


Proliferation-associated long noncoding RNA, *TMPO-AS1*, is a potential therapeutic target for triple-negative breast cancer

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Abstract

Triple-negative breast cancer (TNBC) is an aggressive subtype of breast cancer compared with luminal or epidermal growth factor receptor 2 subtypes, thus effective therapeutic options for TNBC are yet to be developed. Nowadays, oncogenic long noncoding RNAs (lncRNAs) are applied to cancer management as a new class of therapeutic targets. We previously showed that thymopoietin antisense transcript 1 (*TMPO-AS1*) is a proliferation-associated lncRNA that contributes to hormone-dependent breast cancer progression by stabilizing estrogen receptor- α mRNA. We here showed that *TMPO-AS1* is abundantly expressed in basal-like breast cancer subtype based on the transcriptomic data in The Cancer Genome Atlas as well as in TNBC cell lines and patient-derived cells. Small interfering RNA-based loss-of-function analyses showed that *TMPO-AS1* knockdown substantially represses the proliferation and migration of TNBC cells. Expression microarray analysis showed that *TMPO-AS1* alters gene signatures related to transforming growth factor- β signaling in addition to proliferative E2F signaling pathways. *TMPO-AS1*-targeted siRNA treatment through

Abbreviations: ATCC, American type culture collection; CHEK1, checkpoint kinase 1; cRGD, cyclic RGDfK peptide; DDS, drug delivery system; DGL, dendrigraft poly-L-lysine; DMEM, Dulbecco's modified Eagle's medium; ER, estrogen receptor; ESR1, estrogen receptor 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GEO, Gene Expression Omnibus; GO, Gene Ontology; GSEA, Gene set enrichment analysis; HER2, human epidermal growth factor receptor 2; IM, 2-iminothiolane; LAP2, lamina-associated polypeptide 2; lncRNA, long noncoding RNA; MAD2L1, mitotic arrest deficient 2 like 1; MCM6, minichromosome maintenance protein complex component 6; MKI-67, marker of proliferation Ki-67; MPA, 1-(3-mercaptopropyl)amidine; PCNA, proliferating cell nuclear antigen; PEG, poly(ethylene glycol); PI, propidium iodide; PIC/m, polyion complex micelle; PLL, poly(L-lysine); PR, progesterone receptor; rRNA, 18S ribosomal RNA; STR, short tandem repeat; TCGA, The Cancer Genome Atlas; TGFBR1, transforming growth factor beta receptor 1; TGFBR2, transforming growth factor beta receptor 2; TGF- β , transforming growth factor beta; *TMPO-AS1*, thymopoietin antisense transcript 1; TNBC, triple-negative breast cancer; γ -PGA, γ -polyglutamic acid.

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engineered drug delivery systems using cancer-targeted polyion complex micelle or nanoball technology significantly impaired the in vivo growth of primary and metastatic TNBC xenograft tumors. Our findings suggest that *TMPO-AS1* plays a key role in TNBC pathophysiology and could be a potential therapeutic target for TNBC.

KEYWORDS

breast cancer, DDS, lncRNA, *TMPO-AS1*, TNBC

1 | INTRODUCTION

Breast cancer is the most common type of cancer in women and the number of breast cancer patients is on the rise worldwide.¹ Breast cancer is categorized as subtypes by expression markers such as hormone receptors, including ER and progesterone receptor, and HER2.² These expression markers are essential for the development and progression of each type of cancer and utilized for clinical therapies.³ For example, the most predominant type of breast cancer, ER-positive breast cancer, is treated with anti-estrogen reagents such as tamoxifen as a fundamental therapeutic option.⁴ In addition, HER2 Ab is a useful treatment method for HER2-positive breast cancer patients.⁵ However, a breast cancer subtype that does not express these markers, denoted TNBC, accounts for 10%-24% of all breast cancer cases.⁶ Unfortunately, the only fundamental option for the treatment of TNBC is standard chemotherapy, as specific molecular targeted therapy is underdeveloped. Furthermore, TNBC is more aggressive and metastatic compared with other types of breast cancer⁷; therefore, the characterization of new factors involved in the development and progression of TNBC is greatly anticipated.

A number of lncRNAs have been reported to be associated with various biological phenomena, immune reactions, neuronal diseases, and cancer development.⁸⁻¹⁰ Long noncoding RNA, by definition, is longer than 200 nucleotides and does not code for any structured protein.¹¹ Long noncoding RNAs modulate signaling pathways by binding to their target partners, which include protein, DNA, and RNA molecules.^{12,13} Several lncRNAs have been reported to be involved in TNBC cell proliferation and metastasis through elaborate mechanisms.¹⁴⁻¹⁶ In our previous study, we characterized *TMPO-AS1* as an lncRNA strongly associated with cell proliferation markers, including *MKI-67* and *PCNA*.¹⁷ *TMPO-AS1* was originally identified as a downstream lncRNA of E2F signaling.¹⁸ We showed that *TMPO-AS1* promotes ER-positive breast cancer cell proliferation and antiestrogen therapy resistance through stabilizing *ESR1* RNA. However, the role of *TMPO-AS1* in TNBC has not been addressed.

We showed that the intratumoral injection of si*TMPO-AS1* efficiently impairs in vivo growth of s.c. tumors derived from ER-positive breast cancer cells in a mouse xenograft model. RNA interference-mediated medicine is applied to cancer management as an efficient molecular targeting therapy as nucleic acid drugs can be easily designed by targeting specific sequences for individual genes

and RNAs.¹⁹ In the case of siRNA, however, it remains to be solved in terms of its instability and difficulty in delivery to specific target cells. To overcome these drawbacks, DDS has been developed.²⁰ The purpose of DDS includes enhancing the stability of siRNA and the specificity of siRNA delivery, leading to maximized therapeutic impact of siRNA with reduced side-effects.^{21,22}

In the present study, we examine the tumorigenic function of *TMPO-AS1* in TNBC using patient-derived cells as well as known TNBC cell lines. As siRNAs against *TMPO-AS1* could efficiently repress the proliferation and migration of TNBC cells, we evaluated the therapeutic potential of these siRNAs in mouse xenograft models based on recently developed nanoparticulate DDS.

2 | MATERIALS AND METHODS

2.1 | Cell culture and reagents

Human TNBC breast cancer cell lines MDA-MB-231 and MDA-MB-468 (ATCC) were authenticated by short tandem repeat analysis at BEX and cultured in DMEM containing 10% FBS, 50 units/mL penicillin, and 50 µg/mL streptomycin at 37°C in a humidified atmosphere of 5% CO₂. For *TMPO-AS1* overexpression, pcDNA3-empty or pcDNA3-*TMPO-AS1* vectors were transfected in MDA-MB-231 cells with Lipofectamine 2000 (Invitrogen) and used for further experiments. MDA-MB-231 cells were transfected with pGL3-Promoter vector (Promega) and selected with G418 to establish luciferase-expressing MDA-MB-231 cells. The TNBC-PDC were established from tumor tissue of a TNBC patient as described previously^{23,24} and cultured in DMEM containing 10% FBS, 50 units/mL penicillin, and 50 µg/mL streptomycin at 37°C in a humidified atmosphere of 5% CO₂.

2.2 | Bioinformatics

TMPO-AS1 expression data in TCGA breast cancer dataset²⁵ were obtained and analyzed by TANRIC software (<https://www.tanric.org>).²⁶ *TMPO-AS1*-coexpressed genes in TCGA breast cancer dataset were determined at a threshold of greater than 0.4 by Spearman's correlation using cBioportal software (<https://www.cbioportal.org>)^{27,28} The enrichment of pathways among

the retrieved *TMPO-AS1*-coexpressed genes was evaluated by GO analysis with biological process GO terms (<https://david.ncifcrf.gov/>)²⁹

2.3 | Quantitative real-time PCR

Total RNA was extracted from cells using ISOGEN reagent (Nippon Gene), followed by cDNA synthesis using SuperScript III reverse transcriptase (Invitrogen) with random primers. Quantitative RT-PCR was carried out on a StepOnePlus Real-Time PCR System (Thermo Fisher Scientific) using KAPA SYBR FAST qPCR Kit (KAPA Biosystems) and sets of gene-specific primers. RNA expression levels were analyzed by the $\Delta\Delta C_t$ method and normalized to the values of *GAPDH* or 18S ribosomal RNA. The sequences of primers are listed in Table S1.

2.4 | Small interfering RNA transfection

Small interfering RNAs against *TMPO-AS1* were designed using siDirect and purchased from RNAi Inc. A negative control siRNA (siControl) with no homology to known gene targets in mammalian cells was purchased from RNAi Inc. MDA-MB-231 and TNBC-PDC cells were seeded at 200 000–300 000 cells per well in 6-well plates and simultaneously transfected with siRNA at a final concentration of 10 nmol/L using Lipofectamine RNAiMAX (Invitrogen). Cells were harvested 48–72 hours after transfection and used for qRT-PCR, cell cycle analysis, and annexin V and PI staining. The sequences of siRNA are as follows (forward and reverse, respectively): si*TMPO-AS1* #1 (5'-gaagacuaguccuauuu-3' and 5'-uuauaggucacuagucuccu-3') and si*TMPO-AS1* #2 (5'-gagccgaacuacgaaccaacu-3' and 5'-uugguucguaguucggcug-3').

2.5 | DNA assay

Cells were seeded and treated with siRNAs at the indicated densities (3000 cells per well for MDA-MB-231 and 1500 cells per well for TNBC-PDC) in 96-well plates. Cells were harvested at 1, 3, and 5 days, lysed with freeze-and-thaw, and buffered with 5 mmol/L Tris-HCl, pH 7.5, containing 1 mol/L NaCl and 0.5 mmol/L EDTA. Extracted DNA was stained with Hoechst 33 258 pentahydrate (Thermo Fisher Scientific) at a final concentration of 5 μ g/mL. DNA content in each well was measured on ARVO5 (Perkin Elmer) at 355 nm for 0.1 seconds.

2.6 | Cell cycle analysis

The siRNA-treated cells were harvested and fixed with 70% ethanol for 30 minutes. Fixed cells were treated with RNase A and stained with 5 μ g/mL PI. DNA content was measured using FACSCalibur (Becton Dickinson). Data were analyzed by CellQuest software (Becton Dickinson) to determine the percentage of cells in the G₁, S, and G₂/M phases.

2.7 | Annexin V and PI staining

After transfection with siRNAs, cells were harvested, and apoptotic cells were stained using the FITC annexin V apoptosis detection kit (Becton Dickinson) following the manufacturer's instructions. Percentages of apoptotic cells were analyzed on FACSCalibur (Becton Dickinson).

2.8 | Microarray and pathway analysis

For microarray analysis, human Clariom D array (Thermo Fisher Scientific) was used following the manufacturer's instructions. Data were analyzed using Affymetrix Microarray Suite software. All microarray data are available in the Gene Expression Omnibus (GEO) database with the accession number GSE141925. Pathway analyses were undertaken using GSEA.

2.9 | Migration assay

MDA-MB-231 and TNBC-PDC cells were seeded at 100 000 cells per well in a 24-well plate and simultaneously transfected with siRNAs at a final concentration of 10 nmol/L for 48 hours. Cells were reseeded onto a polycarbonate membrane insert in a Transwell apparatus (Costar). Twenty-four hours after incubation, migrated cells were fixed with methanol and stained with Giemsa solution. Stained cells were counted under a microscope in 5 independent fields ($\times 200$).

2.10 | Preparation of siRNA-loaded PIC/m

A cancer-targeted PIC/m was prepared from siRNA and cRGD-installed PEG-block-PLL functionalized with MPA and IM (cRGD-PEG-PLL(MPA/IM)), as described previously.^{30,31} cRGD-PEG-PLL(MPA/IM) (molecular weight of PEG, 12 000; degree of polymerization of PLL, 38) was dissolved in 10 mmol/L HEPES buffer (pH 7.3), mixed with DTT solution, and then incubated for 0.5 hours. Thereafter, the polymer solution was mixed with siRNA dissolved in the same buffer at a molar ratio of cRGD-PEG-PLL to siRNA of approximately 8. The mixture was dialyzed against 10 mmol/L HEPES buffer (pH 7.3) containing 0.5% DMSO for 48 hours and 10 mmol/L HEPES buffer for 48 hours. The obtained PIC/m was characterized to have a hydrodynamic diameter of 35 nm with a polydispersity index of 0.08 by dynamic light scattering.

2.11 | Subcutaneous in vivo tumor formation and siRNA treatment

All animal experiments were approved by the Animal Care and Use Committee of Saitama Medical University, and carried out in accordance with the Guidelines and Regulations for the Care and Use of Experimental Animals of Saitama Medical University. Female BALB/cAJcl-nu/nu mice were purchased from CLEA Japan.

MDA-MB-231 cells (2 million cells per mouse) were mixed with an equal volume of Matrigel matrix (Corning) and injected s.c. into the flank of 10-week-old female mice. When the average tumor volume exceeded 200 mm³, mice were randomly assigned to receive either siControl (n = 5) or si*TMPO-AS1* #2 (n = 6). The siRNA samples (25 µg) encapsulated in PIC/m were i.v. injected into the tail vein twice a week for 4 weeks. Three dimensions of tumor were measured once a week and tumor volumes were estimated according to the formula: $0.5 \times ([\text{smallest diameter}]^2 \times [\text{longest diameter}])$. At the end-point of the experiment, tumors were dissected from the mice.

2.12 | Lung-selective nanoball-siRNA preparation

Lung-selective nanoball was prepared as follows.³² The siRNA solution was mixed with DGL solution and γ -PGA solution by thorough pipetting to construct lung-selective nanoball-siRNA, before being left for 30 minutes at room temperature. The obtained nanoball was characterized to have a hydrodynamic diameter of approximately 100 nm with a polydispersity index of 0.2 by dynamic light scattering. Also, the nanoball had anionic surface charge (approximately -20 mV).

2.13 | Lung metastatic tumor model and siRNA treatment

Female NOD/SCID mice were purchased from CLEA Japan. MDA-MB-231-luc cells stably expressing luciferase (2 million cells per mouse) were i.v. injected into 10-week-old female mice through tail veins. Fifty micrograms of siControl (n = 8) or si*TMPO-AS* #2 (n = 9) were mixed with lung-selective nanoball³⁰ and i.v. injected into tail veins twice a week for 2 weeks. To count fluorescence intensities, mice were i.p. injected with D-luciferin (3 mg per mouse), anesthetized for 5 minutes with isoflurane, and imaged for 2 minutes under anesthesia using the in vivo imaging system (Photon Imager; Biospace Lab). In vivo imaging data were analyzed with the Photo Vision software (Bio Space) for the quantification of data.

2.14 | Statistical analysis

Comparisons between the 2 groups were analyzed by Student's *t* test. Two-way ANOVA was undertaken to analyze differences among siRNA treatment groups.

3 | RESULTS

3.1 | Proliferation-associated lncRNA, *TMPO-AS1*, is abundantly expressed in TNBC cells

We previously reported that, among lncRNAs, *TMPO-AS1* shows the most significant coexpression pattern with proliferation

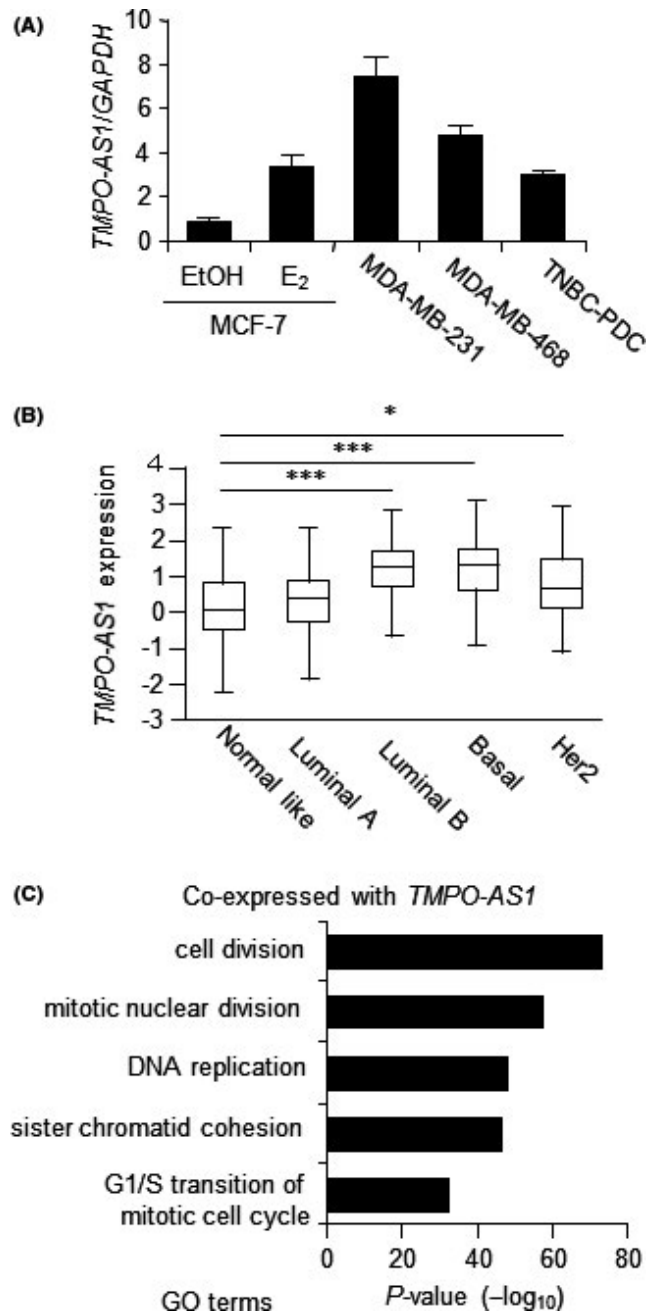


FIGURE 1 Proliferation-associated long noncoding RNA, *TMPO-AS1*, is abundantly expressed in triple-negative breast cancer (TNBC) cells. A, *TMPO-AS1* levels in estrogen (E₂)-treated or -untreated MCF-7 cells and TNBC cells were analyzed by quantitative real-time PCR. Relative RNA levels were determined by the normalization to *GAPDH* levels based on the $\Delta\Delta C_t$ method and each value is presented as mean fold change \pm SD vs MCF-7 cells with vehicle (EtOH) treatment. B, *TMPO-AS1* expression in The Cancer Genome Atlas breast cancer samples was analyzed by TANRIC software. C, *TMPO-AS1*-coexpressed genes were analyzed by gene ontology (GO) pathway analysis. Data show top 5 *TMPO-AS1*-coexpressed genes and their associated pathways

markers (*MKI67* and *PCNA*) in the breast cancer TCGA dataset.¹⁷ We showed that *TMPO-AS1* is important for the proliferation of ER-positive breast cancer cells through the upregulation of *ESR1* mRNA. Because the breast cancer TCGA dataset also includes

TNBC samples, we had a notion that *TMPO-AS1* also plays an important role in TNBC cell proliferation. At first, we assessed *TMPO-AS1* expression levels in TNBC cells compared with those of estrogen-treated and -untreated MCF-7 cells, as *TMPO-AS1* was found as an estrogen-inducible lncRNA (Figure 1A). TNBC cell lines such as MDA-MB-231 or MDA-MB-468 more abundantly express *TMPO-AS1* than estrogen-untreated MCF-7 cells (Figure 1A). In addition, TNBC-patient-derived cells (TNBC-PDC), which were originally established from TNBC tumor cells,^{23,24} also substantially expressed *TMPO-AS1* (Figure 1A). *TMPO-AS1* expression analysis in the TCGA dataset showed that *TMPO-AS1* is highly expressed in basal-type as well as luminal B subtype breast cancer tissues as compared with normal-like tissues (Figure 1B). We also showed that *TMPO-AS1*-coexpressed genes (Table S2) in the TCGA dataset were strongly associated with cell proliferation-related pathways (Figure 1C).

3.2 | *TMPO-AS1* regulates TNBC cell proliferation

As *TMPO-AS1* expression is also elevated in basal subtype breast cancer, we postulated that *TMPO-AS1* is involved in the proliferation of TNBC cells. To test this hypothesis, we introduced si*TMPO-AS1* into TNBC cell line MDA-MB-231 (Figure 2A) and TNBC-PDC (Figure 2B). The siRNA-mediated knockdown of *TMPO-AS1* significantly suppressed the proliferation of both MDA-MB-231 (Figure 2C) and TNBC-PDC (Figure 2D) cells. In addition, *TMPO-AS1* knockdown suppressed cell cycle progression (Figure 2E) and induced apoptotic cell death (Figure 2F). In contrast, *TMPO-AS1* overexpression (Figure S1A) promoted cell proliferation in MDA-MB-231 cells (Figure S1B).

3.3 | *TMPO-AS1* modulates E2F- and TGF- β -associated gene expression in TNBC cells

In the next step, we carried out microarray analysis in the *TMPO-AS1*-silenced MDA-MB-231 cells to understand the transcriptomic effect of *TMPO-AS1* on gene expression in TNBC cells. The GSEA-mediated pathway analysis showed that *TMPO-AS1* knockdown altered cell proliferation-associated gene expression such as "E2F targets" and "G2M checkpoint" (Figure 3A,B). In addition, TGF- β signaling is strongly associated with *TMPO-AS1* (Figure 3A,B). We validated several gene expression profiles in the "E2F target" pathway; *MCM6*, *TMPO*, *MAD2L1*, and *CHEK1* were silenced by si*TMPO-AS1* (Figure 3C). Furthermore, overexpression of *TMPO-AS1* increased these "E2F target" pathway-related genes (Figure S2). We also showed that *TGFBR1* and *TGFBR2* levels were suppressed by *TMPO-AS1* knockdown (Figure 3D).

3.4 | *TMPO-AS1* knockdown suppresses TNBC cell migration

Pathway analysis based on microarray results showed that *TMPO-AS1* is associated with the TGF- β pathway as well as the cell proliferation

pathway (Figure 3A,B). As the TGF- β pathway is known to be associated with the metastasis of broad cancer types including breast cancer,^{33,34} we assume that *TMPO-AS1* is also linked to a metastatic phenotype of TNBC. We showed that *TMPO-AS1* knockdown significantly suppressed the migration of MDA-MB-231 (Figure 4A) and TNBC-PDC cells (Figure 4B). In contrast, *TMPO-AS1* overexpression increased the expression of *TGFBR1* and *TGFBR2* (Figure S3A,B), and promoted MDA-MB-231 cell migration (Figure S3C,D).

3.5 | Polyion complex micelle-*TMPO-AS1* siRNA treatment suppresses s.c. TNBC tumor growth

We next determined whether si*TMPO-AS1* could suppress in vivo growth of TNBC tumors. We previously showed that the intratumoral injection of si*TMPO-AS1* #2 with a conventional lipofection reagent could significantly suppress in vivo tumor growth derived from ER-positive breast cancer cells. In this study, we undertook i.v. siRNA administration to nude mice with s.c. xenograft tumors based on cancer-targeted DDS using cRGD peptide-conjugated PIC/m.^{30,31,35} When the volume of MDA-MB-231-derived tumors in the flank of mice reached more than 200 mm³, i.v. injection of PIC/m-mediated si*TMPO-AS1* (PIC/m-si*TMPO-AS1*) or negative control siRNA (PIC/m-siControl) into the tail veins was carried out twice a week for 4 weeks. Tumor growth was significantly impaired by 8 injections of PIC/m-si*TMPO-AS1* compared to the same number of injections of PIC/m-siControl (Figure 5A,B). To confirm the *TMPO-AS1* knockdown effect in each tumor, we dissected tumors from each mouse and quantified the expression levels of *TMPO-AS1* and its downstream target genes. *TMPO-AS1* and its downstream target gene expression levels were decreased in PIC/m-si*TMPO-AS1*-treated tumors compared with PIC/m-siControl-treated tumors (Figure 5C).

3.6 | Lung delivery of DDS-si*TMPO-AS1* suppresses metastatic tumor growth of TNBC

Because we showed that *TMPO-AS1* is also associated with the migration of TNBC cells, we further examined the therapeutic effect of siRNAs against *TMPO-AS1* based on another lung-selective DDS nanoball technology (nano-si*TMPO-AS1*),³² in a lung metastatic model of TNBC cells. Luciferase-expressing MDA-MB-231 cells were i.v. injected into tail veins of NOD/SCID mice. Nano-si*TMPO-AS1* treatment twice a week for 2 weeks (total of 4 injections) significantly suppressed the viability of MDA-MB-231 cells in the lung (Figure 6A,B). To measure viable MDA-MB-231 cells in detail, we dissected the lung and measured fluorescence intensities. Fluorescence intensities derived from MDA-MB-231 cells stably expressing the luciferase gene were decreased in the nano-si*TMPO-AS1*-treated group compared with the nano-siControl-treated group (Figure 6C). Therefore, *TMPO-AS1* is important for the proliferation and metastasis of TNBC cells, and could serve as a potential therapeutic target of TNBC.

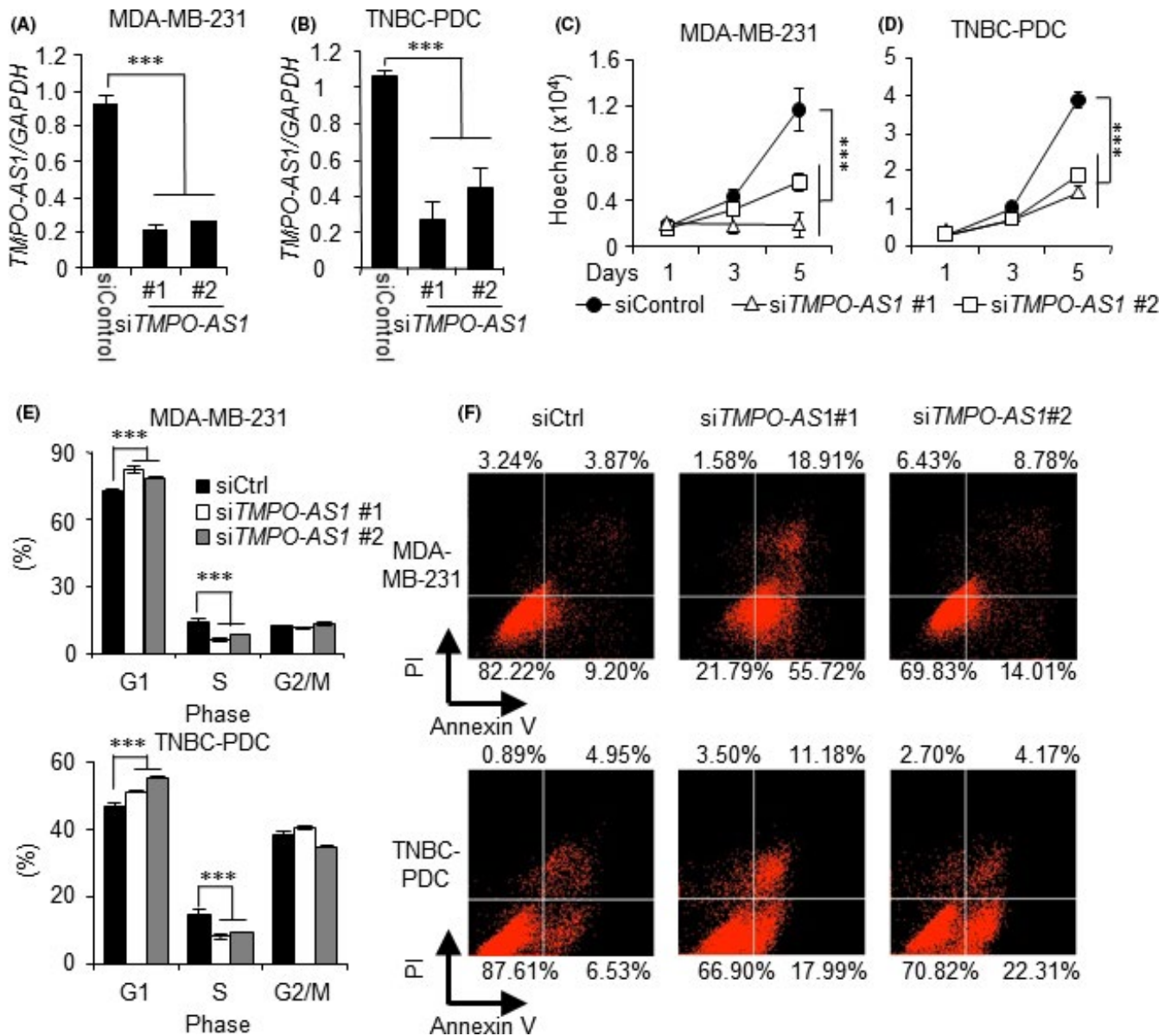


FIGURE 2 *TMPO-AS1* knockdown suppresses triple-negative breast cancer (TNBC) cell proliferation. A, B, Knockdown efficiency of *TMPO-AS1* siRNAs in MDA-MB-231 (A) and TNBC-patient-derived (PDC) (B) cells analyzed by quantitative real-time PCR. Relative RNA levels are presented as mean fold change \pm SD vs negative control siRNA (siCtrl) in each cell type ($n = 3$). C, D, Viability of MDA-MB-231 (C) and TNBC-PDC (D) cells on indicated days after siRNA treatment analyzed by DNA assay. Values are presented as mean \pm SD vs siControl in each cell type ($n = 5$). E, Cell cycle profiles with propidium iodide (PI) of MDA-MB-231 (upper panel) and TNBC-PDC (lower panel) cells treated with indicated siRNAs analyzed by flow cytometry. Percentages of cell populations in G₁, S, and G₂/M phases are shown ($n = 3$). F, Percentages of annexin V-positive populations in MDA-MB-231 cells (upper panel) and TNBC-PDC (lower panel) cells treated with indicated siRNAs analyzed by flow cytometry. * $P < .05$; ** $P < .01$; *** $P < .001$

4 | DISCUSSION

In the present study, we showed that *TMPO-AS1* is expressed abundantly in TNBC cells as well as in ER-positive breast cancer cells. Knockdown of *TMPO-AS1* significantly impaired the proliferation, cell cycle progression, and migration of TNBC cells, and promoted apoptosis of TNBC cells. Microarray-based global expression analysis showed that *TMPO-AS1* is strongly associated with the E2F and TGF- β signaling pathways. Two distinct DDS-mediated siRNA

treatment methods efficiently impair in vivo tumor growth of primary and metastatic TNBC models in mice.

We previously showed that *TMPO-AS1* is inducible by estrogen treatment¹⁷; the present study showed that *TMPO-AS1* is also abundantly expressed in TNBC cells lacking ER expression. *TMPO-AS1* was originally identified as a LAP2 α antisense transcript that is inducible by serum treatment.¹⁸ Luciferase assay using putative promoter regions of *TMPO/LAP2 α* and *TMPO-AS1* showed that ectopic E2F3 expression induced both promoter activities.¹⁸

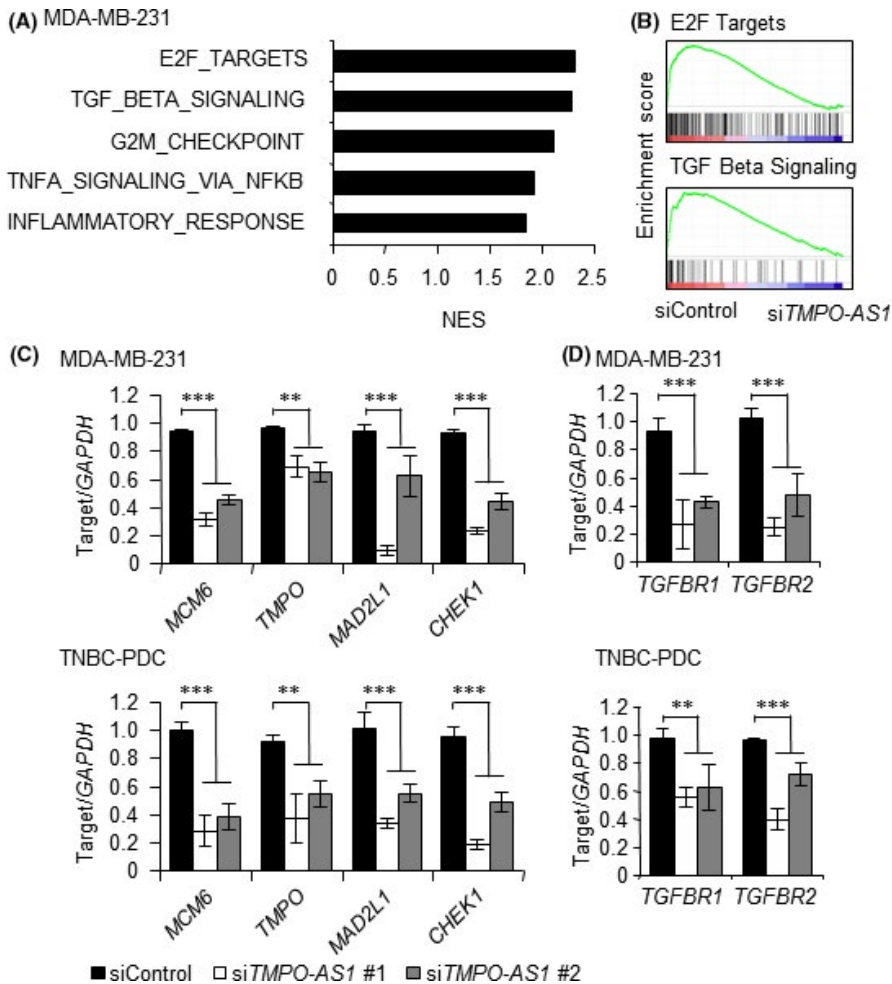


FIGURE 3 *TMPO-AS1* modulates E2F- and transforming growth factor- β (TGF- β)-associated gene expression in triple-negative breast cancer (TNBC) cells. A, Top 5 pathways enriched in genes downregulated by si*TMPO-AS1* vs negative control siRNA (siControl) determined by gene set enrichment analysis (GSEA) in MDA-MB-231 cells. B, GSEA enrichment plots for dominant pathways in MDA-MB-231 cells treated with si*TMPO-AS1*, including “E2F targets” and “TGF- β signaling”. C, Effects of *TMPO-AS1* knockdown on *MCM6*, *TMPO*, *MAD2L1*, and *CHEK1* levels in MDA-MB-231 (upper panel) and TNBC-patient-derived (PDC) (lower panel) cells. Relative RNA levels are presented as mean fold change \pm SD vs siControl ($n = 3$). D, Effects of *TMPO-AS1* knockdown on *TGFBR1* and *TGFBR2* levels in MDA-MB-231 (upper panel) and TNBC-PDC (lower panel) cells. Relative RNA levels are presented as mean fold change \pm SD vs siControl ($n = 3$). * $P < .05$; ** $P < .01$; *** $P < .001$. NES, normalized enrichment score.

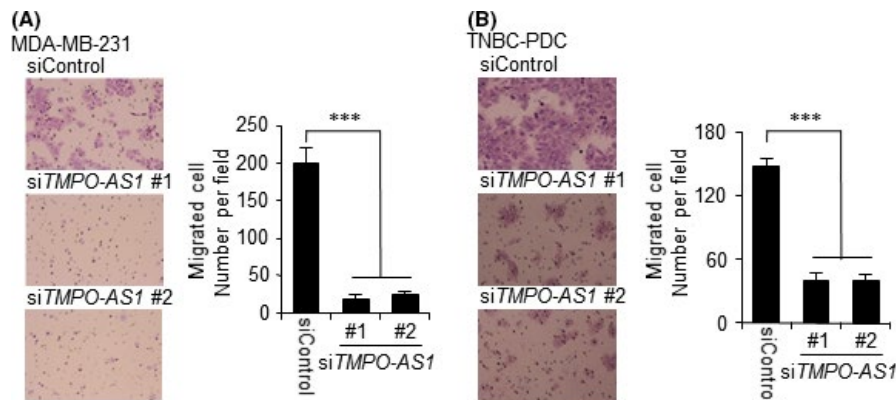
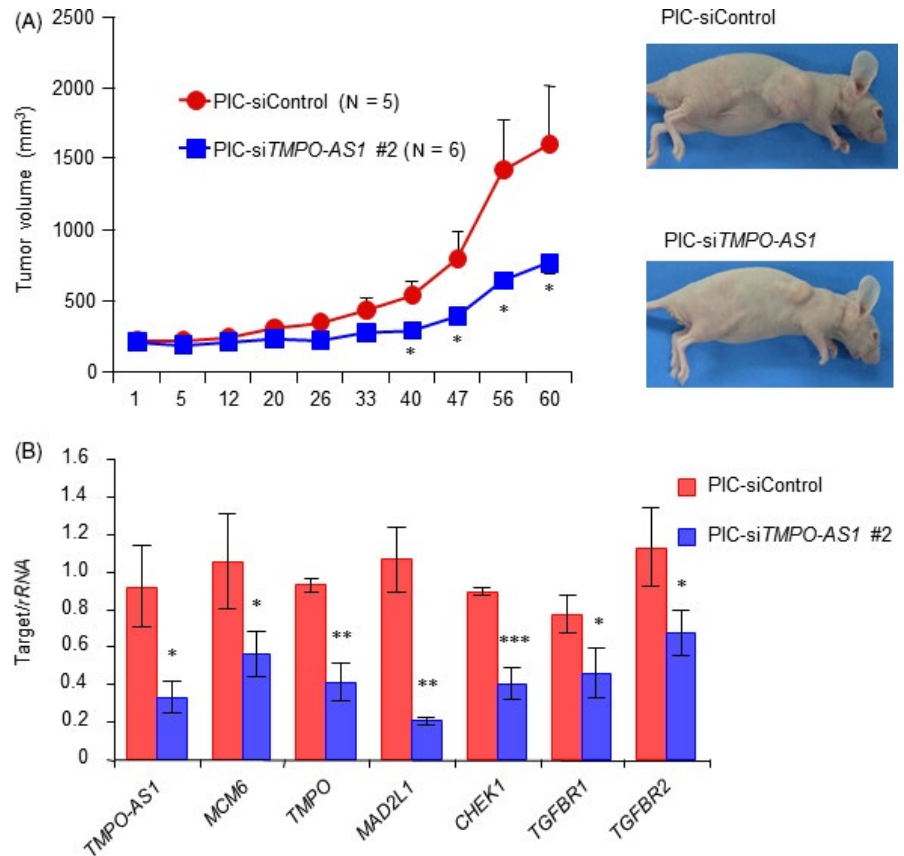


FIGURE 4 *TMPO-AS1* knockdown suppresses triple-negative breast cancer (TNBC) cell migration. A, B, si*TMPO-AS1*-treated cells were seeded onto the Transwell membrane. Migrating cells were stained with Giemsa and counted under a microscope in 5 independent fields. Representative photograph of migrating MDA-MB-231 (A, left panel) and TNBC-patient-derived (PDC) (B, left panel) cells is shown. Summarized data of MDA-MB-231 (A, right panel) and TNBC-PDC (B, right panel) cells are presented as mean \pm SD ($n = 5$). * $P < .05$; ** $P < .01$; *** $P < .001$. siControl, negative control siRNA

In addition, high *TMPO-AS1* expression was reported in prostate³⁶ and lung³⁷ cancers. We speculate that *TMPO-AS1* could be regulated by the E2F signaling pathway in TNBC. Our microarray analysis showed that *TMPO-AS1* knockdown significantly attenuates the E2F signaling pathway, suggesting that *TMPO-AS1* promotes

the E2F signaling pathway in TNBC cells. *TMPO-AS1* knockdown decreased the expression of typical E2F target genes, *MCM6* and *MAD2L1*, in both MDA-MB-231 and TNBC-PDC cells. Notably, we previously showed that *MCM6* and *MAD2L1* were downregulated by *ESR1* or *TMPO-AS1* knockdown in ER-positive breast cancer

FIGURE 5 Polyion complex micelle (PIC/m)-*TMPO-AS1* siRNA treatment suppresses s.c. triple-negative breast cancer tumor growth. A, B, Progression of MDA-MB-231-derived xenograft tumors treated with PIC/m loaded with siRNAs (PIC/m-siRNA) in nude mice. PIC/m-negative control siRNA (siControl) or PIC/m-si*TMPO-AS1* #2 were i.v. injected into mice inoculated with MDA-MB-231 cells through tail veins twice a week (PIC/m-siControl, n = 5; PIC/m-si*TMPO-AS1* #2, n = 6). Tumor volumes are presented as mean \pm SE (A). Representative photographs of xenografted mice are shown (B). C, *TMPO-AS1*, *ESR1*, *MCM6*, *MAD2L1*, *CHEK1*, *TGFBR1*, and *TGFBR2* levels in tumors treated with PIC/m-siControl or PIC/m-si*TMPO-AS1* #2. Tumors were dissected from mice 60 days after the beginning of siRNA administration. Data are presented as mean fold change \pm SD vs siControl (n = 3). * $P < .05$; ** $P < .01$; *** $P < .001$



cells, suggesting that the transcription of these genes is also regulated by estrogen signaling. Overall, *TMPO-AS1* maintains the expression of proliferation-related factors *MCM6*³⁸ and *MAD2L1*³⁹ by modulating both estrogen and E2F signaling pathways in breast cancer. Consistently, *TMPO-AS1* overexpression promoted MDA-MB-231 cell proliferation and migration, further supporting our findings that *TMPO-AS1* regulates TNBC tumorigenesis.

Intriguingly, we found that *TMPO-AS1* knockdown downregulated its sense gene *TMPO*. A lung cancer study showed that *TMPO-AS1* contributes to cancer proliferation by regulating *TMPO* expression.³⁷ A nuclear membrane-associated protein,⁴⁰ *TMPO*, or *LAP2 α* , has been reported to be associated with Hutchinson-Gilford progeria⁴¹ through binding telomeres. A cultured cell study suggested that *TMPO* could be a therapeutic target for glioblastoma;⁴² future studies will reveal the precise molecular mechanism of *TMPO* in tumorigenesis.

The present study showed that *TMPO-AS1* is also associated with the TGF- β signaling pathway. The TGF- β signaling pathway plays an essential role in the development of metastatic phenotype, including cell-cell interactions and angiogenesis.³³ Transforming growth factor- β is a cytokine and basically activates its downstream Smad signaling pathway through interaction with TGF- β receptors.³³ Decreased expression of TGF- β receptor by si*TMPO-AS1* could attenuate TGF- β reactivity and signaling, leading to the suppression of breast cancer cell proliferation in mouse models of metastatic tumors. The contribution of TGF- β signaling in breast cancer metastasis has been previously reported as the overexpression of dominant-negative mutant *TGFBR2* in MDA-MB-231 cells or other breast

cancer cells that suppresses metastatic phenotype or proliferation of these cells.^{43,44}

In the present study, we identified new *TMPO-AS1* target genes and pathways in TNBC cells. In lung cancer, *TMPO-AS1* stabilizes *TMPO* RNA and promotes cell proliferation.²⁷ We have shown that *TMPO-AS1* stabilizes *ESR1* mRNA through binding to the 3'-UTR of *ESR1* mRNA in ER-positive breast cancer.¹⁷ In TNBC, it is likely that *TMPO-AS1* is associated with RNA stability or the decay process; however, further study is required to clarify the precise functions of *TMPO-AS1* in the disease.

Our findings indicate that the inhibition of *TMPO-AS1* could be a useful therapeutic option for TNBC patients, as *TMPO-AS1* targets both the proliferative E2F and metastatic TGF- β pathways. We used recent powerful DDS technologies, cRGD-conjugated PIC/m and nanoball, to improve siRNA efficacy through i.v. injection targeting in vivo tumor growth of TNBC cells. Polyion complex micelle-si*TMPO-AS1* significantly suppressed tumor formation in the s.c. tumor model. The present PIC/m was designed to possess cRGD ligands on its surface, which specifically bind to $\alpha_v\beta_3$ integrin, for enhanced cancer cell-binding and accelerated endocytosis.^{30,31,35} Of note, MDA-MB-231 cells are reported to express high levels of $\alpha_v\beta_3$ integrin on their cell surface.⁴⁵ Thus, the significant tumor growth inhibition by PIC/m-si*TMPO-AS1* is probably dependent on higher accumulation of si*TMPO-AS1*, which efficiently represses *TMPO-AS1* expression in MDA-MB-231 cells.

Moreover, lung-selective nanoball containing si*TMPO-AS1* notably suppresses tumor formation in a lung metastatic tumor model. Nanoball was able to deliver siRNA into cancer cells through a specific

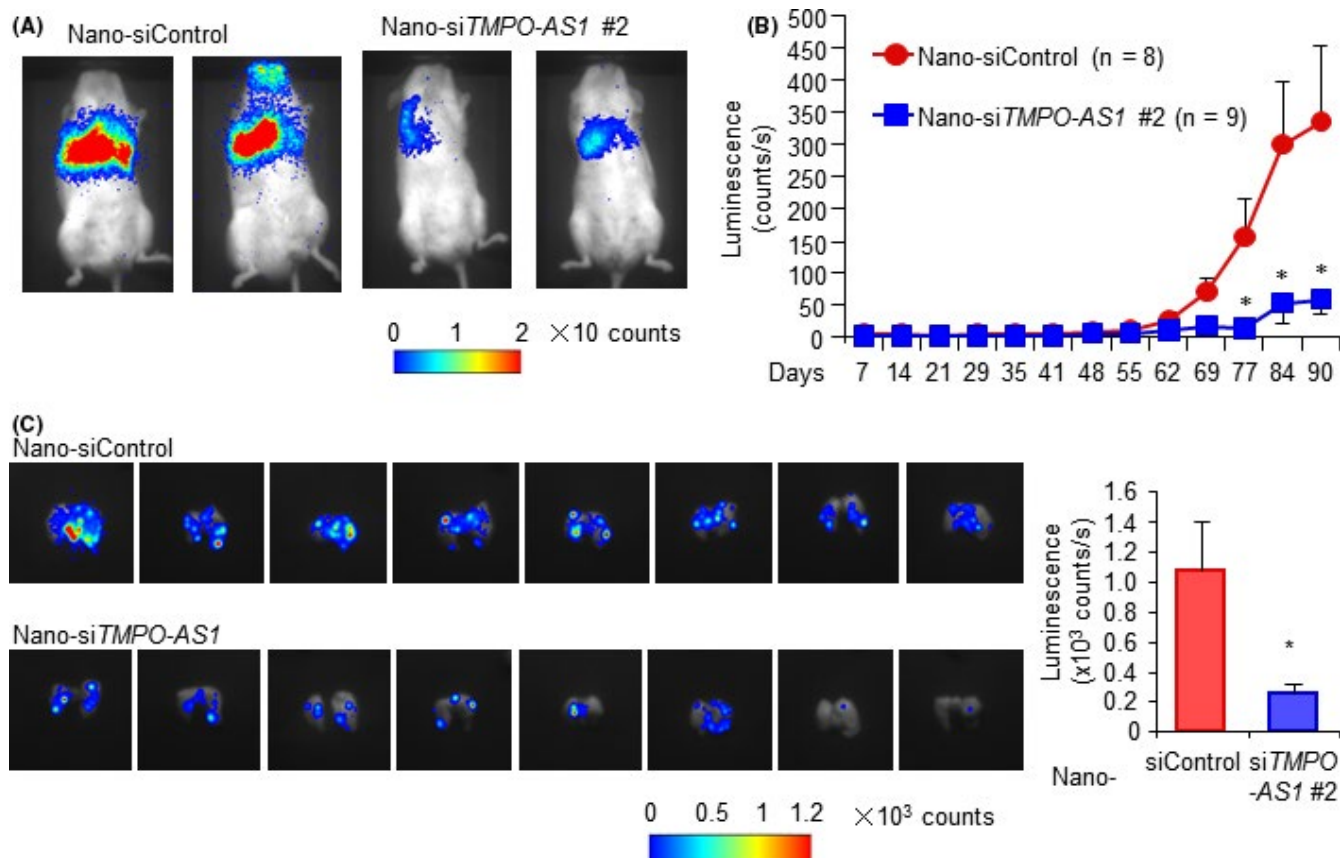


FIGURE 6 Lung delivery of drug delivery system-siTmPO-AS1 suppresses metastatic tumor growth of triple-negative breast cancer. A, B, Progression of MDA-MB-231-derived xenograft tumors treated with nanoball-conjugated siRNAs (Nano-siRNA) in nude mice. Nano-negative control siRNA (siControl) or nano-siTmPO-AS1 #2 were i.v. injected to mice inoculated with MDA-MB-231 cells through tail veins twice a week (Nano-siControl, $n = 8$; Nano-siTmPO-AS1 #2, $n = 9$). Representative photographs of xenografted mice with fluorescence are shown (A). Tumor volumes are measured with fluorescence and presented as mean \pm SE (B). C, MDA-MB-231 cells were measured by fluorescence in dissected lung tissues. Data are presented as mean fold change \pm SD vs Nano-siControl (Nano-siControl, $n = 8$; Nano-siTmPO-AS1 #2, $n = 9$). * $P < .05$; ** $P < .01$; *** $P < .001$

pathway regardless of its anionic charge.³² Also, Nanoball shows no hematotoxicity or cytotoxicity, unlike in cationic commercial vectors, because of their anionic surface charge. Dendrigrift poly-L-lysine consists entirely of lysine, including its central core, and is completely biodegradable, water-soluble, thermally stable, and nonimmunogenic.^{32,46} Synthesized γ -PGA is a biodegradable polymer that causes no inflammatory or immune reactions.⁴⁷ Thus, early clinical use of nanoball is expected not only because of efficacy and safety but also biocompatibility. These results suggest that these DDS technologies are useful for exhibiting *in vivo* gene silencing activity of siTmPO-AS1 in xenograft tumor models, and could be clinically applied to cancer management.

In conclusion, TmPO-AS1 plays important roles in the proliferation and migration of TNBC cells, and could be considered as a candidate for molecular targeting therapy in TNBC patients.

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DISCLOSURE

The authors have declared that no competing interests exist.

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REFERENCES

- Ghoncheh M, Pournamdar Z, Salehiniya H. Incidence and mortality and epidemiology of breast cancer in the world. *Asian Pac J Cancer Prev*. 2016;17:43-46.

2. Sharma GN, Dave R, Sanadya J, Sharma P, Sharma KK. Various types and management of breast cancer: an overview. *J Adv Pharm Technol Res*. 2010;1:109-126.
3. Waks AG, Winer EP. Breast cancer treatment: a review. *JAMA*. 2019;321:288-300.
4. Zwart W, Terra H, Linn SC, Schagen SB. Cognitive effects of endocrine therapy for breast cancer: keep calm and carry on? *Nat Rev Clin Oncol*. 2015;12:597-606.
5. Bilous M, Dowsett M, Hanna W, et al. Current perspectives on HER2 testing: a review of national testing guidelines. *Mod Pathol*. 2003;16:173-182.
6. Carey L, Winer E, Viale G, Cameron D, Gianni L. Triple-negative breast cancer: disease entity or title of convenience? *Nat Rev Clin Oncol*. 2010;7:683-692.
7. Bianchini G, Balko JM, Mayer IA, Sanders ME, Gianni L. Triple-negative breast cancer: challenges and opportunities of a heterogeneous disease. *Nat Rev Clin Oncol*. 2016;13:674-690.
8. Kotzin JJ, Spencer SP, McCright SJ, et al. The long non-coding RNA Morrbid regulates Bim and short-lived myeloid cell lifespan. *Nature*. 2016;537:239-243.
9. Faghihi MA, Modarresi F, Khalil AM, et al. Expression of a non-coding RNA is elevated in Alzheimer's disease and drives rapid feed-forward regulation of beta-secretase. *Nat Med*. 2008;14:723-730.
10. Mitobe Y, Takayama KI, Horie-Inoue K, Inoue S. Prostate cancer-associated lncRNAs. *Cancer Lett*. 2018;418:159-166.
11. Kopp F, Mendell JT. Functional Classification and Experimental Dissection of Long Noncoding RNAs. *Cell*. 2018;172:393-407.
12. Wang KC, Chang HY. Molecular mechanisms of long noncoding RNAs. *Mol Cell*. 2011;43:904-914.
13. Schmitt AM, Chang HY. Long noncoding RNAs in cancer pathways. *Cancer Cell*. 2016;29:452-463.
14. Lin A, Li C, Xing Z, et al. The LINK-A lncRNA activates normoxic HIF1 α signalling in triple-negative breast cancer. *Nat Cell Biol*. 2016;18:213-224.
15. Hu Q, Ye Y, Chan L-C, et al. Oncogenic lncRNA downregulates cancer cell antigen presentation and intrinsic tumor suppression. *Nat Immunol*. 2019;20:835-851.
16. Li C, Wang S, Xing Z, et al. A ROR1-HER3-lncRNA signalling axis modulates the Hippo-YAP pathway to regulate bone metastasis. *Nat Cell Biol*. 2017;19:106-119.
17. Mitobe Y, Ikeda K, Suzuki T, et al. ESR1-stabilizing long noncoding RNA TMPO-AS1 promotes hormone-refractory breast cancer progression. *Mol Cell Biol*. 2019;39:e00261-19.
18. Parise P, Finocchiaro G, Masciadri B, et al. Lap2alpha expression is controlled by E2F and deregulated in various human tumors. *Cell Cycle*. 2006;5:1331-1341.
19. Devi GR. siRNA-based approaches in cancer therapy. *Cancer Gene Ther*. 2006;13:819-829.
20. Singh A, Trivedi P, Jain NK. Advances in siRNA delivery in cancer therapy. *Artif Cells Nanomed Biotechnol*. 2018;46:274-283.
21. Barata P, Sood AK, Hong DS. RNA-targeted therapeutics in cancer clinical trials: Current status and future directions. *Cancer Treat Rev*. 2016;50:35-47.
22. Cabral H, Miyata K, Osada K, Kataoka K. Block copolymer micelles in nanomedicine applications. *Chem Rev*. 2018;118:6844-6892.
23. Tominaga K, Shimamura T, Kimura N, et al. Addiction to the IGF2-ID1-IGF2 circuit for maintenance of the breast cancer stem-like cells. *Oncogene*. 2017;36:1276-1286.
24. Tominaga K, Minato H, Murayama T, et al. Semaphorin signaling via MICAL3 induces symmetric cell division to expand breast cancer stem-like cells. *Proc Natl Acad Sci USA*. 2019;116:625-630.
25. The Cancer Genome Atlas Network. Comprehensive molecular portraits of human breast tumours. *Nature*. 2012;490:61-70.
26. Li J, Han L, Roebuck P, et al. TANRIC: an interactive open platform to explore the function of lncRNAs in cancer. *Cancer Res*. 2015;75:3728-3737.
27. Cerami E, Gao J, Dogrusoz U, et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer Discov*. 2012;2:401-404.
28. Gao J, Aksoy BA, Dogrusoz U, et al. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. *Sci Signal*. 2013;6:pl1.
29. Huang DW, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res*. 2009;37:1-13.
30. Christie RJ, Matsumoto YU, Miyata K, et al. Targeted polymeric micelles for siRNA treatment of experimental cancer by intravenous injection. *ACS Nano*. 2012;6:5174-5189.
31. Nishida H, Matsumoto Y, Kawana K, et al. Systemic delivery of siRNA by actively targeted polyion complex micelles for silencing the E6 and E7 human papillomavirus oncogenes. *J Control Release*. 2016;231:29-37.
32. Kodama Y, Kuramoto H, Mieda Y, et al. Application of biodegradable dendrigraft poly-L-lysine to a small interfering RNA delivery system. *J Drug Target*. 2017;25:49-57.
33. Massagué J. TGF β signalling in context. *Nat Rev Mol Cell Biol*. 2012;13:616-630.
34. Bellomo C, Caja L, Moustakas A. Transforming growth factor β as regulator of cancer stemness and metastasis. *Br J Cancer*. 2016;115:761-769.
35. Katsushima K, Natsume A, Ohka F, et al. Targeting the Notch-regulated non-coding RNA TUG1 for glioma treatment. *Nat Commun*. 2016;7:13616.
36. Huang W, Su X, Yan W, et al. Overexpression of AR-regulated lncRNA TMPO-AS1 correlates with tumor progression and poor prognosis in prostate cancer. *Prostate*. 2018;78:1248-1261.
37. Qin Z, Zheng X, Fang Y. Long noncoding RNA TMPO-AS1 promotes progression of non-small cell lung cancer through regulating its natural antisense transcript TMPO. *Biochem Biophys Res Commun*. 2019;516:486-493.
38. Issac MSM, Yousef E, Tahir MR, Gaboury LA. MCM2, MCM4, and MCM6 in breast cancer: clinical utility in diagnosis and prognosis. *Neoplasia*. 2019;21:1015-1035.
39. Yuan B, Xu Y, Woo JH, et al. Increased expression of mitotic checkpoint genes in breast cancer cells with chromosomal instability. *Clin Cancer Res*. 2006;12:405-410.
40. Dechat T, Vlcek S, Foisner R. Review: lamina-associated polypeptide 2 isoforms and related proteins in cell cycle-dependent nuclear structure dynamics. *J Struct Biol*. 2000;129:335-345.
41. Chojnowski A, Ong PF, Wong ESM, et al. Progerin reduces LAP2 α -telomere association in Hutchinson-Gilford progeria. *Elife*. 2015;4:e07759.
42. Zhang L, Wang G, Chen S, et al. Depletion of thymopoietin inhibits proliferation and induces cell cycle arrest/apoptosis in glioblastoma cells. *World J Surg Oncol*. 2016;14:267.
43. Yin JJ, Selander K, Chirgwin JM, et al. TGF-beta signaling blockade inhibits PTHrP secretion by breast cancer cells and bone metastases development. *J Clin Invest*. 1999;103:197-206.
44. Tang B, Vu M, Booker T, et al. TGF-beta switches from tumor suppressor to prometastatic factor in a model of breast cancer progression. *J Clin Invest*. 2003;112:1116-1124.
45. Bauer K, Mierke C, Behrens J. Expression profiling reveals genes associated with transendothelial migration of tumor cells: a functional role for α v β 3 integrin. *Int J Cancer*. 2007;121:1910-1918.
46. Kodama Y, Nakamura T, Kurosaki T, et al. Biodegradable nanoparticles composed of dendrigraft poly-L-lysine for gene delivery. *Eur J Pharm Biopharm*. 2014;87:472-479.

47. Ye H, Jin L, Hu R, et al. Poly(γ -L-glutamic acid)-cisplatin conjugate effectively inhibits human breast tumor xenografted in nude mice. *Biomaterials*. 2006;27:5958-5965.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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