Blocking TGF-β inhibits breast cancer cell invasiveness via ERK/S100A4 signal

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Abstract. – **OBJECTIVE:** Targeted down-regulation of TGF-β expression inhibits invasion and metastasis in breast cancer cells. However, the mechanism that TGF-β functions by remains largely unknown. In the present study we report the mechanism of ERK1/2 dependant S100A4 regulation by TGF-β and its possible role in TGF-β-mediated tumour invasion *in vitro*.

MATERIALS AND METHODS: Small interfering RNA targeting TGF-β1 (TGF-β1 siRNA) were stably transfected into the breast cancer cell line MDA231. The TGF-β1 siRNA/9MDA231 cells were then treated with TGF-β1 (5 ng/ml) or treated with PD98059 (25 μM) or transfected into S100A4 siRNA before TGF-β1 treatment. The cells were used in several *in vitro* analyses, including migration, invasion, angiogenesis, and signaling assays. A wound-healing assay was used to determine migration of the cells in culture and a Boyden chamber transwell assay was used for invasion. *In vitro* angiogenesis studies using conditioned medium in HMEC-1 cells.

RESULTS: Inhibition of TGF-β1 expression by TGF-β1 siRNA transfection in MDA231 cells showed significant decrease migration, invasion and angiogenesis *in vitro*. TGF-β1 siRNA/MDA231 cells treated with 5 ng/ml TGF-β1 for 24 hs restored the invasive ability of TGF-β1 siRNA/MDA231 cells. TGF-β1 treatment could not increase migration, invasion and angiogenesis in TGF-β1 siRNA/MDA231 cells when treated with 25 μM PD98059 or transfected with S100A4 siRNA before TGF-β1 treatment. Analysis of TGF-β1 signaling pathways showed a decrease in p-ERK1/2 activation and an decrease in S100A4 expression. Interestingly, TGF-β1 regulated S100A4 via ERK1/2 signalling.

CONCLUSIONS: Our findings showed that blocking TGF-β inhibits breast cancer cell invasiveness, migration and angiogenesis via ERK/S100A4 signalling. Therapies targeting the TGF-β signaling pathway may be more effective to prevent progression in breast cancer.

Key Words:
    Breast cancer, Invasion, Angiogenesis, TGF-β, ERK1/2, S100A4.

Introduction

Transforming growth factor (TGF)-β is involved in physiologic processes, such as wound healing, tissue development, and remodeling. TGF-β has also been implicated in many pathologic conditions, including cancer, and has been shown to regulate a number of events such as angiogenesis, immune suppression, and cell migration[1-4]. TGF-β was overexpressed in breast cancers[5]. Several models have shown correlations between TGF-β expression and increased tumorigenicity, increased invasion, and increased drug resistance[6-8]. In mouse models of breast cancer, TGF-β signaling has been shown to promote lung[9,10] and bone metastasis[9-11]. However, the mechanisms of TGF-β function is not clear.

It has proved that TGF-β first binds to TBRII, which recruits and activates TBRI[12]. The latter then activates Smad2/3. The activated Smad2/3 combines with Smad4 and migrates to the nucleus to regulate transcription[13]. In addition to the Smad pathway, TGF-β also signals through a number of non-canonical pathways, including m-TOR, RhoA, Ras, MAPK, PI3K/AKT, PP2A/p70s6K, and JNK[14]. The relative importance and interplay of these pathways of TGF-β signaling is still under investigation[15,16].

Growth inhibitory function is among the most important features of TGF-β, and it plays a central role in homoeostasis of normal cells. Accordingly, TGF-β is considered a tumour suppressor. The loss of a cytostatic response to TGF-β is indeed a hallmark of various kinds of cancer[17,18]. Once cancer is established, however, the same cytokine paradoxically favours tumour progression and metastasis through increasing activities of invasion, mitogenesis and angiogenesis[19,20].

Extracellular signal-regulated kinase 1/2 (ERK1/2) has been reported to play a role in invasion, migration and angiogenesis of many can-

cer cells *in vivo* and *vitro*[21-27]. It is responsible for a host of oncogenic signaling events including NF-kB activation[28], promotion of angiogenesis[29], evasion of immune surveillance[30,31], stimulation of cancer cell proliferation, inhibition of cancer cell apoptosis[32], and facilitation of epithelial-to-mesenchymal transition (EMT) and metastasis[28].

It is well known that TGF-β is able to activate ERK in cancer cells[27,28,33-34]. TGF-β mediated ERK activation in cancer cells will lead to up-regulates DNA methyltransferases (DNMTs)[35]. Targets of DNMTs promoter methylation in many tumor suppressor genes are TBRs[35,36]. The biological, consequence of a down-regulated TBR will be an attenuate Smad2/3 activation and an elevated ERK1/2 activation in advanced cancer cells.

S100A4, also known as mts1, p9Ka, FSP1, CAPL, calvasculin, pEL98, metastasin, 18A2, and 42A, was cloned in the 1980s and early 1990s from various cell systems[37,38]. The calcium-binding protein S100A4 promotes metastasis in several experimental studies[39-47], and S100A4 protein expression is associated with patient outcome in a number of tumor types[48-55]. S100A4 possesses a wide range of biological functions, such as regulation of angiogenesis, cell survival, motility, and invasion[56-59].

Xie et al[60] has found that TGF-beta1 stimulated endometrial cancer cell migration and invasion with a concomitant increase in S100A4 protein. Induction of S100A4 was associated with the activation of Smads. TGF-beta1-mediated endometrial cancer cell motility was inhibited by S100A4 siRNA, which suggest that S100A4 is a critical mediator of invasion in endometrial cancer and is upregulated by the TGF-beta1 signaling pathway. Luo et al[61], Matsuura et al[62] and Tomcik et al[63] also reported that S100A4 was positively regulated by TGF-beta signaling. Recent study reported that S100A4 was positively regulated by extracellular signal-regulated kinase 1/2 (ERK1/2)[64-65]. Thus, although the molecular mechanisms by which TGF-beta induces a metastatic phenotype may not be fully understood, modulation of the function of its targets appears to be an important part of the role of TGF-beta.

In the present study, we have identified TGF-β as a new ERK1/2 target. Interaction of TGF-β with ERK1/2 augments TGF-β-mediated up-regulation of S100A4, leading to an increase in cell invasion ability. The findings of the present study provide a new molecular mechanism for the regulation of metastatic ability by TGF-β/ERK/S100A4 signal.

Materials and Methods

Cell culture

MDA-MB-231 (MDA231) cells was obtained from the American Type Culture Collection (ATCC, Shanghai, China). The cell line was chosen because it has been previously shown that the MDA231 cells have high endogenous levels of TGF-β[65], and high endogenous levels of p-ERK and S100A4[66], and is highly invasive. Cells were maintained in Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco-BRL, Grand Island, NY, USA) and Dulbecco's-modified Eagle's medium/F-12 (Gibco-BRL) with 10% fetal bovine serum (Gibco-BRL) and 1% penicillin-streptomycin-glutamine (Gibco-BRL) at 37°C in 5% CO_2 with constant humidity.

Agents

Antibodies against ERK1/2 and S100A4 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against TGF-β and phospho-ERK1/2 (Thr202/Tyr204) (p-ERK1/2) was obtained from Millipore (Bedford, MA, USA). β-actin antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The ERK kinase inhibitor PD98059 was purchased from CalBiochem (La Jolla, CA, USA). Peprotech Recombinant Human TGF-β1 was obtained from sjhskj.com, Shanghai, China.

Establishment of Stable siRNA-Expressing Clones of MDA231 Cells

The pCDNA3.1 plasmid expressing TGF-β1 siRNA (pCDNA3.1- TGF-β1 siRNA) was constructed by ligating the short hairpin RNA sequence containing both sense and antisense strands. An TGF-β1 sequence that was 23 bases in length with a 9 base loop region with BamHI and HindIII sites (5'-GATCCCGTTTAACTTGAGCCTCAGCA-GACGCAGCTTCAAGAGAGCTGCGTCTGCT-GAGGCTCAAGTTAAATTTTTTCCAAA-3') incorporated at the ends was used. The self-annealed oligo was ligated into the BamHI site of pcDNA-3 vector. MDA231 cells were transfected at 80% confluency with a pCDNA3.1-TGF-β1 siRNA plasmid using Lipofectamine 2000 (Invitrogen, Guangzhou, China) as per the manufacturer's instructions. Twenty-four hours after transfection, the cells were split into 96-well plates and cultured in medium containing 1 mg/mL geneticin (G418) for stable transfection. Single-cell clones and a mixed pool of 10 clones, constitutively expressing TGF-β1 siRNA, were expanded and screened using reverse tran-

scription-PCR (RT-PCR) as described below to determine transcriptional silencing of TGF-β1.

Transient S100A4 siRNA Transfection

S100A4 siRNAs and its controls were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). 1×106 TGF-β1 siRNA/MDA231cells were plated in 100 mm Petri dishes for the transfection experiment. The cells were transfected in serum-free L-15 media using 10 μg of lipofectamine reagent (Invitrogen, Guangzhou, China) as per manufacturer's instructions. The S100A4 siRNA constructs were used for transfection. No plasmid was introduced in the control plates. After allowing 12 h for transfection, the serum-free media was replaced with serum-containing media and left in the incubator at 37°C for 24 h.

Treatment of Cells with TGF-β or PD98059

To study the activity of TGF-β to p-ERK1/2, pCDNA3.1-TGF-β1 siRNA transfected MDA231 clones (TGF-β1 siRNA/MDA231) were cultured and serum starved for 24 h, then treatment with TGF-β1 (R&D Systems, Minneapolis, MN, USA) at a concentration of 5 ng/ml for 48 hours to activite TGF-β.

To study the TGF-β-dependent p-ERK1/2-S100A4 activity, TGF-β1 siRNA-S100A4 siRNA/MDA231 cells were treatment with TGF-β1 (5 ng/ml) for 24 hours to activite TGF-β, or treated with PD98059 at a concentration of 25 μM for 2-3 h prior to treatment with TGF-β1 24 hours.

Western Blot Analysis

After treatment in virous conditions, MDA231cells were rinsed once with PBS and scrapped in 50 μl of sample buffer supplemented with complete protease inhibitor cocktail and phosphatase inhibitors at 4 °C. Samples were immediately heated at 95 °C for 8 min and then maintained at –20 °C until use. Aliquots of 40 μl of samples were electrophoretically separated in 10% polyacrylamide gels and transferred to nitrocellulose (0.45 μm pore size, BioScience, San Jose, CA, USA). The nitrocellulose was incubated with blocking solution (5% w/v nonfat dry milk, 0.1% tween-20 in Tris-buffered saline (TBS), pH 7.4) at room temperature for 1 h. The blots were probed with primary antibodies against TGF-β1, p-ERK1/2, ERK1/2, S100A4 according to the manufacturer's protocol, and then incubated with the secondary antibody, goat anti-rabbit HRP-conjugated IgG (1:2000; Cal-

biochem, San Diego, CA, USA) at room temperature for 1 h. Bands were visualized by chemiluminescence (PerkinElmer, Shanghai, China).

Wound Closure Assay

MDA231cells in different conditions at different time point were plated at 105 per well in a six-well plate coated with fibronectin. Once the cells reached 90% confluency, they were serum starved for 12 h. Following serum starvation, 10 uL/mL mitomycin C were added to the medium for 2 h in complete medium and a "wound" was created using a sterile 200 uL pipette tip. Photographs of the wounded area were taken at the time of wounding and thereafter every 24 hours for 3 d to determine the rate of wound closure. Percent migration was calculated by measuring the length and width of the cell-free area. The width was measured at five points along the scratch area and then averaged to get an accurate representation of the entire scratch. Percent migration was determined by using the following formula: [D area/area (day 0)] × 100.

In vitro Angiogenesis

Conditioned media collected from MDA231 cells in different conditions at different time point was tested for induction of angiogenesis in human microvascular endothelial cells (HMEC) plated onto 8-well chamber slides (4 × 104/well). Once the cells had attached to the surface of the slide, the media was removed and replaced with conditioned media collected from MDA231cells in different conditions at different time point. After 72 hours in conditioned media, cells were washed gently with phosphate buffered saline (PBS) and stained with hematoxylin and eosin (H&E). Angiogenic network formation was visualized under the microscope and Image Pro software was used for quantification of angiogenesis. The degree of angiogenesis was measured by the following method: number of branch points and the total number of branches per point were counted at random (per 10 fields), with the product indicating the degree of angiogenesis as compared to the controls.

Migration Assays

Migration assays was done as described[67]. MDA231 cells in different conditions at different time point were plated on a cell culture insert coated with fibronectin (8-mm pore size, 24-well format; Becton Dickinson Labware, Franklin Lakes, NJ, USA) in serum-free medium and a
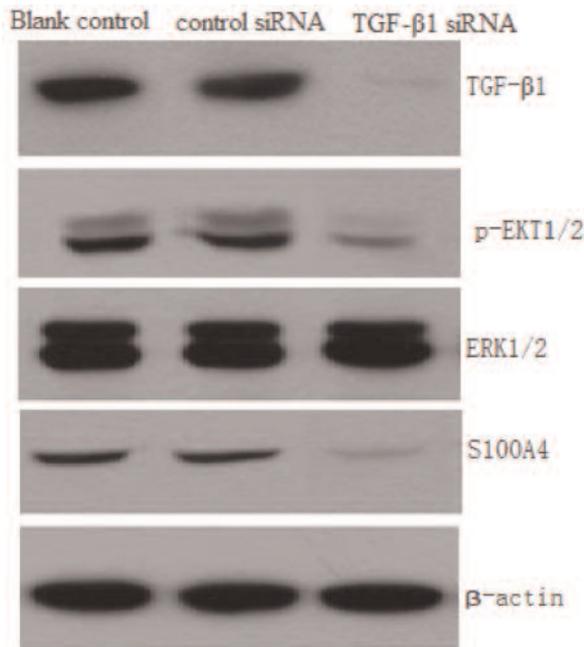
Figure 1. Knockdown of TGF-β1 expression by siRNA transfection. MDA231 cells were stably transfected with TGF-β1 siRNA and control siRNA. Whole-cell extracts were collected for Western blot analysis using specific antibodies against TGF-β1, phosho-ERK1/2, ERK1/2 and S100A4.

chemoattractant (5% fetal bovine serum) was added to the lower chamber. To determine the amount of invasion, cells were incubated for 24 h and then removed from the upper chamber using a cotton swab. The cells on the underside of the chamber were fixed to the membrane using methanol for 5 min. Once fixed, the cells were stained with crystal violet for 10 min and rinsed with PBS. The chambers were then photographed to compare the amount of invasive cells on the underside of the membrane. Quantitation of the invasive cells was done using a semiquantitative colorimetric analysis of the crystal violet-stained cells. To remove the crystal violet dye from the cells, the membranes were submerged in 0.01% sodium dodecyl sulphate (SDS) solution for 20 min. Fifty microliters of the solution were then pipetted into a 96-well plate and absorbance measured at 560 nm. Values were then normalized to the unmodified MDA231 cells.

Statistical Analysis

A Student's *t*-test and a one-way ANOVA were used to determine the statistical significance of differences between values for the various experimental and control groups. Data are expressed as mean±standard errors (SEM) and the results are

taken from at least three independent experiments performed in triplicate. *p* values of 0.05 or less were considered to be statistically significant.

Results

Knockdown of TGF-β1 Inhibits p-ERK and S100A4 Expression in MDA231Cells

MDA-MB-231 (MDA231) cells has been previously shown to have high endogenous levels of TGF-β[65], and high endogenous levels of p-ERK and S100A4[66]. Immunoblot analysis showed that in the TGF-β1 siRNA stably transfected MDA231 cells (TGF-β1 siRNA /MDA231), TGF-β1 protein was completely inhibited, followed by decreased p-ERK and S100A4 expression (Figure 1).

TGF-β1 stimulates p-ERK1/2 and S100Aa Expression in MDA231 Cells.

In the study above, we found that knockdown of TGF-β1 expression inhibited phosho-ERK1/2 and S100A4 expression. However, when the TGF-β1 siRNA/MDA231 cells were treated with TGF-β1 (5 ng/ml) for 24 hours to activite TGF-β, phosho-ERK1/2 and S100A4 was significantly increased (Figure 2).
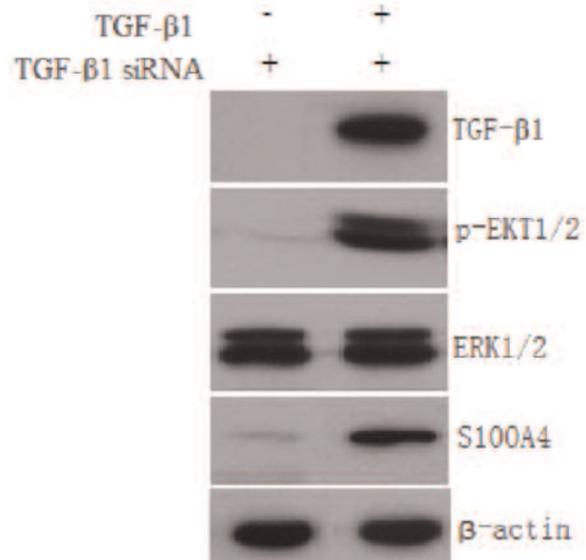
Figure 2. TGF-β1 promotes p-ERK1/2 and S100A4 expression. TGF-β1 siRNA/MDA231 cells were treated withTGF-β1 (5 ng/ml) for 24 hours. Whole-cell extracts were collected for Western blot analysis using specific antibodies against TGF-β1, phosho-ERK1/2, ERK1/2 and S100A4.
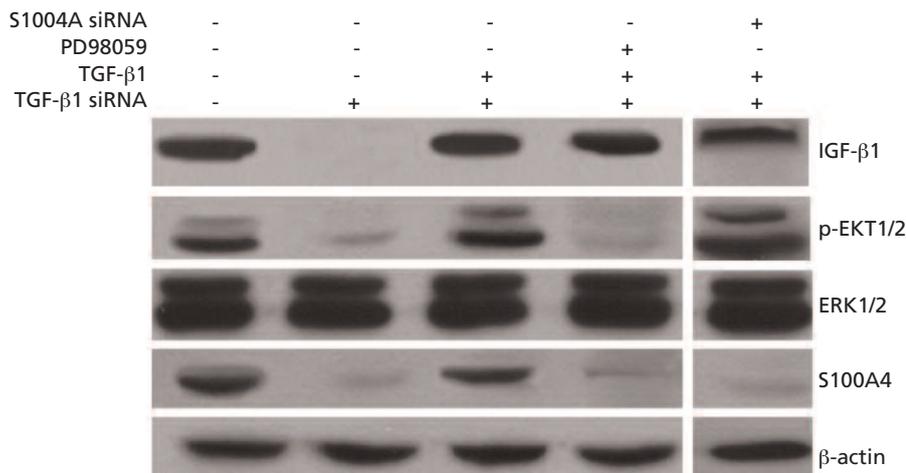
Figure 3. Role of TGF-β1 signalling pathway in the regulation of S100A by ERK. TGF-β1 siRNA/MDA231 cells or the TGF-β1 siRNA-S100A4 siRNA/MDA231 cells were treated with 25 βM PD98059 or/and 5 ng/ml TGF-β1. Immunoblot with antibodies against TGF-β1, phosphorylated ERK1/2 (p ERK1/2), total ERK1/2, S100A4, and β-actin.

TGF-β1 Mediates S100A4 Induction Through Activation of ERK1/2 Signalling

To study the TGF-β-dependant p-ERK1/2-S100A4 activity, TGF-β1 siRNA/MDA231 cells were first treated with PD98059 at a concentration of 25 μM for 2-3 h prior to treatment with TGF-β1. Treatment with PD98059 resulted in the decreased p-ERK1/2 and S100A4 expression, through TGF-β1 was upregulated by TGF-β1 treatment (Figure 3). Furthermore, after TGF-β1 siRNA/MDA231 cells were transfected into S100A4 siRNA, treatment with TGF-β1 upregulated p-ERK1/2, but not S100A4 (Figure 3).

Knockdown of TGF-β Inhibits Angiogenesis in HMEC-1 Cells via Inhibition of S100A4

HMEC, grown in conditioned media from TGF-β1 siRNA, TGF-β1 siRNA/TGF-β1, and TGF-β1 siRNA/TGF-β1/S100A4 siRNA treated MDA231 cells were stained with H&E. The results clearly demonstrated that TGF-β1 siRNA alone significantly inhibited tumor cell-induced angiogenesis. After treatment with TGF-β1 in TGF-β1 siRNA transfected MDA231 cells, tumor cell-induced angiogenesis was significantly increased. However, when the TGF-β1 siRNA transfected MDA231 cells were transfected with S100A4 siRNA, TGF-β1 treatment did not increase tumor cell-induced angiogenesis (Figure 4).

Knockdown of TGF-β Inhibits Migratory Potential of MDA231 Cells via Inhibition of S100A4

In vitro studies were done to determine the effects of TGF-β silencing on both migration and invasion of these cell clones. Cell migration was first determined using a wound-healing assay in which cells were scratched and allowed to migrate into the wound area. The amount of migration closure was enumerated 72 hours after disruption. Compared with the normal MDA231cells that showed 91% wound closure by 72 hours, clones expressing the TGF-β siRNA showed 39% wound closure in the same period (Figure 5A). After treatment with TGF-β1 in TGF-β1 siRNA transfected MDA231 cells, clones showed 90% wound closure in the same period (Figure 5A). However, when the TGF-β1 siRNA transfected MDA231 cells were transfected with S100A4 siRNA, TGF-β1 treatment did not increase the wound closure in the same period (Figure 5A).

Using a Boyden chamber, coated with fibronectin, we determined changes in cell invasiveness after 24 hours. Cells were fixed and stained with crystal violet to determine the number of cells that invaded across the membrane. Cell invasiveness has the same results as the wound-healing assay (Figure 5B).

Discussion

TGF-β has emerged as a promising new target for treatment of cancer metastasis. Most of the
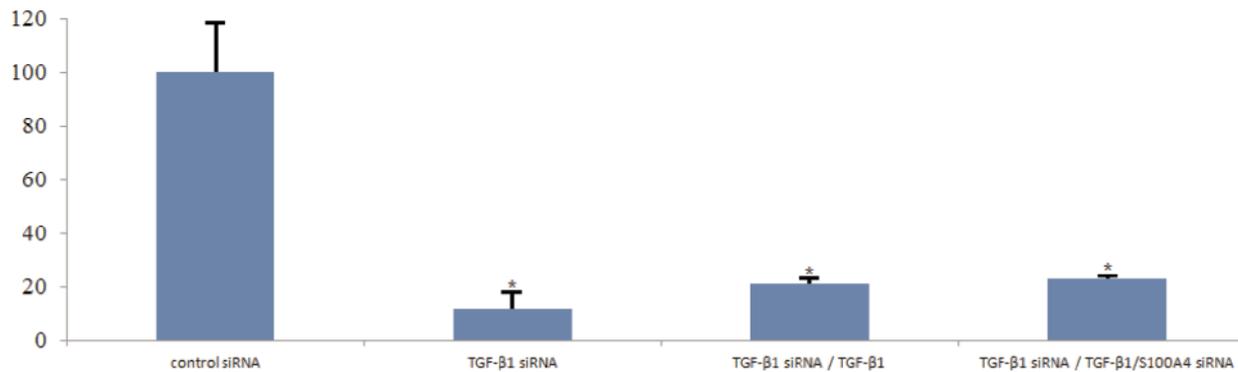
Figure 4. *In vitro angiogenesis.* Network formation by human microvascular endothelial cells in conditioned media from TGF-β1 siRNA, TGF-β1 siRNA/TGF-β1, and TGF-β1 siRNA/TGF-β1/S100A4 siRNA treated MDA231 cells. HMEC-1 cells grown in conditioned media were stained with H&E after 72 hours and examined under a confocal scanning laser microscope. Quantification of angiogenesis in co-cultures as described in Methods. Values are mean ± SD from three different experiments. Vs control, $*p < 0.05$.

studies to date have examined the effects of TGF-β receptor inhibition on tumor metastasis, including breast cancer[67-70]. Through the TGF-β signalling pathway plays a pivotal role in diverse cellular processes ranging from development to carcinogenesis. However, its role and mechanisms in cancer progression is highly complex.

TGF-β functions as a tumour suppressor in normal or dysplastic cells and as a tumour promoter in advanced cancers[71]. TGF-β mediates its
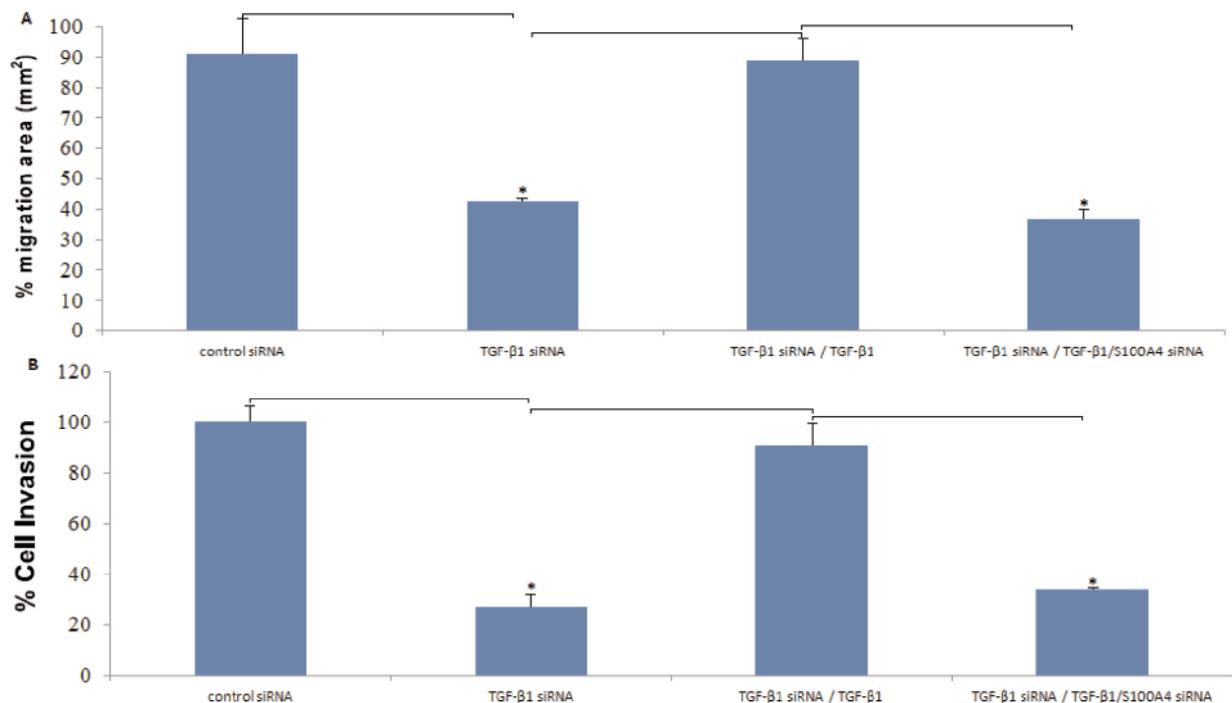
Figure 5. Silencing of TGF-β affects migration and invasion of MDA231 cells *in vitro* via inhibition of S100A4. MDA231 cells, TGF-β siRNA/MDA231 cells, TGF-β siRNA/TGF-β/MDA231 cells and TGF-β1 siRNA/S100A4 siRNA/TGF-β/MDA231 cells were analyzed for cell migration using the wound-healing scratch assay. *A,* Cells were "wounded" and monitored every 24 hours for 3 d to determine the rate of migration into the scratched area. *B,* Invasiveness of cells was determined using a Boyden chamber assay. Cells were plated in the upper chamber of the apparatus and allowed to grow for 24 hours in serum-free medium. A chemoattractant (5% fetal bovine serum) was placed in the lower chamber. Cells were fixed to the membrane and stained after 24 h to determine invasion. $*, p < 0.01$; all assays were done in triplicate.

growth inhibitory or tumour-promoting actions by regulating the expression of its target genes in a cellular context-dependent manner. This has led to the identification and characterization of several novel TGF-β effectors, such as c-Myb[72], Pin1[73] and ERK[67] which are shown to mediate pro-tumorigenic actions of TGF-β.

Members of the S100 family of proteins, including S100A4, show altered expression in several cancers. Recently, studies reported that S100A4 may be as a prognostic marker or a predictor of distant metastasis in patients with cancers. It could also be a target for cancer treatment *in vivo* and *vitro*[39-45,50]. In a previous report, S100A4 was identified as a downstream target of TGF-β signaling[60,61,73]. In the present study, we found that knockdown of TGF-β inhibited migration, invasion and angiogenesis in the MDA231 cells, followed by decreased S100A4 expression. TGF-β treatment restored the invasive ability of the TGF-β siRNA/MDA231 cells, followed by increased S100A4 expression. However, TGF-β treatment did not restore the invasive ability of the TGF-β siRNA/MDA231 cells when S100A4 siRNA was transfected into the TGF-β siRNA/MDA231 cells. We, therefore, suggested that TGF-β regulated S100A4-mediated migration, invasion and angiogenesis in the MDA231 cells. However, the mechanism of regulation for S100 A4 proteins by TGF-β has not been studied so far.

It has been reported that ERK plays a critical role in the process of EMT (74). During the course of our assays, phosphorylation of ERK (pERK1/2) was found to be much in MDA231 cells. When the TGF-β was knockdown by siRNA transfection, pERK1/2 was significantly inhibited. However, TGF-β treatment could restore the pERK1/2 level in the TGF-β siRNA/MDA231 cells. Thus, our data suggest that the ERK1/2 signaling pathway is regulated by TGF-β.

In the current study, we found TGF-β siRNA inhibited MDA231 cells invasion, and TGF-β treatment restored the invasive ability. However, when TGF-β siRNA/MDA231 cells were treated with the MEK1 inhibitor PD98059, TGF-β treatment did not increase the migratory and angiogenesis formation ability in the TGF-β siRNA/MDA231 cells. We, therefore, suggested that TGF-β1 enhanced cell motility and invasion in MDA231 cells via ERK1/2 regulation.

Interestingly, the protein levels of pERK1/2 and S100A4 were reduced after TGF-β1 siRNA transfection. Treatment with TGF-β restored the pERK1/2 and S100A4 level. However, S100A4 protein was significantly decreased upon exposure to the ERK inhibitor PD98059, indicating that ERK is biochemically important for S100A4 regulation.

Up-regulation of TGF-β exhibits enhanced invasive ability in the MDA231 cells and enhanced pERK1/2 and S100A4 level, and vice versa. Interestingly, siRNA targeting S100A4 suppressed TGF-β induced migration, invasion and angiogenesis in the MDA231 cells. These results suggest that blocking TGF-β inhibits breast cancer cell invasiveness via ERK/S100A4 signal.

Conclusions

We demonstrate a novel synergistic regulation of S100A4 by TGF-β-induced ERK signalling, and involvement of S100AA in TGF-β-mediated prometastasis and proangiogenesis formation actions.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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