HEK-293 cells together with TOP-flash-*firefly* luciferase reporter and pRL-CMV *renilla* luciferase control reporter vectors. Cells were stimulated with Wnt3A conditioned medium or control medium as indicated. (B-D) Parental and TMEPAI knockout of MDA-MB-231 (B), Hs578T (C) and BT-549 (D) cells were transfected with TOP-flash-*firefly* luciferase reporter and pRL-CMV *renilla* luciferase control reporter vectors. Cells were stimulated with TGF-β ligand (1 ng/ml) and Wnt3A conditioned medium treatment for 18 h. The results are presented as means ± SDs. Data were analyzed using student *t*-test (*$p < .05$ compared with control).

3.5. Both TMEPAI PY motifs are essential for Wnt signaling inhibition

Previous reports suggested that TMEPAI works as a regulator of intracellular signaling pathway through its PY and SIM domain [4,7,8]. We tested whether TMEPAI PY motifs (PY1 and PY2) and a SIM contribute to Wnt/β-catenin signaling suppression using the TOP-flash-luciferase reporter assay (Fig. 5A). Comparable suppression of TCF/LEF luciferase reporter activity was observed in mutant TMEPAI PY1 and SIM-overexpressing cells compared to TMEPAI wild-type cells. In contrast, the TMEPAI PY2 mutant had a modest loss in its suppressive effect on TOP-flash activity and the double PY motifs mutant (PY1 plus PY2) could not inhibit Wnt signaling at all (Fig. 5B). Next, we examined the interaction between TMEPAI and β-catenin by co-immunoprecipitation assay. The result showed that TMEPAI WT and SIM mutant bound to β-catenin. However, double PY motifs mutant diminished the interaction with β-catenin (Fig. 5C). These data indicate that the PY motifs of TMEPAI are essential for the interaction with β-catenin and cooperation of both PY motifs are necessary for inhibition

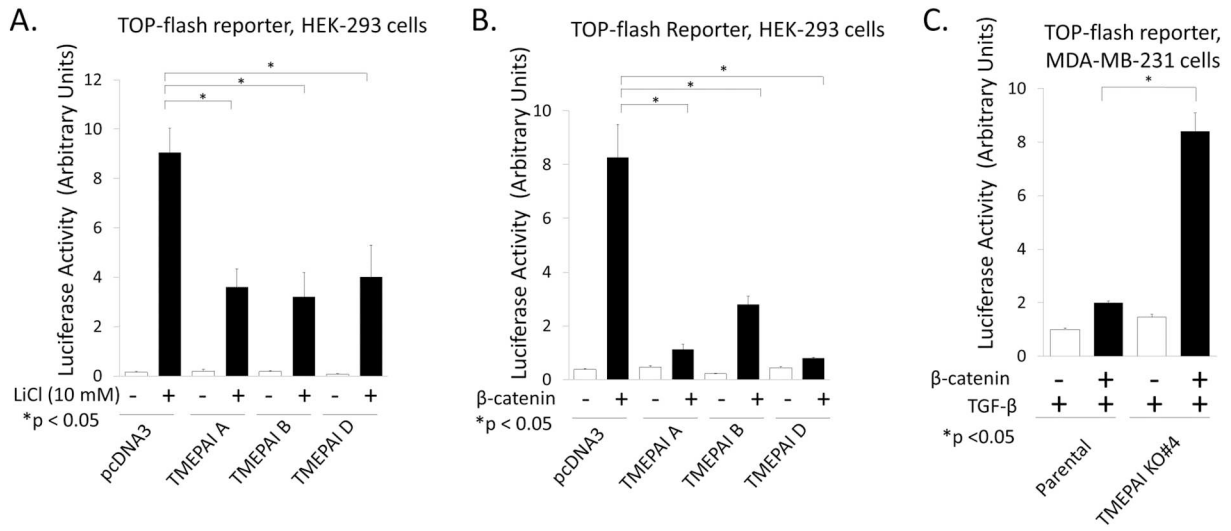

Fig. 2. TMEPAI interferes Wnt signaling at downstream of β-catenin stabilization. (A, B) TMEPAI isoform A, B, and D expression plasmids were transfected into HEK-293 cells together with TOP-flash-*firefly* luciferase reporter and pRL-CMV *renilla* luciferase control reporter vectors. Cells were stimulated with (A) 10 mM LiCl for 18 h, or (B) co-transfection of β-catenin expression plasmid as indicated. (C) MDA-MB-231 parental and TMEPAI knockout cells were transfected with TOP-flash-*firefly* luciferase reporter, pRL-CMV *renilla* luciferase control reporter, and β-catenin expression plasmid vectors. Cells were stimulated with TGF-β ligand (1 ng/ml) for 18 h then subjected to luciferase assay. The results are presented as means ± SDs. Data were analyzed using student *t*-test (*p < .05 compared with control).

of Wnt signaling.

3.6. TMEPAI affects Wnt signaling target genes AXIN2 and c-MYC

As activation of Wnt signaling results in transcriptional regulation of Wnt target genes [20–22], we next examined the effect of TMEPAI on the transcription of these targets. TMEPAI knockout MDA-MB-231 cells (KO#2 and KO#4) showed higher expression of Wnt target genes *AXIN2* and *c-MYC* than parental cells after treatment with Wnt3A conditioned medium (Fig. 6A and B). We also evaluated the AXIN2 mRNA level in Hs578T parental and knockout cells. The Wnt3A-induced expression of AXIN2 was moderately increased by TGF-β treatment and TMEPAI knockout further enhanced Wnt3A-induced AXIN2 expression compared with parental cells regardless of TGF-β stimulation (Fig. 6C). Although the expression of TMEPAI in the absence of exogenous TGF-β was marginal, endogenous TMEPAI might work in

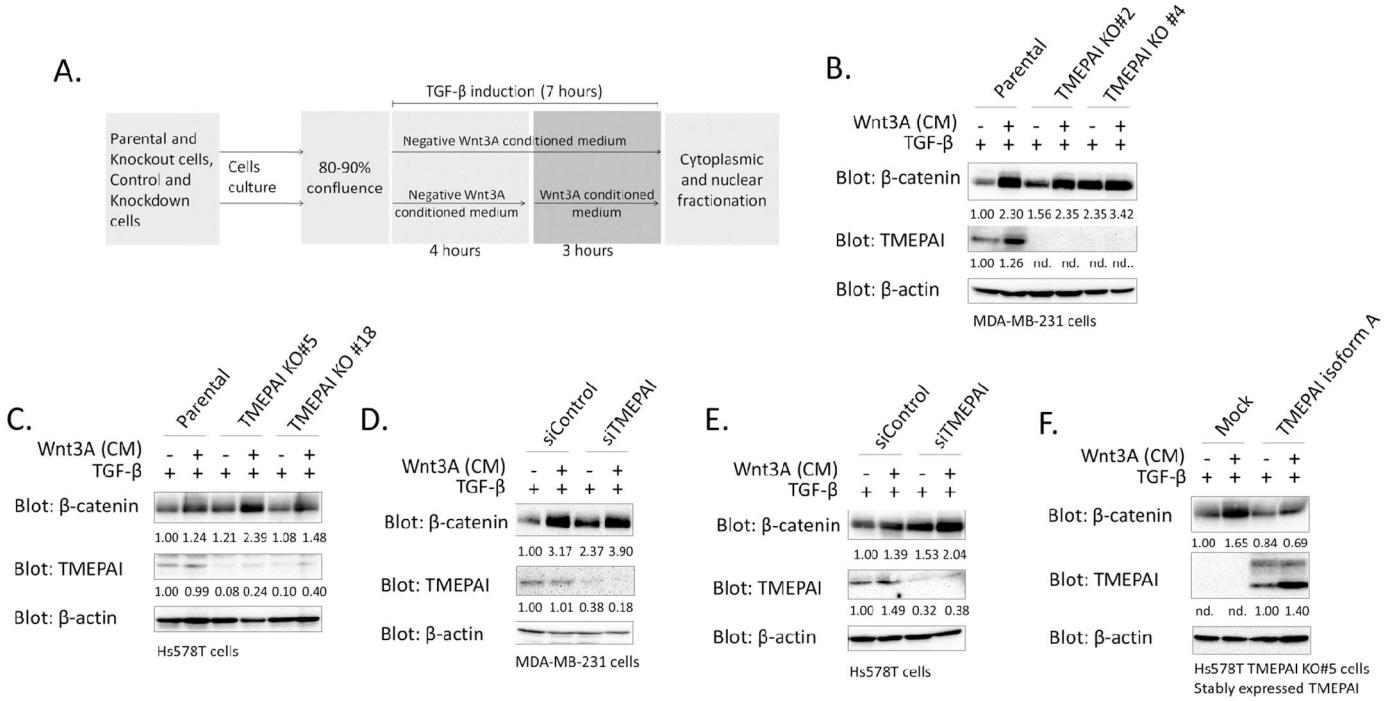

Fig. 3. TMEPAI depletion increased β-catenin stability. (A) MDA-MB-231 and Hs578T cells were stimulated with control or Wnt3A conditioned medium for last 3 h in the presence of TGF-β ligand (1 ng/ml) for 7 h. (B, C) Total β-catenin and TMEPAI level in MDA-MB-231, Hs578T and its TMEPAI knockout cells were detected by western blotting. Quantity of proteins were measured by densitometry analysis and normalized with corresponding β-actin band intensity. (D, E) Cells were transfected with TMEPAI siRNA or control siRNA. 48 h after transfection, cells were stimulated with TGF-β and Wnt3A as indicated in Fig. 3A. (F) Total β-catenin and TMEPAI level in Hs578T TMEPAI KO#5 cells and TMEPAI isoform A stably expressing cells were detected by western blotting. Protein intensity was measured and normalized with corresponding β-actin.

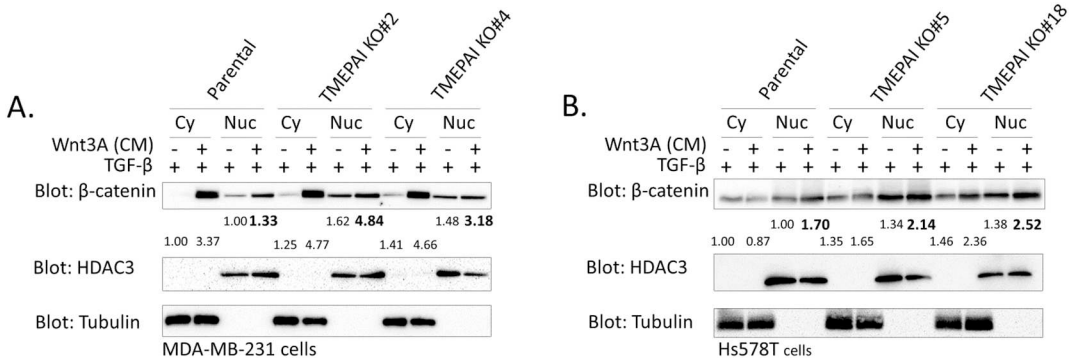

Fig. 4. TMEPAI knockout increases β-catenin stability and nuclear translocation. Cells were treated with control or Wnt3A conditioned medium for the last 3 h of a 7-h TGF-β incubation (1 ng/ml). The cytoplasmic and nuclear fractions of the MDA-MB-231 (A) and Hs578T (B) cells were subjected to western blotting. β-catenin proteins in each fraction were measured and normalized with HDAC3 (nuclear loading control) and tubulin (cytoplasmic loading control).

inhibition of Wnt signaling. These data further support the inhibitory role of TMEPAI in Wnt signaling.

Previous reports have suggested that TGF-β/Smad signaling activates Wnt/β-catenin signaling [23–27] and, since TMEPAI is a negative regulator of TGF-β/Smad signaling [4], we considered the possibility that TMEPAI inhibition of Wnt signaling is dependent on TGF-β signaling. Blocking TGF-β signaling in TMEPAI knockout MDA-MB-231 cells using SB431542, a TGF-β type I kinase inhibitor, did not change the enhanced expression of *AXIN2* mRNA induced by Wnt3A conditioned medium (Fig. 6D). Moreover, the TMEPAI SIM mutant, which does not inhibit TGF-β signaling, could suppress Wnt signaling comparably to TMEPAI wild type (Fig. 5B). Collectively, these data suggest that TMEPAI inhibits Wnt signaling independent of TGF-β signaling.

3.7. TMEPAI affects Wnt signaling-mediated biological processes

Wnt signaling has been implicated in diverse biological processes, such as cell fate determination, cell proliferation, differentiation, apoptosis, and stem cell maintenance during embryogenesis and adult tissue homeostasis. Here, we focused on adherent cell proliferation enhanced by Wnt3A treatment and performed cell proliferation assay. Compared with parental

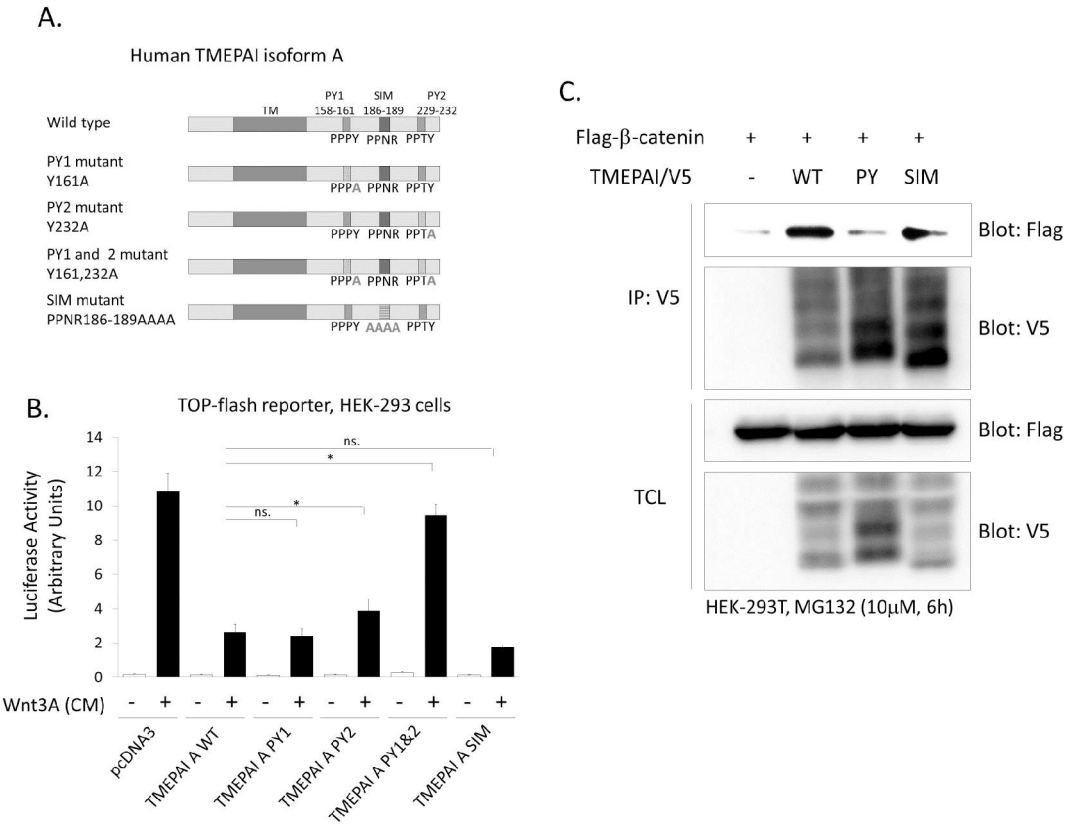

Fig. 5. TMEPAI PY motifs mutant diminishes inhibitory function of TMEPAI in Wnt signaling and are binding site for β-catenin. (A) The schematic figure of TMEPAI isoform A mutants. PY (PPxY) motif was replaced with PPxA and SIM (PPNR) was replaced with AAAA. (B) TMEPAI wild type and mutant expression plasmids were transfected together with TOP-flash-*firefly* luciferase reporter and pRL-CMV *renilla* luciferase control reporter vectors. Thirty hours after transfection, Cells were stimulated with control or Wnt3A conditioned medium for 18 h. The results are presented as means ± SDs. Data was analyzed using student *t*-test (*p < .05 compared with control). (C) HEK-293 cells were transfected with Flag-β-catenin, TMEPAI-V5 and its mutants expression plasmid vectors as indicated then incubated for 40 h. MG132 (10 mM) was added into media 6 h before harvest. Cell lysates were subjected to co-immunoprecipitation assay with anti-V5 antibody and each protein was detected by western blotting with anti-Flag and anti-V5 antibodies. PY: PY1 and 2 double PY mutant. SIM: SIM mutant.

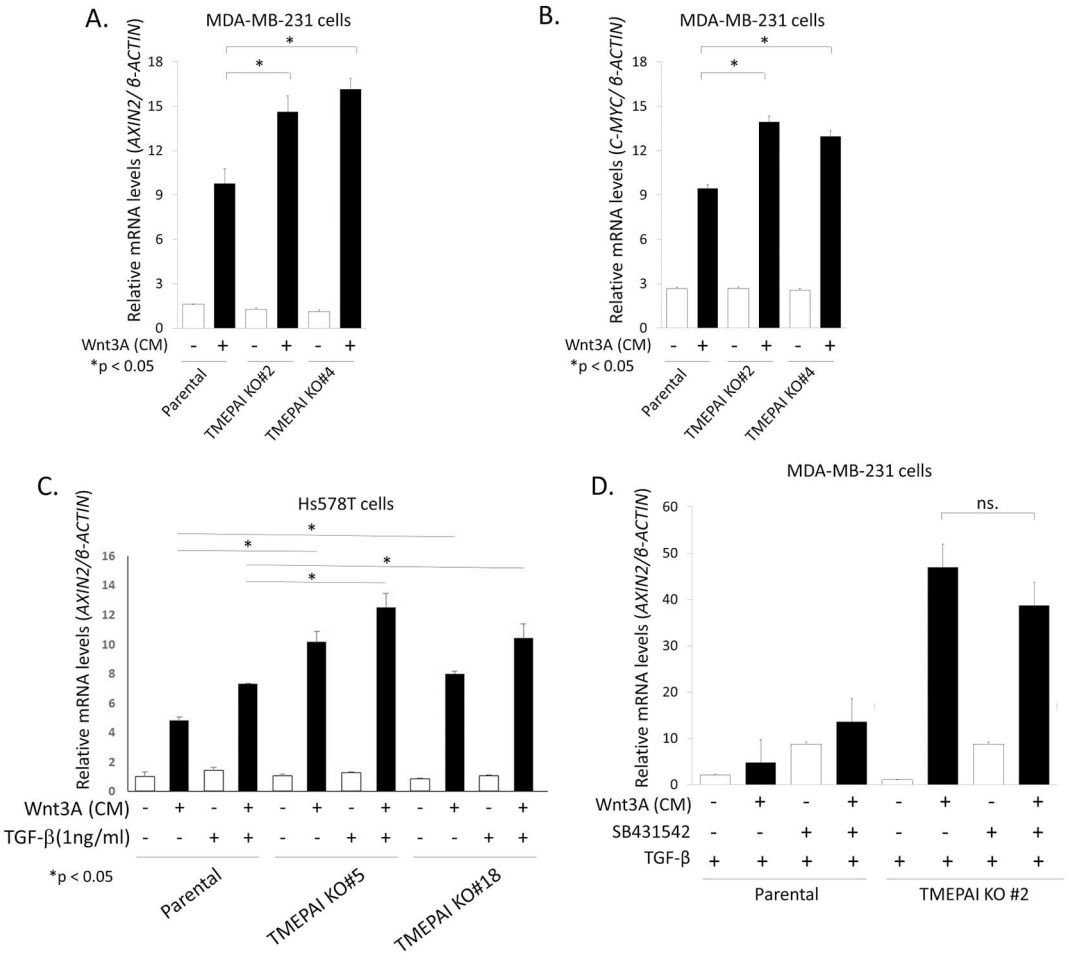

Fig. 6. TMEPAI knockout increases Wnt target gene expression. (A, B) MDA-MB-231 parental and TMEPAI knockout cells (#2 and #4) were treated with Wnt3A conditioned medium for 3 h in the presence of TGF-β ligand (1 ng/ml) for 7 h. Total RNAs were isolated and converted to cDNA. Relative expression of *AXIN2* (A) and *c-MYC* (B) were measured by real time PCR and normalized with corresponding *β-ACTIN* expressions. (C) Hs578T parental and TMEPAI knockout cells (#5 and #18) were treated with TGF-β (1 ng/ml) for in total 7 h together with Wnt3A conditioned medium at last 3 h before harvest. Total RNAs were isolated and converted to cDNA. Relative expression of *AXIN2* was measured by real time PCR and normalized with corresponding *β-ACTIN* expressions. (D) MDA-MB-231 parental and TMEPAI knockout #2 cells were stimulated with control or Wnt3A conditioned medium for 3 h in the presence of TGF-β (1 ng/ml) for 7 h. As indicated, cells were also treated with TGF-β type I receptor kinase inhibitor, SB431542 (10 μM) for 3 h. Graph shows relative *AXIN2* mRNA expression which is normalized with *β-ACTIN* expression.

cells, MDA-MB-231 TMEPAI knockout #4 showed comparable cell proliferation rate compared although MDA-MB-231 TMEPAI knockout #2 exhibited mildly slower cell proliferation in control medium condition. The Wnt3A treatment did not affect cell proliferation rate in MDA-MB-231 parental cells. In contrast, Wnt3A significantly increased cell proliferation rate in both knockout cell clones (Fig. 7A, B). Furthermore, we investigated the correlation of altered TMEPAI expression and Wnt signaling target gene expression signature in clinical database. We listed 1212 samples from 1093 TCGA invasive breast cancer cases (BRCA) in a descending order by RSEM-normalized RNA-seq values of PMEPA1/TMEPA1 to examine the enriched biological processes in the top 220 samples (TMEPAI.High) compared with the bottom 220 samples (TMEPAI.Low) (Supplementary Table S1). After filtering the biological processes that represent regulation of WNT signaling in one direction (positive or negative) (Supplementary Table S2), TMEPAI.High samples were significantly enriched in target genes of GO_NEGATIVE_REGULATION_OF_WNT_SIGNALING_PATHWAY and of GO_POSITIVE_REGULATION_OF_WNT_SIGNALING_PATHWAY. But on specifying the canonical Wnt signaling, TMEPAI.High samples were significantly enriched in target genes of GO_NEGATIVE_REGULATION_OF_CANONICAL_WNT_SIGNALING_PATHWAY (Fig. 7C, Supplementary Table S2) suggesting an association between high expression of TMEPAI and suppression of β-catenin-dependent Wnt signaling.

4. Discussion

TGF-β and Wnt signaling pathways are important for cellular function and activation of both pathways is required for many developmental and patterning events [28]. Multiple studies have described the signaling crosstalk between TGF-β and Wnt pathways by showing that Axin can associate with the TGF-β-regulated Smad2 and Smad3 [22–27]. Mouse models have indicated the cooperation of TGF-β and Wnt signaling in cancer [28], although much remains to be understood on how loss of TGF-β signaling along with concomitant Wnt pathway activation modifies tumor progression.

Our group previously demonstrated that TMEPAI is induced by TGF-β and Wnt signaling [12] and is highly expressed in several cancers, suggesting its role as an oncogene. It has been reported that overexpression of TMEPAI promotes PC-3 prostate cell proliferation [29] while depletion of TMEPAI inhibits the cell growth, migration and invasion potential of breast cancer cell line [8]. Moreover, reduction of TMEPAI expression was found to significantly decrease xenograft tumor growth in studies using PC-3, MDA-MB-231 and lung cancer cell lines Calu3 and NCI-H23 [7,9,29].

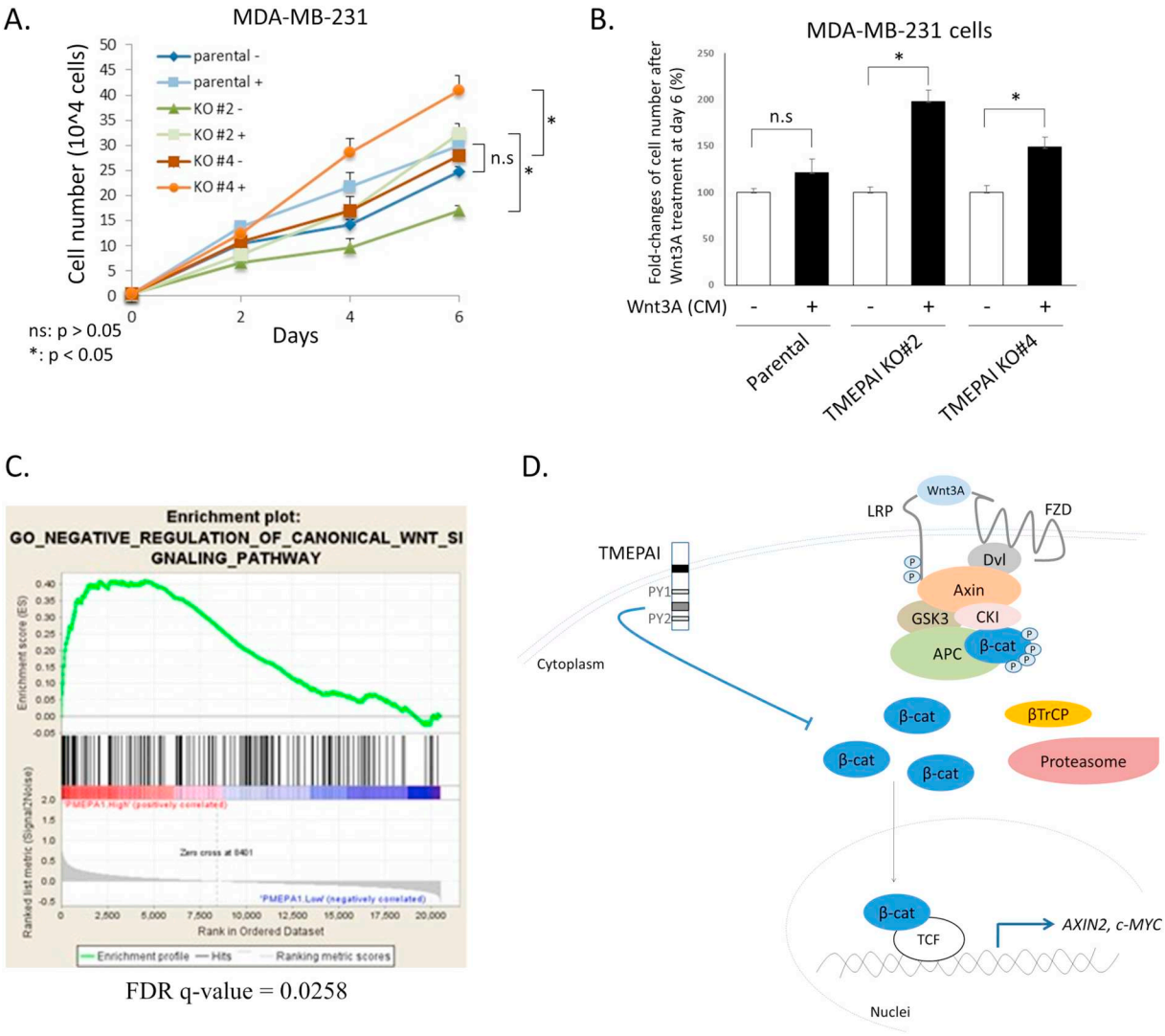

Fig. 7. TMEPAI is involved in Wnt signaling-mediated physiological events. (A) Cell proliferation rate of MDA-MB-231 parental and TMEPAI knockout cell lines (#2 and #4) in monolayer culture condition without (−) or with (+) Wnt3A conditioned medium treatment. (B). Percentage of cells number increasement on day 6. The results are presented as means ± SDs. Data were analyzed using the student's *t*-test. *p < .05 compared with control (C) Enrichment plot of GO_NEGATIVE_REGULATION_OF_CANONICAL_WNT_ SIGNALING_PATHWAY showing profile of the running enrichment score (ES) and positions of gene set members on the rank ordered list of upregulated genes in PMEPA1.High samples in TCGA invasive breast cancer cases (BRCA). (D) Suggested model of TMEPAI action in Wnt/β-catenin signaling. TMEPAI inhibits β-catenin stability and nuclear accumulation resulting decrease of Wnt target genes, *AXIN2* and *c-MYC* expression in TNBC cells.

Herein, we examined the roles of TMEPAI in Wnt signaling and found inhibition of Wnt signaling and downstream transcription of Wnt targets through regulation of β-catenin stability. We employed TMEPAI-expressing breast cancer cell lines and showed that loss of TMEPAI increased β-catenin stability and nuclear accumulation while TMEPAI overexpression suppressed β-catenin stability. Moreover, TMEPAI regulated endogenous Wnt signaling target genes such as *AXIN2* and *c-MYC,* possibly by affecting the stability and translocation of β-catenin to the nucleus. Therefore, our findings propose a novel role of TMEPAI in Wnt signaling.

TMEPAI is a direct target of TGF-β signaling and can interact with Smad2 and Smad3 via its Smad interaction motif (SIM) to sequester Smads from TGF-β/Smad signaling [4]. On the other hand, TGF-β induces nuclear translocation of β-catenin in mesenchymal stem cells (MSCs) in a Smad3-dependent manner [23]. Likewise, TGF-β/Smad3 activates canonical Wnt/β-catenin signaling, leading to proliferation in smooth muscle cells [24], and TGF-β/Smad3 signaling activates Wnt-TCF/LEF in a β-catenin-dependent manner during myoblast proliferation, but in a β-catenin-independent manner during muscle stem cell quiescence [27]. Although we initially thought that TMEPAI was affecting Wnt signaling through regulation of TGF-β signaling, this is unlikely to be the case because our TMEPAI SIM mutant that lacks the ability on TGF-β signaling also displayed inhibitory action on Wnt signaling comparable in potency to wild-type TMEPAI. Furthermore, TGF-β type I kinase inhibitor does not affect the inhibitory function of TMEPAI on Wnt signaling, indicating TMEPAI inhibition of Wnt signaling as independent from TGF-β signaling. Moreover, we found that PY motifs are required for inhibition of Wnt signaling and are the site of interaction with β-catenin although mechanistic determinations remain elusive. A PY motif is well known as binding site for the WW domain-containing proteins. Since β-catenin does not have WW domain in its structure, other proteins may facilitate the interaction between TMEPAI and β-catenin. HECT type E3 ubiquitin ligases (e.g., NEDD4) which possess WW domain are known to be partners of TMEPAI and involved in the degradation of androgen receptor and PTEN in a TMEPAI-dependent manner [8,30]. Thus, we presumed the involvement of NEDD4 in the instability of β-catenin by TMEPAI. However, overexpression of NEDD4 together with TMEPAI does not change β-catenin expression

levels compared with TMEPAI alone (data not shown). Further experiments are needed to clarify how TMEPAI affects β-catenin instability.

TMEPAI is highly expressed in many human cancers and knockdown of TMEPAI suppresses tumorigenic activities [7–9]. Although our previous results suggested that TMEPAI induces tumorigenic activity, here, we report that TMEPAI inhibits Wnt/β-catenin signaling. Considering Wnt signaling as a promoter of tumor progression, TMEPAI involvement in both signaling pathways could play a paradoxical role in tumorigenesis. However, Fournier et al. showed that low TMEPAI expression tends to coincide with increased incidence of prostate cancer metastasis to bone [31] whereas Jang et al. showed that inhibition of Wnt/β-catenin signaling could suppress breast cancer metastasis by inhibiting a cancer stem cell-like phenotype [32]. Our data suggested the involvement of TMEPAI in Wnt3A-induced cell proliferation in monolayer culture condition and correlation between TMEPAI expression and canonical Wnt signaling signature in breast cancer cells. However, TMEPAI may have context-dependent roles in tumor-promotion or tumor-suppression in primary or metastatic cancers through the regulation of many intracellular signaling.

Our proposed action of TMEPAI on Wnt/β-catenin signaling provides a partial elucidation on the complex roles of TMEPAI in cancer progression even though we were unable to fully resolve its paradoxical role. We therefore propose that, in the cancer microenvironment, high TMEPAI expression keeps TGF-β signaling to a minimum level for tumor growth promotion but, on the other hand, inhibits Wnt/β-catenin signaling. These conflicting roles potentially place TMEPAI as a protein involved in TGF-β and Wnt/β-catenin crosstalk.

5. Conclusions

Collectively, our data support the inhibitory role of TMEPAI in Wnt signaling. TMEPAI regulates β-catenin stability, nuclear accumulation, and the resultant transcriptional activity that affects the expression of Wnt target genes *AXIN2* and *c-MYC*.

Conflict of interest

The authors declare that there are no conflicts of interest.

Author contributions

Conception and design of the work: R.A., Y.W., M.K. Acquisition of data: R.A., M.A., M.U.P., J.H., F. A., Y.W. Analysis and interpretation of data: R.A., M.A., M.U.P., J.H., F. A., Y.W., M.K. Drafting or correcting the manuscript: R.A., Y.W., M.K. All authors read and approved of the final article.

Acknowledgements

This work was supported by Grant-in-Aid for Young Scientists (B) [JP25870093 (Y.W.), JP16K19100 (Y.W.)], for Scientific Research (B) [JP25293092 (M.K.)], for Scientific Research on Innovative Area [JP26116707 (M.K.)] and Research Grant from the Uehara Memorial Foundation (Y.W.). The authors thank George Church for the kind gifts of hCas9 expression vector (Addgene plasmid #41815) and gRNA_cloning vector (Addgene plasmid #41824). We also thank Dr. Bryan J. Mathis of the Medical English Communication Center (Univ. of Tsukuba Faculty of Medicine) for English editing.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellsig.2019.03.016.

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