# Transmembrane 7 superfamily member 4 regulates cell cycle progression in breast cancer cells

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**Abstract.** – OBJECTIVE: TM7SF4 (transmembrane 7 superfamily member 4) gene encodes a seven-pass transmembrane protein that is primarily expressed in dendritic cells called as dendritic cell-specific expressed seven transmembrane protein (DC-STAMP). This protein regulates immunological functions, osteoclastogenesis and myeloid differentiation. Although the roles of TM7SF4 have been currently studied on Paget's disease of bone and papillary thyroid cancers, it is unclear whether TM7SF4 plays a role in breast cancer. In current study, we investigated the expression of TM7SF4 in human breast cancer cell lines.

MATERIALS AND METHODS: In this study, five breast cancer lines were cultured. Small hairpin RNA against TM7SF4 using a lentiviral vector was generated and transfected into MCF-7 breast cancer cells. Effects of down-regulating TM7SF4 in transfected cells were examined by Western blot, RT-PCR, apoptotic rate, colony formation, and cell cycle analyses.

RESULTS: The results demonstrated that down-regulation of TM7SF4 led to a decrease in colony formation in MCF-7 cells compared to the control group. This is likely due to a decrease in proliferation and cell cycle and an increase in apoptosis.

CONCLUSIONS: To our knowledge, our data demonstrate for the first time that TM7SF4 plays an essential role in regulating cell cycle progression in breast cancer.

Key Words:
  TM7SF4, DC-STAMP, Breast cancer, Cell proliferation.

## Abbreviations

DC-STAMP = dendritic cell-specific expressed seven-membrane protein; GFP = green fluorescent protein; DMEM = Dulbecco's Modified Eagle Medium; PVDF = polyvinylidene fluoride; ECL = electrochemiluminescence; PBS = phosphate buffered saline; GAPDH = glyceraldehyde 3-phosphate dehydrogenase.

## Introduction

A novel 470-amino acid protein, transmembrane 7 superfamily member 4 (TM7SF4), was initially identified in dendritic cells (DC). TM7SF4 is also referred to as DC specific transmembrane protein (DC-STAMP) and its encoded gene is localized on chromosome 8q23[1,2]. While a previous study using a TM7SF4-GFP fusion protein in human embryonic kidney 293 cells showed that TM7SF4 is localized at the cell membrane with an intracellular carboxyl terminus[3], the protein has no sequence homology with any other proteins or multimembrane-spanning receptors. In addition, TM7SF4 in humans and mice resides in the endoplasmic reticulum[3]. These studies suggested that DC-STAMP may be localized in different compartments.

In macrophages and osteoclasts, expression of TM7SF4 is up-regulated in response to interleukin 4 (IL-4)[4-5]. Receptor activator of nuclear factor kappa-B ligand (RANKL) also facilitates the overexpression of TM7SF4, which results in enhanced osteoclastogenesis. Furthermore, TM7SF4 directly induces the expression of an osteoclast marker tartrate-resistant acid phosphatase and is required for cell to cell fusion of osteoclasts and foreign body giant cells[6]. These data demonstrated that TM7SF4 plays a critical role in osteoclastogenesis.

Recently, TM7SF4 was also found to be overexpressed in papillary thyroid carcinoma (PTC) harboring the V600E BRAF mutation[7]. TM7SF4 was one of 96 genes whose expression levels were increased greater than 2-fold in PTC tissues compared to paired normal tissues[8]. In addition, TM7SF4 is one of risk factors for Paget's disease of bone (PDB), as characterized by a genome wide association study[9]. The association with PDB was further confirmed in a study including seven inde-

pendent populations with odds ratio of 1.40 ($p = 7.38 \times 10^{-17}$)[10]. Taken together, these studies provide new insights into potential roles of TM7SF4 in physiological and pathological conditions.

Breast cancer is the most common neoplasia in women, with an estimated 226,000 new cases in American women each year[11,12]. Over the past decade, several improvements in understanding the molecular pathology of breast cancer have been achieved. However, due to the complexity of the disease, including genomic and histological subtypes, the molecular mechanisms underlying this malignancy are largely unclear. In this report, we examined the expression of TM7SF4 in human breast cancer cell lines and studied its role in regulating cell cycle. We found that TM7SF4 was predominantly expressed in human breast cancer cells, and down-regulation of TM7SF4 led to an increase in apoptosis accompanied by a decrease in proliferation. These results suggest that TM7SF4 may play a critical role in breast cancer progression.

## Materials and Methods

### Cell Culture

The human breast cancer cell lines SKBR-3, MCF-7, T-47D, HCC-1937, and MDA-MB-231 were obtained from the cell bank at Chinese Academy of Sciences and maintained in DMEM complete medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (GIBCO), 2 mM glutamine, 100 U/mL penicillin and 100 □g/mL streptomycin at 37°C in an incubator with 5% $CO_2$. The medium was changed every 3 days.

### Semi-quantitative RT-PCR

Total RNA from human breast cancer cell lines was extracted using Trizol reagent (Invitrogen Inc., Carsbad, CA, USA) according to the manufacturer's instructions. Subsequently, 2 □g of total RNA was reverse transcribed to single-stranded cDNA. One □L of cDNA was used as template for the PCR reaction. The primers used for TM7SF4 was 5'-TCCAGCATTTGGGAGTTTG-3' and 5'-AGCATTCCTGCCTTCACG-3' to get product of 203 bp, for GAPDH 5'-TGACTTCAACAGCGACACCCA-3' and 5'-CACCCTGTTGCTGTAGCCAAA-3' of product size 121 bp. The semi-quantitative RT-PCR comprised an initial denaturation at 95°C for 15s, then 22 cycles at 95°C for 5s and 60°C for 30s. The PCR products were separated on 2% agarose gel.

### Generation of Small Hairpin RNA Against TM7SF4 Using a Lentiviral Vector

Lentiviral vector pGCL-GFP was purchased from GeneChem (Shanghai, China) and used to express small hairpin RNAs (shRNA) targeting the TM7SF4 ORF sequence (Genbank No. NM_030788) (TM7SF4-shRNA). A non-targeting sequence was used as a negative control and was purchased from GeneChem (Shanghai, China). The sequence of shRNA is 5'-CTGTTGTC-CTCTATCCTTA-3' and the sequences were cloned into the pGCL-GFP to generate the lentiviral vectors expressing control shRNA and shRNA against TM7SF4. Human embryonic kidney 293T cells were infected with lentivirus expressing TM7SF4-shRNA and control shRNA. The knockdown efficiency of TM7SF4 protein expression was detected by Western blot analysis.

### Western Blotting

MCF-7 cells were harvested in RIPA buffer supplemented with protease and phosphatase inhibitor cocktails. Protein lysates were separated by SDS-PAGE, transferred onto PVDF membranes, and incubated with the following antibodies: anti-TM7SF4 1:4000 (Novus International Inc., Saint Charles, MO, USA), anti-GAPDH 1:5000 (Santa-Cruz Biotechnology, Santa-Cruz, CA, USA). Secondary antibodies conjugated to horseradish peroxidase and ECL Western blotting reagents were used for detection.

### MCF-7 Cells were Infected with TM7SF4-shRNA Lentivirus

Human breast cancer MCF-7 cells were infected with TM7SF4-shRNA lentivirus and control lentivirus. Uninfected cells were also included as a control. GFP expression was examined by fluorescent microscopy three days post infection to ensure infection efficiency. After five days of infection, cells were harvested to determine knockdown efficiency by real-time quantitative PCR.

### Cell Growth Analysis

Cell growth was measured by multi-parametric high-content screening (HCS). Cell number was analyzed every day for up to 5 days following transfection using Cellomics (Thermo, Pittsburg, PA, USA). In short, human breast cancer MCF-7 cells 10 days after being infected with either control lentivirus or TM7SF4-shRNA lentivirus were seeded in 96-well plates (2000 cells per well). Cells were, then, incubated at 37°C with 5% $CO_2$ for five days. Plates were

processed with the ArrayScan™ HCS software (Cellomics Inc., Pittsburg, PA, USA) and kept at cool temperature of 4°C for up to 24h before analysis on each day. The system is an automated, computerized fluorescence-imaging microscope that can automatically identify stained cells and record the intensity and distribution of fluorescence in each individual cell. Images were acquired for each fluorescence channel, using suitable filters and 20× objective. In each well, at least 800 cells were analyzed.

### Flow Cytometric Analysis of Cell Cycle Distribution

Cell cycle distribution of MCF-7 cells post-infection was determined by flow cytometry using a propidium iodide (PI) detection kit (Sigma, St. Louis, MO, USA), according to the manufacturer's recommendation. MCF-7 cells ($1×10^5$ cells) were washed twice in PBS, trypsinized, and collected. The cell pellets were resuspended in 100 $\mu$L of binding buffer followed by the addition of 2 mg/mL PI staining solution. The cells were analyzed in the FACS Calibur cytometer equipped with Cell Quest Pro software (BD Biosciences, Franklin Lakes, NJ, USA).

### Detection of Apoptosis by Flow Cytometry

Phosphatidylserine externalization was detected by flow cytometry using an Annexin V-APC apoptosis detection kit (eBioscience, Franklin Lakes, NJ, USA), according to the manufacturer's instruction. MCF-7 cells ($1 \times 10^5$ cells) were washed twice in phosphate buffered saline (PBS), trypsinized, and collected. The cell pellets were resuspended in 100 $\mu$L of binding buffer followed by the addition of 5 $\mu$L of Annexin V-APC for 10 min at room temperature in the dark. The cells were analyzed in the FACS Calibur cytometer equipped with Cell Quest Pro software (BD Biosciences, Franklin Lakes, NJ, USA).

### Colony Formation Analysis

Lentivirus-infected MCF-7 cells in the logarithmic phase were harvested by pancreatic enzyme digestion. Eight hundred cells were seeded in each well of 6-cell plates, with each group having at least three representative wells. Cells were cultured for 14 days. The medium was changed every 3 days. Cells were observed by fluorescence microscope before the end of the experiment. Cells were washed with PBS, followed by fixation by 1 ml of paraformaldehyde per cell for 30-60 min.

Subsequently, 500 $\mu$L of GIEMSA was added into each cell. Cells were washed with water several times and cell colonies were observed by microscope. Clones that consisted of greater than 50 cells were counted.

### Statistical Analysis

Statistical analyses were performed with SPSS 17.0 (SPSS Inc., Chicago, IL, USA). The data were expressed as mean ± SD and statistical analyses were performed with multivariate analysis of variance. A value of $p < 0.05$ was reported as statistically significant.

## Results

### TM7SF4 is Differentially Expressed in Breast Cancer Cell Lines

We analyzed expression levels of TM7SF4 mRNA in five breast cancer cell lines (SKBR-3, MCF-7, T-47D, HCC1937, MDA-MB-231) by semi-quantitative RT-PCR analysis. Representative results are shown in Figure 1A. The amount of each cDNA product was normalized to GAPDH (Figure 1B). The results revealed that TM7SF4 was expressed in SKBR-3, MCF-7, T-47D and HCC1937 cells, but not in MDA-MB-231 cells. Consistent with RNA expression analysis, expression levels of TM7SF4 protein in four breast cancer cell lines (SKBR-3, MCF-7, T-47D, HCC1937) were markedly higher as
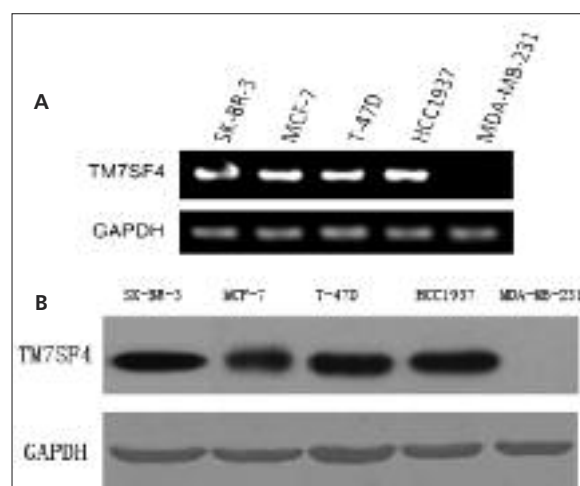


**Figure 1.** *A,* Expression of TM7SF4 mRNA in the five breast cancer cell lines measured by semi-quantitative RT-PCR. The house keeping gene GAPDH was used as an internal control. *B,* Expression of TM7SF4 protein showed in the five breast cancer cell lines.

compared to MDA-MB-231 (Figure 1C). We chose the MCF-7 cell line that stably expressed TM7SF4 for further study.

## Downregulation of TM7SF4 Expression

To test short hairpin RNA (shRNA)-mediated down-regulation of TM7SF4 expression, human breast cancer cells MCF-7, which have high transduction efficiency, were infected with lentivirus expressing control shRNA or TM7SF4-shRNA. As shown in Figure 2, TM7SF4 protein level, detected by Western blot, was greatly reduced in TM7SF4-shRNA infected MCF-7cells, indicating effective knockdown of the target sequence ($p < 0.005$).

## Downregulation of TM7SF4 in MCF-7 Cells Results in Decreases in Cell Growth and Colony-Forming Capability

To explore role of TM7SF4, we further investigated whether TM7SF4 regulates cell growth. Cell numbers and colony forming ability of TMFSF4-shRNA infected MCF-7 cells were ex-
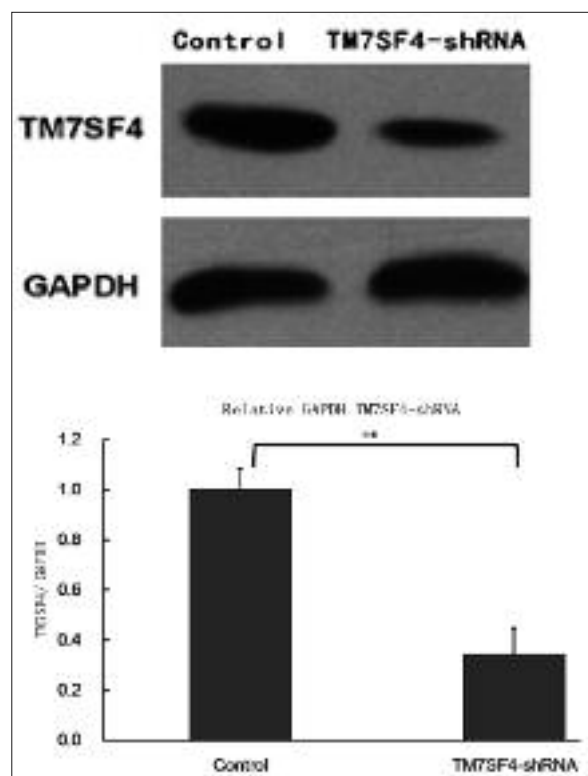
**Table I.** Cell numbers counted for 5 days after infected with TM7SF4-shRNA lentivirus and control lentivirus. There was statistical significance between two groups with $p < 0.05$.

| AV | Control | TM7SF4-shRNA |
|---|---|---|
| Day 1 | 382.8 ± 38.0 | 23.09 ± 37.7 |
| Day 2 | 1115.0 ± 155.9 | 508.4 ± 52.8 |
| Day 3 | 1426.2 ± 192.4 | 282.2 ± 84.0 |
| Day 4 | 2468.4 ± 232.6 | 308.0 ± 167.1 |
| Day 5 | 2903.0 ± 165.7 | 131.6 ± 82.6 |

amined during five consecutive days (Table I). Cell growth rate monitored by high-content screening (HCS) was defined as cell count of $n^{th}$ day/cell count, where n = 1, 2, 3, 4 and 5. Figure 3A, 3B, and Table I showed that TM7SF4-shRNA infected MCF-7 cells had significantly decreased cell numbers as compared to control cells. The results of the study show that cell growth was significantly inhibited over the course of five days.

Down-regulation of TM7SF4 also resulted in a marked reduction in colony formation in MCF-7 cells (Table II and Figure 3C and D, control cells: 30 ± 1; TM7SF4-shRNA cells; 11 ± 3, $p = 0.004$).

## Downregulation of TM7SF4 Results in a Change in Cell Cycle Progression and Apoptosis in MCF-7 Cells

Because TM7SF4-shRNA infected MCF-7 cells grew slower and formed less colonies than control cells, we hypothesized that TM7SF4 may regulate cell cycle progression. Indeed, while the negative control group displayed a normal cell cycle distribution: (G0/G1 57.06%, S 30.11%, G2/M12.3%), the TM7SF4-shRNA group displayed aberrant cell cycle progression (G1 61.87%, S 20.31%, G2/M 17.83%) as the G0/G1 and G2/M phases of TM7SF4-shRNA infected MCF-7 cells were significantly increased, and the S phase was decreased ($p = 0.0004$) (Figure



**Figure 2.** TM7SF4 expression in breast cancer cells MCF-7 was measured by Western blot. Compared with control, the expression level of TM7SF4-shRNA protein in MCF-7 cells was markedly decreased. GAPDH is used a loading control.

**Table II.** Colony numbers of MCF-7 cells expressing control shRNA and TM7SF4-shRNA.

| Group | Control | TM7SF4-shRNA |
|---|---|---|
| Numbers of | 29.0 | 9.0 |
| colony numbers | 31.0 | 14.0 |
| | 30.0 | 9.0 |

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4A and 4B). Taken together, these data suggest that TM7SF4 regulates cell growth and blocks cell cycle progression.

To test whether TM7SF4 regulated apoptosis in MCF-7 cells, apoptosis of MCF-7 cell expressing TM7SF4 shRNA was determined by Annexin V staining followed by flow cytometry (Figure 4C).

As shown in Figure 4D, cell apoptosis was significantly increased in the TM7SF4-shRNA cells compared to the negative control cells (control $5.82 \pm 0.23\%$; TM7SF4 -shRNA $9.84 \pm 0.57\%$, $p = 0.0025$). These results demonstrated that TM7SF4 plays an important role in regulation of cell cycle and apoptosis in MCF-7 cells.
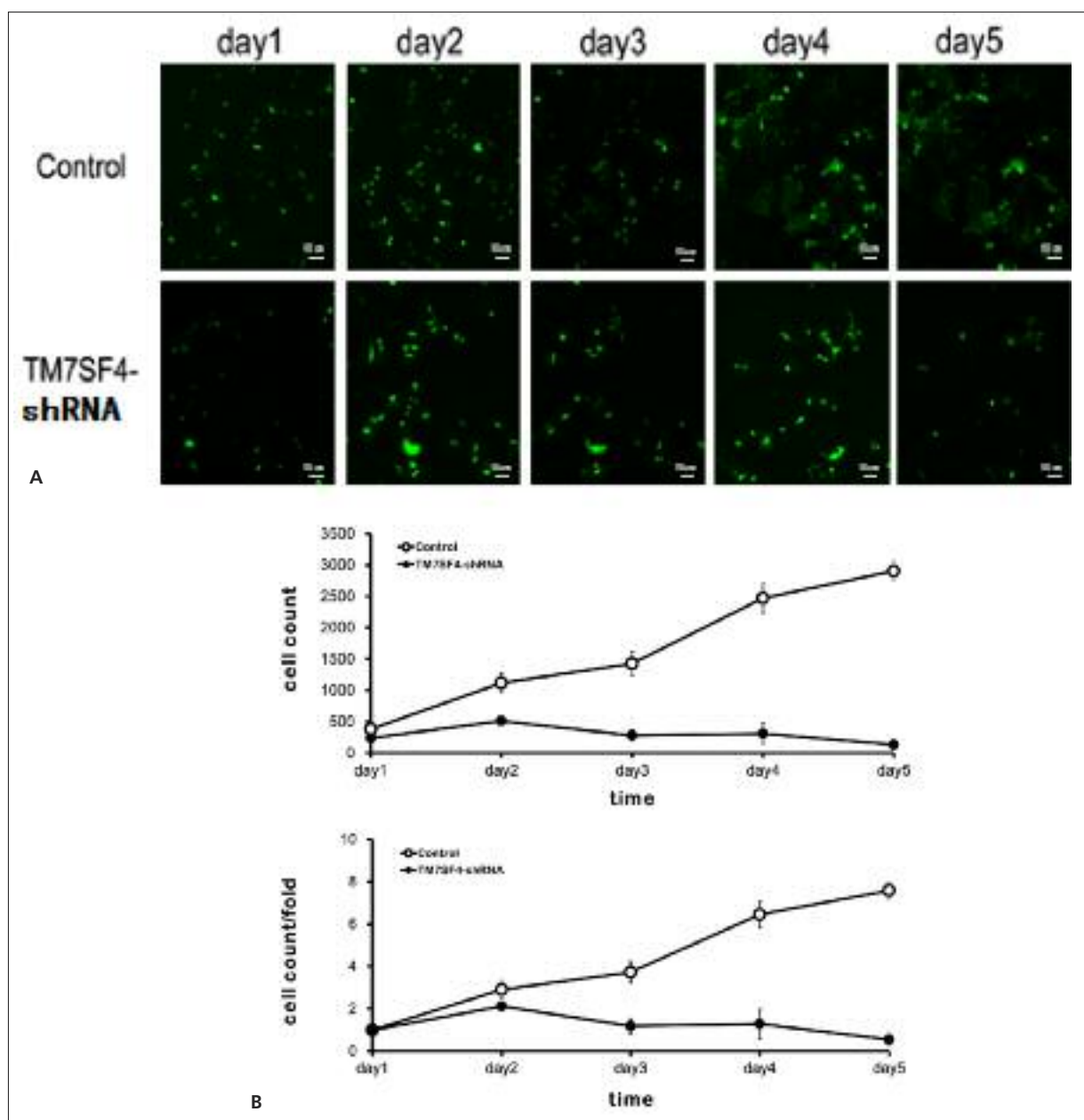


**Figure 3. A,** Analysis of cell numbers in MCF-7 cells transfected with control shRNA and TM7SF4-shRNA. Representative images of cells for both control group and TM7SF4-shRNA group during 5 days in culture. **B,** Fewer TM7SF4-shRNA transfected MCF-7 cells grew than control cells during 5 days in culture. Quantitative data of cells/well or ratio of relative cell number normalized to day 1 were shown. MCF-7 cells transfected with TM7SF4-shRNA showed a significant decrease in cell number ($p < 0.0001$, multivariate analysis of variance).
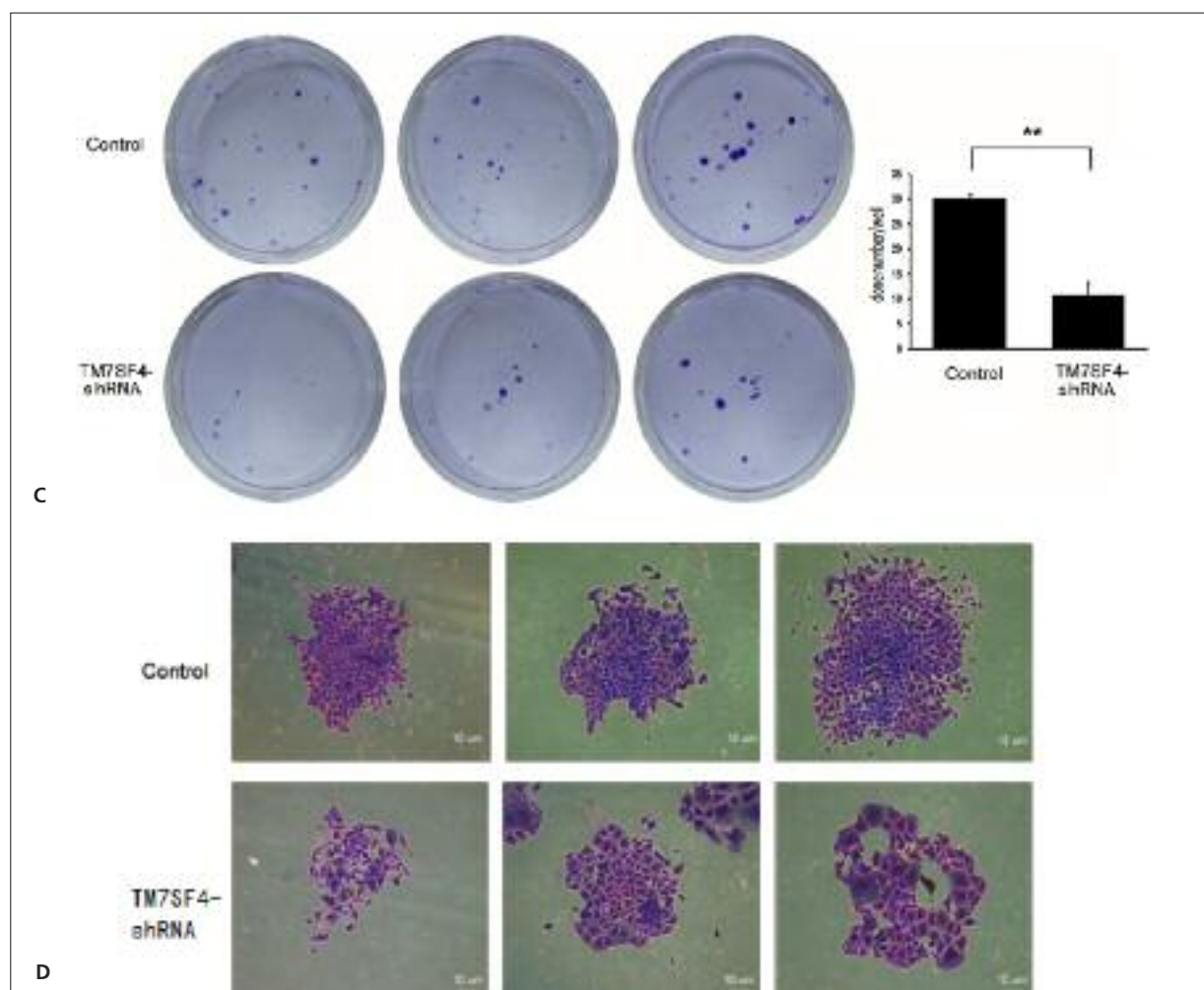
**Figure 3 (Continued). C,** and **D,** Analysis of colony formation in MCF-7 cells transfected with control shRNA and TM7SF4-shRNA. Down-regulation of TM7SF4 in MCF-7 cells resulted in a marked decrease in the number of colonies ($p$ = 0.004). The numbers of TM7SF4-shRNA transfected MCF-7 cells in single colony were also less than those in control cells.

## Discussion

Breast cancer is the leading cause of cancer-related mortality in women, and is the most common cancer diagnosed among women in the United States. The American Cancer Society has provided an overview of female breast cancer statistics in the USA, including data on incidence, mortality, survival, and screening. Approximately 232,340 new cases of invasive breast cancer and 39,620 breast cancer deaths are expected to occur among US women in 2013. One in 8 women in the USA will develop breast cancer in her lifetime[12]. In spite of the high frequency, however, more than 90% of the breast cancer patients will survive if cancer is detected at an early stage and if treatment is begun promptly. Therefore, early detection is extremely crucial for successful treatment and favorable prognosis.

Transmembrane 7 superfamily member 4 (TM7SF4) was initially discovered as dendritic cell-specific transmembrane protein (DC-STAMP), which appears to be preferentially expressed in myeloid DC[1-3,7]. In macrophages and osteoclasts, expression of DC-STAMP is up-regulated in response to interleukin 4 (IL-4)[5]. The TM7SF4 gene is required for fusion of osteoclast precursors and known to play a critical role in osteoclast differentiation and function. Some studies have demonstrated that TM7SF4 plays a key role in osteoclastogene-
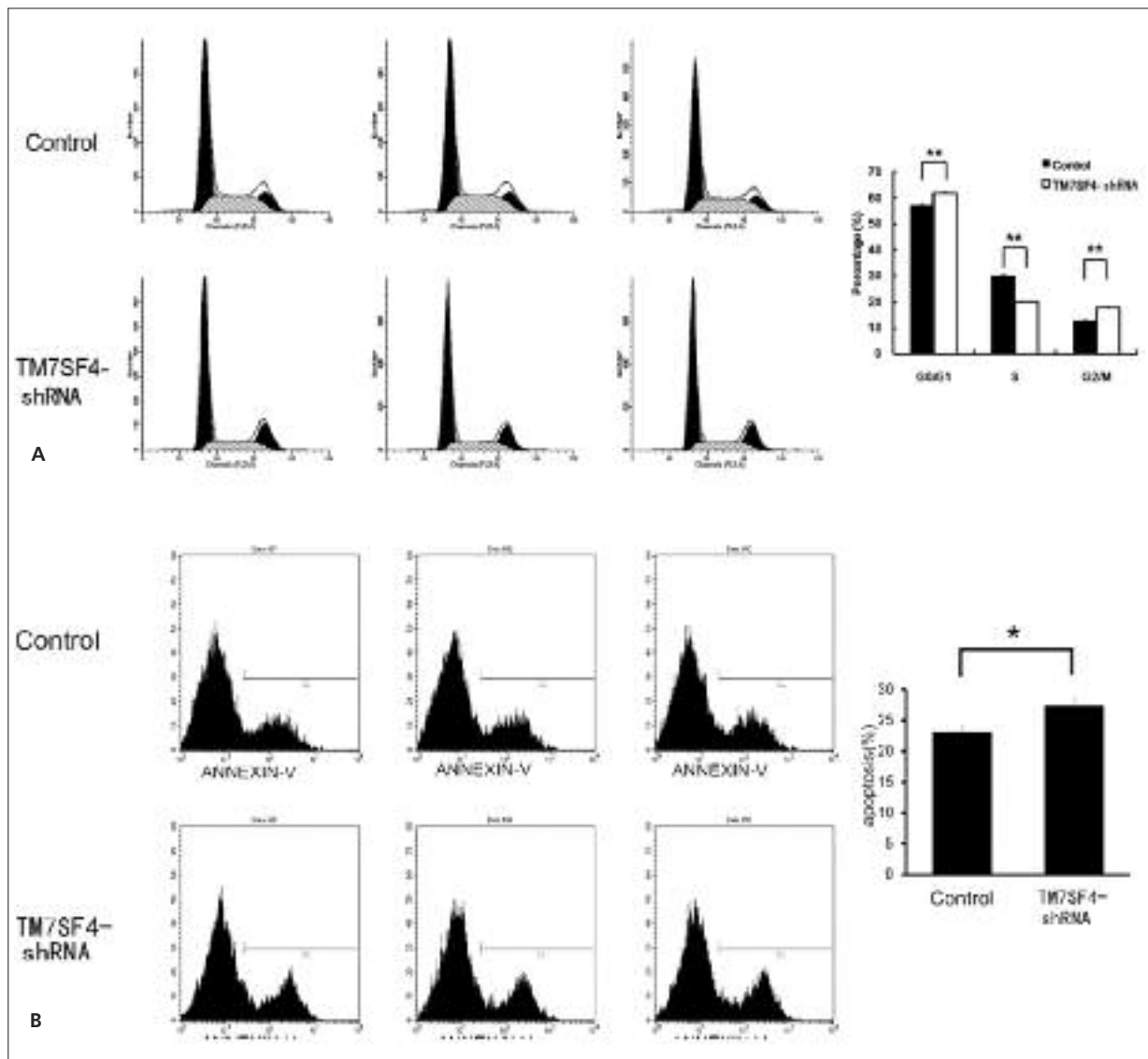
**Figure 4.** TM7SF4 regulates cell cycle and apoptosis in MCF-7 cells. *A,* (*Left*). The G1 and G2/M phases of TM7SF4-shRNA infected MCF-7 cells increased significantly while S phase decreased (PG0/G1 = 0.001, PS = 0.000, PG2/M = 0.001). *A,* (*Right*). Compared with control cells, TM7SF4-shRNA cells showed a significant decrease in S phase by flow cytometry (S, 20.31 ± 0.11, $p$ = 0.0004), while a distinct increase in G0/G1 and G2/M phases (G0/G1, 61.87 ± 0.27; G2/M, 17.83 ± 0.35). *B,* (*Left*). There was a significant increase in apoptosis observed in TM7SF4-shRNA infected MCF-7 cells compared to control cells ($p$ = 0.01). *B,* (*Right*). Percentage of apoptosis was plotted in cells expressing control shRNA and TM7SF4-shRNA. There was a greater amount of apoptosis in the TM7SF4 down-regulated group of human breast cancer MCF-7 cells ($p$ = 0.013, $p$ < 0.05).

sis[14]. Receptor activator of nuclear factor kappa-B ligand (RANKL) also facilitates overexpression of TM7SF4 which results in enhanced osteoclastogenesis[15]. Furthermore, TM7SF4 directly induces the expression of an osteoclast marker tartrate-resistant acid phosphatase and is required for cell–cell fusion of osteoclasts and foreign body giant cells[6].

TM7SF4 also plays an important role in other organs, in addition to bone. Hyo Young Kim et al[17], found that 39 genes were associated with hepatic biomarkers AST and ALT in a study involving 8,842 Korean individuals, and TM7SF4 gene was expressed at highest level in the liver. Recent research has shown that TM7SF4 is important and highly expressed in several tumors.

For example, it is evident that TM7SF4 plays a role in the pathogenesis of Paget's disease of bone and papillary thyroid cancer[8,9,14,15]. It has been shown that expression of TM7SF4 (20.22-fold), SLC34A2 (14.56-fold), KCNJ2 (5.69-fold), and KLK7 (7.01-fold), among other 96 genes, was increased more than 2-fold in papillary thyroid cancer (PTC) tissues compared with paired normal tissues[8]. Many studies have suggested that TM7SF4 plays a critical role in PTC progression. Interestingly, a study showed that TM7SF4 was a novel methylation biomarker candidate, among a group of new potential methylation biomarkers identified by microarray data analysis. While those molecules might help elucidate the underlying mechanisms of breast tumorigenesis, TM7SF4 may be suggestive of an important role in the progression of breast cancer[12]. However, the specific role of TM7SF4 in breast cancer remains unclear. In this report, we found that TM7SF4 protein was overexpressed in breast cancer cell lines except for MDA-MB-231 cells.

Down-regulation of TM7SF4 by RNA interference in MCF-7 cells led to an increase in numbers of cells in the G0/G1 phase (61.87 ± 0.27, $p = 0.001$) and G2/M phase (17.83 ± 0.35, $p = 0.0005$), and a decrease in number of cells in S phase (20.31 ± 0.11, $p = 0.0004$) (Figure 4A, 4B). It also resulted in a significant increase in apoptosis compared to normal control cells (control 5.82 ± 0.23%; TM7SF4-shRNA 9.84 ± 0.57%, $p = 0.0025$ (Figure 4C, 4D).

## Conclusions

These results suggested that TM7SF4 plays an important role in cell cycle regulation. To our best knowledge, this is the first study showing the potential role of TM7SF4 in breast cancer. Further studies are warranted to elucidate the mechanism by which TM7SF4 regulates cell cycle in breast cancer cells.

### Conflict of Interest

The Authors declare that there are no conflicts of interest.

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