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## Knock-out transmembrane prostate androgen-induced protein gene suppressed triple-negative breast cancer cell proliferation

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### ABSTRAK

**Latar belakang:** Kanker payudara triple negative (TNBC) cenderung tumbuh lebih cepat dan memiliki prognosis yang buruk dibanding sub-tipe lain. Ekspresi transmembrane prostate androgen-induced protein (TMPEAI) yang tinggi berhubungan dengan prognosis yang buruk pada pasien TNBC. Namun demikian, mekanisme TMPEAI pada proses tumorigenik belum diketahui. Penelitian ini bertujuan mengetahui pengaruh TMPEAI terhadap proliferasi sel TNBC.

**Metode:** CRISPR-Cas9 telah digunakan sebelumnya sebagai teknik genome editing untuk membuat galur sel knock-out (KO) TMPEAI pada galur sel TNBC, Hs587T. Galur sel Hs587T wild-type (WT) dan Hs587T knock-out TMPEAI dikultur pada Dulbecco's modified eagle medium (DMEM) yang disuplementasi dengan 10% fetal bovine serum, 1% penicillin-streptomycin, dan amfoterisin B. Kedua jenis sel dikultur pada 24-well plates dan dihitung setiap dua hari, kemudian dibuat plot kecepatan proliferasi sel. Setelah itu, total RNA dari kedua sel tersebut diisolasi untuk pemeriksaan tingkat ekspresi mRNA marker proliferasi, Ki-67 dan TGF- $\beta$ .

**Hasil:** Laju proliferasi sel menunjukkan galur sel WT-TMPEAI tumbuh lebih cepat dibandingkan dengan KO-TMPEAI. Hasil ini didukung dengan penurunan tingkat ekspresi mRNA dari marker proliferasi, Ki-67 dan TGF- $\beta$  galur sel KO-TMPEAI.

**Kesimpulan:** Knock-out TMPEAI menurunkan proliferasi sel pada kanker payudara triple negative.

### ABSTRACT

**Background:** Triple negative breast cancer (TNBC) tends to grow more rapidly and has poorer prognosis compared to others. High expression of transmembrane prostate androgen-induced protein (TMPEAI) correlates with poor prognosis in TNBC patients. However, the mechanistic role of TMPEAI in tumorigenic remains unknown. This study aimed to knock-out TMPEAI in TNBC cell line to determine its function further in cells proliferation.

**Methods:** CRISPR-Cas9 has been used previously to knock-out TMPEAI in Hs857T TNBC cell line. Hs587T TNBC parental cell line (wild-type/WT) and TMPEAI knock out Hs 586T cell lines were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin and amphotericin B. Both cell lines were seeded in 24-well plates and counted every two days, then proliferation rates were plotted. Afterwards, total RNA were isolated from the cells and Ki-67, and TGF- $\beta$  mRNA expression levels as proliferation markers were determined.

**Results:** Cell proliferation rates as displayed in growth curve plots showed that WT-TMPEAI cell line grew more rapidly than KO-TMPEAI. In accordance, mRNA expression levels of Ki-67 and TGF- $\beta$  were significantly decreased in KO-TMPEAI cell line.

**Conclusion:** Knock-out of TMPEAI attenuates cell proliferation in TNBC.

**Keywords:** proliferation, TGF- $\beta$ , TMPEAI, triple negative breast cancer

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Triple negative breast cancer (TNBC) presents in a small number of patients of 15–25% out of the total breast cancer patients.<sup>1,2</sup> However, this type of breast cancer is related to shorter survival, poorer prognosis, and metastasize more frequently in lungs and brains compared to other breast cancer subtypes.<sup>1-3</sup> Until now, there is no available targeted therapy indicated for TNBC due to lack expressions of estrogen, progesterone, and human epidermal receptors. The current modality of treatment is chemotherapy such as doxorubicin and paclitaxel, with no specific targets.<sup>3</sup> Unfortunately, this type of breast cancer is more prone to develop resistance, either intrinsic or acquired to cytotoxic agents.<sup>4</sup> Therefore, a deep and thorough understanding for the pathogenesis as well as chemo-resistance mechanism is urgent in order to determine a novel drug targets for TNBC patients.

Transmembrane prostate androgen-induced protein (TMEPAI) is remarkable in TNBC. It found highly expressed in 68,8% TNBC patients.<sup>2</sup> Moreover, high expression of TMEPAI correlates to poorer prognosis and shorter relapse-free survival.<sup>5</sup> The importance of TMEPAI in TNBC started when Singha et al suggested it as a responsible converter for TGF- $\beta$  from tumor suppressor into promotor.<sup>6</sup> Further study by Singha et al<sup>2</sup> discussed about the mechanism of TMEPAI in TNBC. TMEPAI inhibits Smad-dependent pathways of TGF- $\beta$  and instantly promotes non-Smad pathways via PI3K/Akt signaling. Previously, Watanabe et al proved how TMEPAI works in Smad-dependent pathways to inhibit TGF- $\beta$  signaling. TMEPAI disrupts interaction between R-Smad and SARA through Smad interaction motif (SIM). Afterwards, TMEPAI traps phosphorylated Smad2 and Smad3 by preventing their translocation into the nucleus. Consequently, transcription and translation of TGF- $\beta$  target genes will be stopped.<sup>7</sup>

In addition, TMEPAI promotes degradation of PTEN, thus promotes PI3K/Akt pathways to be activated. It resulted in an enhancement of cell growth.<sup>2</sup> Furthermore, TMEPAI has been proven to increase tumorigenic properties in lung cancer.<sup>8</sup> However, the mechanism of how TMEPAI enhances tumorigenic activities in cancer remains unknown. Hence, we aimed to investigate the role TMEPAI in TNBC to determine its function further in cell proliferation, using our previous knock out TMEPAI TNBC.

## METHODS

### Cell culture and cell proliferation rate

Triple negative breast cancer cell line, Hs578T was obtained from the American Type Culture Collection (ATCC). Hs578T with TMEPAI knock-out (KO) cells was developed from Hs578T wild-type (WT) previously using CRISPR-Cas9 system.<sup>9</sup> Both of Hs578T, WT and KO, were cultured in Dulbecco's modified essential medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Gibco), 10  $\mu$ g/mL insulin, and 100 units/mL of penicillin G and 0.1 mg/mL of streptomycin sulfate (Wako). Cells were maintained in 5% CO<sub>2</sub> incubator at 37°C. Both cell lines were seeded in same number in 24-well plates and counted every two days using hemocytometer in 0.4% trypan blue cell suspension up to 8 days. Area under the curve (AUC) of cell proliferation was calculated by connecting the dots which represents number of cells in different time points. AUC were calculated automatically using GraphPad Prism v. 7.0 software (GraphPad Prism, USA). Doubling time for each cell line were also calculated using online doubling time calculator.<sup>10</sup> All of the experiments were conducted three times and were duplicated.

### RT-PCR

We isolated total RNA using Total RNA Mini Kit (Geneaid) for cultured cells, and afterwards, converted total RNA into cDNA using Transcriptor First Strand cDNA Synthesis Kit (Roche). RT-PCR were performed using FastStart Essential DNA Green Master (Roche). All primers were purchased from the Integrated DNA Technologies, Singapore. Primer sequences all of the mRNA evaluated were as below: Ki-67 Fwd: 5'-TCCTTTGGTGGGCACCTAAGACCTG-3'; Ki-67 Rev: 5'-TGATGGTTGAGGTCGTTCCCTTGATG-3'; TGF- $\beta$  Fwd: 5'-TGAACCGGCTTTTCCTGCTTCTCATG-3'; TGF- $\beta$  Rev: 5'-GCGGAAGTCAATGTACAGCTGCCGC-3'.  $\beta$ -actin was used as a house-keeping gene with primer sequences:  $\beta$ -actin Fwd: 5'-GCTGGAAGGTGGACAGCGA-3' and  $\beta$ -actin Rev: 5'-GGCATCGTGATGGACTCCG-3'.

### Statistical analysis

Data were shown as mean  $\pm$  standard deviation (SD). Statistical analysis was performed with independent t-test comparing area under

the curve (AUC) for proliferation rate, mRNA expressions of Ki-67, and TGF- $\beta$  of knock-out cell line versus wild-type. Differences were considered statistically significant at  $p < 0.05$ . All the statistical analysis and graphs presented were calculated and drawn using GraphPad Prism v 7.0 software (GraphPad Prism, USA).

## RESULTS

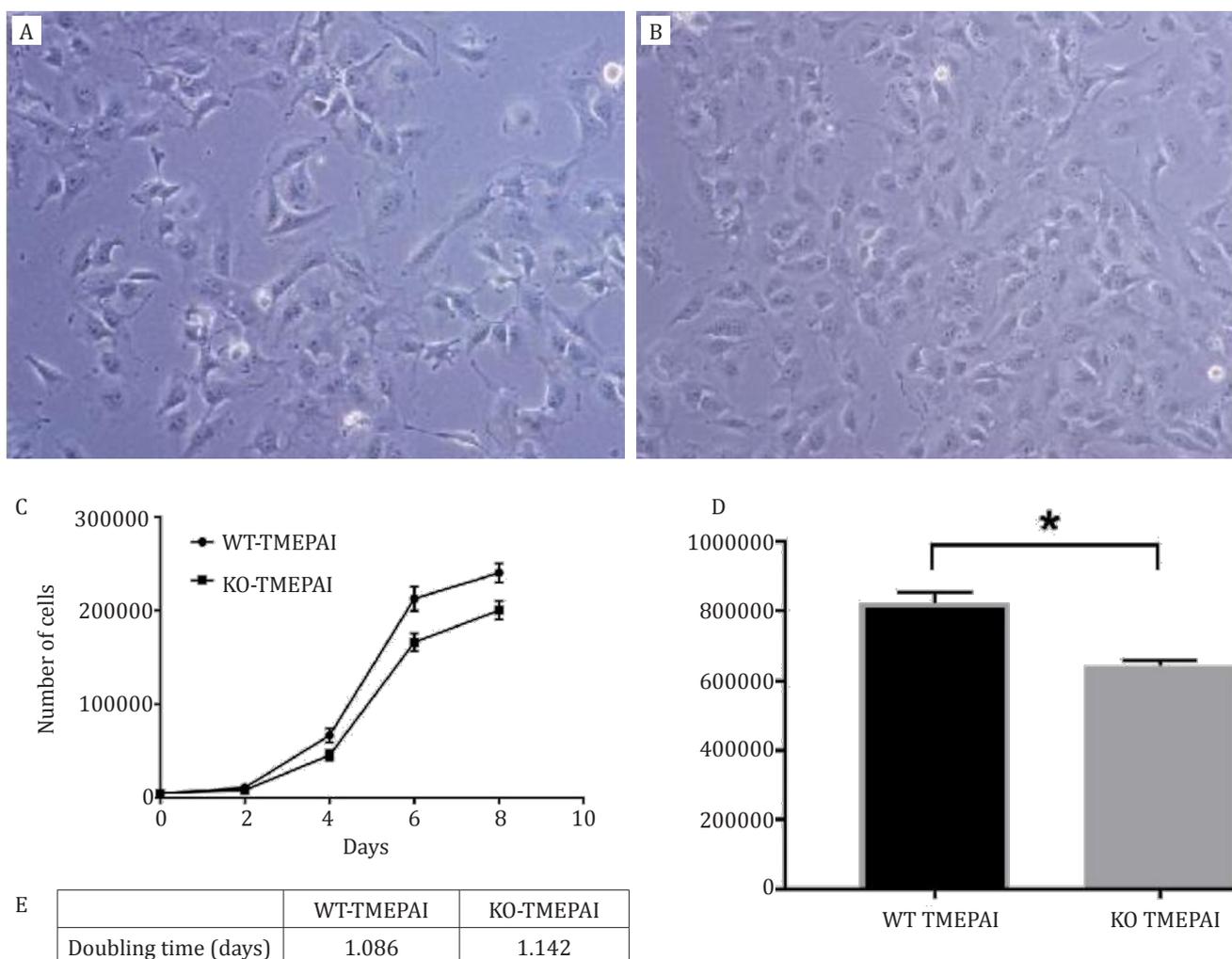
### Decreased cell proliferation rate in KO-TMEPAI versus WT-TMEPAI TNBC

KO-TMEPAI (Figure 1A) had lower cell confluency

compared to WT-TMEPAI Hs587T cells (Figure 1B). In accordance, cell proliferation rate of KO-TMEPAI was slower than those of WT-TMEPAI Hs587T cells (Figure 1C), along with lower area under curve (Figure 1D). Doubling time for KO-TMEPAI was longer than WT-TMEPAI cells as shown in (Figure 1E).

### Proliferation marker and TGF- $\beta$ were attenuated in the knock-out TMEPAI Hs587T cells

Figure 2A and 2B show alterations of mRNA expressions of Ki-67 and TGF- $\beta$ . Proliferation marker, Ki-67 decreased remarkably in KO-TMEPAI Hs587T cells as well as TGF- $\beta$ .



**Figure 1.** WT-TMEPAI cells grew more rapidly than KO-TMEPAI Hs587T cells. A) TNBC cell line, Hs578T, with wild-type TMEPAI (WT-TMEPAI). B) TNBC cell line, Hs578T, with knock-out TMEPAI (KO-TMEPAI). Cells were taken picture under inverted microscope with 10x magnification. C) Cell proliferation rates of WT-TMEPAI and KO-TMEPAI Hs587T cells were determined using cell growth curve plot up to 8 days. D) Area under curve of WT-TMEPAI and KO-TMEPAI cells proliferation rate. E). Doubling time for KO-TMEPAI was longer compared with WT-TMEPAI Hs587T cells. All experiments were conducted three times in duplicate. WT: wild-type. KO: knock-out. \*)  $p < 0.05$  after independent t-test comparing wild-type versus knock-out

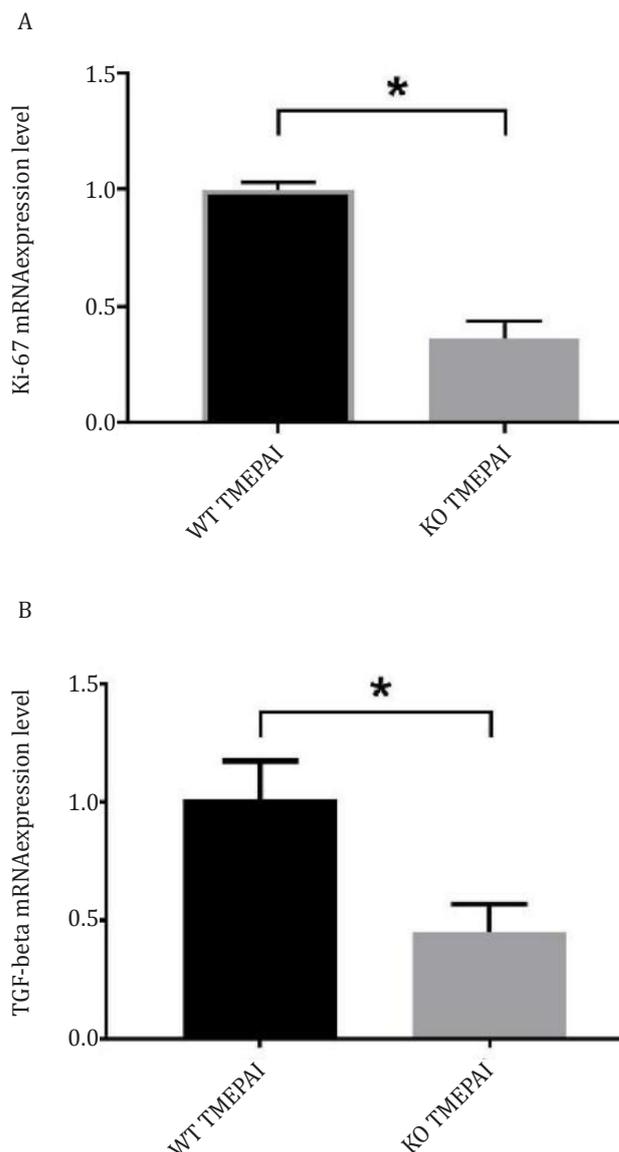
## DISCUSSION

It is noteworthy that currently there is no targeted therapy registered for triple negative breast cancer (TNBC) worldwide. TNBC is counted due to its more aggressive behavior, and thus, resulted in poorer prognosis as compared to other types of breast cancer. Moreover, TNBC frequently occurs in young woman.<sup>3</sup> Many researchers aimed to elucidate the signaling pathways which involved in TNBC as a target for novel therapy.

TMEPAI has been suggested to enhance tumorigenic activity in breast cancers. Previously, there were two reports from Singha<sup>2,6</sup> which used knock-down TMEPAI TNBC cells to elucidate the role of TMEPAI in oncogenic and tumorigenic activities of TNBC. However, their model, that used shRNA, could not fully omitted TMEPAI in TNBC cells. Therefore, in this study we aimed to determine cell proliferation alteration in a fully knock-out TMEPAI TNBC cells.

We established knock-out TMEPAI cells in previous study using CRISPR-Cas9 systems in Hs578T wild-type, one of triple negative cell line.<sup>9</sup> In this study, we showed that KO-TMEPAI cells had longer doubling time compared to WT-TMEPAI cells. KO-TMEPAI cells also showed a slower proliferation rate than that of wild-type TMEPAI cell line. This strongly indicated that TMEPAI had an important role in cell proliferations of triple negative breast cancer. In accordance, previous study by Singha et al<sup>2</sup> reported that knock-down TMEPAI attenuated tumorigenic activity. TMEPAI was reported by Azami et al. as a pro-tumorigenic factor in lung cancer induced by TGF- $\beta$  signaling. It also dominantly depended on activated autocrine TGF- $\beta$  signaling.<sup>11</sup>

Furthermore, we also evaluated mRNA expression levels of TGF- $\beta$ . It was previously known that TGF- $\beta$  induced TMEPAI expression in genomic transcript, as well as proteomic levels.<sup>2,6-8</sup> Our study differed with previous study which were conducted by Singha et al<sup>2,6</sup> and Ngunyen et al<sup>8</sup> Those study used TGF- $\beta$  induction in cell medium. In our study, we did not add TGF- $\beta$  exogenously. This condition might be due to the basal conditions that corresponded to autocrine signaling of TGF- $\beta$  in KO and WT-TMEPAI TNBC cells. Our results in KO-TMEPAI cells confirmed



**Figure 2.** Decreased mRNA expressions of A) Ki-67 (normalized to  $\beta$ -actin as housekeeping gene) and B) TGF- $\beta$  (normalized to  $\beta$ -actin as housekeeping gene) in KO-TMEPAI as compared to WT-TMEPAI. All experiments were performed three times in duplicate. WT: wild-type. KO: knock-out. \*)  $p < 0,05$  after independent t-test comparing wild-type versus knock out

that mRNA expression levels of TGF- $\beta$  was lower than that of wild-type cells. This indicated that the loss of TMEPAI resulted in decreased proliferation rates, followed by decreased mRNA expressions of TGF- $\beta$ . The expression of TMEPAI was controlled by TGF- $\beta$  constitutively. However, TMEPAI suppressed TGF- $\beta$  signaling by binding to R-Smad competitively with SARA (Smad anchor for receptor activation), to refrain R-Smad binding and inhibit activation of TGF- $\beta$  receptor kinase.<sup>7</sup> It is remain unclear how TMEPAI affects cell proliferation.

In this study, the decrease of TGF- $\beta$  mRNA expression levels was still inadequate to describe the activation of TGF- $\beta$  that was related to cell proliferation signaling. Further studies on TMEPAI downstream targets are still needed. On the other hand, xenograft models from KO-TMEPAI triple negative breast cancer cells should be generated in order to determine the role of TMEPAI in TNBC.

Previous study by Singha et al<sup>2</sup> reported that tumor volumes and weights of triple negative xenografts models were less in knock-down TMEPAI group compared to the wild-type group after 28 days. The result was followed by the decrease in proliferation marker, Ki67. In accordance with that finding, our KO-TMEPAI TNBC also showed a decrease in Ki-67 mRNA expressions compared to wild-type cells. Cell proliferation rate and confluency supported that finding. As indicated before, TMEPAI has pivotal role to alter cancer proliferation in triple negative breast cancer.

Therefore, we concluded that TMEPAI attenuates cell proliferation in TNBC cells. In support our finding, Singha et al<sup>2</sup>, Watanabe et al<sup>7</sup> and Ngunyen et al<sup>8</sup> already suggested role of TMEPAI in TGF- $\beta$  signaling pathway through Smad-dependent and PI3K/Akt pathways. Although we have not investigated in xenograft models and determine downstream of TGF- $\beta$  signaling due to the loss of TMEPAI, we suggest that TMEPAI has a potency to affect cancer progression in triple negative breast cancer, so that it potentially becomes a novel targeted therapy.

#### Conflict of interest

Melva Louisa and Rianto Setiabudy are editorial board members but were not involved in the review or decision for the article.

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