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S100A4 silencing suppresses proliferation, angiogenesis and invasion of thyroid cancer cells through downregulation of MMP-9 and VEGF

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Abstract. – BACKGROUND AND AIM: It is well documented that S100A4 is upregulated in many cancers and plays a pivotal role in tumor proliferation, invasion, metastasis and angiogenesis. However, the precise role and mechanism S100A4 exerts in the thyroid cancer have not been fully elucidated to date. In the present study, we investigated the effect of S100A4 on proliferation, invasion, metastasis and angiogenesis in thyroid cancer cells.

MATERIALS AND METHODS: A plasmid construct was made that expressed full long S100A4 cDNA. The construct was stably transfected into BCPAP and ML-1 thyroid cancer cells (BCPAP/S100A4 cDNA, ML-1/S100A4 cDNA). S100A4 siRNA was transiently transfected into the DRO cells (DRO/S100A4 siRNA). MMP-9 siRNA or VEGF siRNA was transiently transfected into the BCPAP/S100A4 cDNA, ML-1/S100A4 cDNA cells (BCPAP/S100A4 cDNA/VEGF siRNA, ML-1/S100A4 cDNA/MMP-9 siRNA).

RESULTS: We found that the down-regulation of S100A4 by small interfering RNA decreased cell invasion, metastasis, and angiogenesis by using chicken chorioallantoic membrane (CAM), whereas S100A4 overexpression by cDNA transfection led to increased tumor cell invasion, metastasis, and angiogenesis. Consistent with these results, we found that the down-regulation of S100A4 reduced VEGF and MMP-9 expression. Furthermore, Knockdown of MMP-9 by MMP-9 siRNA inhibited cell invasion and metastasis in the BCPAP/S100A4 cDNA, ML-1/S100A4 cDNA cells. Knockdown of VEGF by VEGF siRNA inhibited cell angiogenesis in the BCPAP/S100A4 cDNA, ML-1/S100A4 cDNA cells.We also found that downregulation of S100A4 by small interfering RNA resulted in enhanced cell growth inhibition and apoptosis, and vice versa. Our data suggest S100A4 could be an effective approach for the regulation of proliferation, invasion and angiogenesis. Downregulation of S100A4 could inhibit angiogenesis, proliferation and invasion by regulating the expression of MMP-9 and VEGF.

CONCLUSIONS: Our results provide evidence that the downregulation of S100A4 using RNAi technology may provide an effective tool for thyroid cancer therapy.

Key Words:
Thyroid cancer, S100A4, Angiogenesis, Metastasis, Apoptosis.

Abreviations

VEGF = vascular endothelial growth factor
MMP-2,9 and 13 = Matrix metallopeptidase 2,9,13
siRNA = Small interfering RNA
CAM = Chicken chorioallantoic membrane;
DMEM = Dulbecco's Modified Eagle's medium
HUVEC = Human Umbilical Vein Endothelial Cells
MTT = 4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
FACS = Fluorescence-activated cell sorting
PI:Propidium iodide
PBS:phosphate-buffered saline
TUNEL = Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling

Introduction

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[1-2]. It belongs to the S100 protein family, which consists of at least 21 different members[3]. S100 proteins are Ca^{2+} binding, low molecular mass proteins (10 to 12 kDa) that generally exist as homo- or heterodimers within cells[4]. S100A4 is localized in the nucleus, cytoplasm, and extracellular space and possesses a wide range of biological functions, such as regulation of angiogenesis, cell

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survival, motility, and invasion. The calcium-binding protein S100A4 promotes metastasis[5-9], and knockdown of S100A4 inhibited metastasis in several experimental animal models[10-15]. Furthermore, S100A4 protein expression is associated with patient outcome in a number of tumor types[16-20].

S100A4 is a well established marker and mediator of metastatic disease, but the exact mechanisms responsible for the metastasis promoting effects are less well defined. In human prostate cancer and osteosarcoma cells, the invasive capability is stimulated by S100A4, at least partly through transcriptional activation of MMP-9[12,20-21]. In esophageal squamous cell carcinoma cells, S100A4 mediated cell invasion and metastasis of esophageal squamous cell carcinoma via the regulation of MMP-2 and E-cadherin activity[22]. Based on observations in transgenic mice, S100A4 has been identified as a potent stimulator of angiogenesis. The mechanisms that participate in this process are only starting to become unraveled. Augmented MMP-13 expression in endothelial cells was associated with S100A4-mediated stimulation of capillary-like growth in three-dimensional Matrigel cultures *in vitro*[23].

Thyroid cancer,the most common type of endocrine malignancy, accounts for the majority of endocrine cancer related to death each year[24]. Well-differentiated thyroid cancers arise from follicular cells and encompassespapillary, follicular and hurthle carcinomas. Other histological types of thyroid cancer are medullary and anaplastic[25-28]. Papillary thyroid cancer is the most common type of thyroid tumors, making up about 70-80% of all thyroid cancers,and can occur at any age[27]. Involvement of the lymph node is relatively common in papillary carcinoma and lymphatic spread is the major target of the metastasis[28]. It has found S100A4 was overexpressed in thyroid tumour, and overexpression of S100A4 is associated with thyroid tumour invasion and metastasis[29-32]. Previous study shown S100A4 expression knockdown by RNA interference inhibited metastasis and growth of human anaplastic thyroid carcinoma cells *in vivo*[33]. However, the mechanism by S100A4 signaling regulates invasion and metastasis remains to be elucidated.

Angiogenesis is a process of new blood vessel development from preexisting vasculature. The vascular endothelial growth factor (VEGF) is one of the most potent endothelial cell mitogens and plays a crucial role in both angiogenesis and lymphogenesis[34]. The microvascular density is increased in the thyroid malignancy compared with normal thyroid tissue and benign thyroid tumors[35]. Growing evidence from *in vitro* and *in vivo* experiments have shown that increased VEGF expression promotes thyroid cancer cell growth, subsequent lymph node metastasis, local invasion, and distant metastasis, whereas the inhibition of VEGF signaling results in suppression of the tumor growth[36]. Recent study found significant relation was shown between S100A4 and VEGF expression in clear cell renal cell carcinoma, gastric carcinoma and pancreatic cancer[37-39]. Whether VEGF was regulated by S100A4 is not clear.

To further investigate its role in thyroid tumor metastasis and its potential as a therapeutic target, in the present study, we introduced into thyroid cancer cell line cells a plasmid vector expressing S100A4 siRNA to inhibit S100A4. The effects of reduced S100A4 gene expression by RNA interference on tumor growth and metastasis were examined. Furthermore, the effect of S100A4 on MMP-9 and VEGF was investigated.The results suggest that S100A4 gene knockdown could significantly suppress thyroid carcinoma cell growth, angiogenesis and metastasis and S100A4 gene controls the invasive potential and angiogenesis of human thyroid cancer cells through regulation of MMP-9 and VEGF.

Materials and Methods

Cell Line and Cell Culture

BCPAP (human papillary TCa cell line) was purchased from DSMZ (Braunsch, Germany). It was cultured in Rosswell Park Memorial Institute (RPMI)-1640 (Mediatech, Herndon, VA, USA) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA, USA), penicillin 10,000 IU/mL, streptomycin 10,000 µg/mL (Mediatech), and 2 mM L-glutamine (Mediatech). DRO, a human anaplastic thyroid carcinoma cell, ML-1, a human follicular thyroid cancer, were purchased from ATCC, was propagated in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum, 2 mm l-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO_2.

Experimental Reagents

S100A4 siRNA were commercially purchased from Qiagen (Valencia, CA, USA), and siRNA

control were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). VEGF siRNA and MMP-9 siRNA were obtained from Santa Cruz Biotechnology. LipofectAMINE 2000 was purchased from Invitrogen (Carlsbad, CA, USA). Primary antibodies for S100A4, VEGF, MMP-9 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All secondary antibodies were obtained from Pierce (Rockford, IL, USA). Chemiluminescence detection of proteins was done with the use of a kit from Amersham Biosciences (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Protease inhibitor cocktail and all other chemicals were obtained from China.

Plasmids and Transfections

The pcDNA3.1-S100A4 cDNA Plasmid was constructed as the method reported previously. DRO cells were transfected with S100A4 siRNA and control siRNA for 36 hours, respectively, using LipofectAMINE 2000. BCPAP and ML-1 cells were stably transfected with human S100A4 cDNA or vector alone (pcDNA3) were selected with 400 μg/mL G418 for 14 d. Stably transfected BCPAP and ML-1 cells were transfected with MMP-9 siRNA, or VEGF siRNA and control siRNA for 36 hours using LipofectAMINE 2000.

Cell Invasion and Migration Assays

Cell invasion and migration assays were done in chemotaxis chambers (Neuro Probe, Inc., Gaithersburg, MD, USA). Cells (6×10^4) were suspended in 50 μL serum-free medium and added to the upper chamber. The lower chamber was filled with complete medium. Polycarbonate membrane (8 μm) coated with culture medium containing 20 μL/mL Matrigel was placed between the two chambers. Cells were invaded at 37°C for 36 hours. After removing noninvaded cells, the membrane was fixed in methanol and stained with Giemsa. Invaded cells were photographed and quantified in 10 random fields per membrane. Each sample was assayed in triplicate. As analyzed by Student's *t* test, $p < 0.05$ indicated statistical significance. A similar system, with uncoated polycarbonate membrane, was used for migration assay.

Angiogenesis Assay on the CAM

Fertilized White Leghorn chicken eggs were incubated at 37°C under conditions of constant humidity. The developing CAM was separated from the shell by opening a window at the broad end of the egg above the air sac on day 9. The opening was sealed and the eggs were returned to the incubator. To investigate tumor angiogenesis, the S100A4 cDNA or pcDNA3.1 transfected BCPAP and ML-1 cells, S100A4 cDNA/MMP-9 siRNA or/VEGF siRNA transfected BCPAP and ML-1 cells and S100A4 siRNA transfected DRO cells were suspended in medium containing 50% Matrigel (BD Biosciences, Bedford, MA, USA). Aliquots (30 μl) of the mixture were then applied onto the CAM of 9-day-old embryos. The area DROund the implanted Matrigel was photographed 4 days after the implantation, and the blood vessels were counted by two observers in a double-blind manner. Assays for each treatment were carried out using 8-10 chicken embryos.

Matrigel in vitro HUVEC Tube Formation Assay

The S100A4 cDNA or pcDNA3.1 transfected BCPAP and ML-1 cells, S100A4 cDNA/MMP-9 siRNA or/VEGF siRNA transfected BCPAP and ML-1 cells and S100A4 siRNA transfected DRO cells were cultured in serum-free RPMI 1640 for 24 h. The conditioned media were collected, centrifuged, transferred to fresh tubes, and stored at -20°C. HUVECs were trypsinized and seed ed (5×10^4 per well) in Matrigel-coated well with 250 uL of conditioned medium from the S100A4 cDNA or pcDNA3.1 transfected BCPAP and ML-1 cells, S100A4 cDNA/MMP-9 siRNA or/VEGF siRNA transfected BCPAP and ML-1 cells and S100A4 siRNA transfected DRO cells.The tube formation was assayed as described earlier[40].

Cell Proliferation and Viability Assay

The S100A4 cDNA or pcDNA3.1 transfected BCPAP and ML-1 cells and S100A4 siRNA transfected DRO cells were seeded in 96-well plates with a concentration of 1×10^4 cells per well in triplicate. Cell proliferation was determined after 72 h by bromodeoxyuridine (BrdU) incorporation assay using Promega's BrdU cell proliferation assay kit. Cell viability was determined using MTT assay. The results were plottedas mean ± SD of three separate experiments having six determinations per experiment for each experimental condition.

In vitro Apoptosis Analysis by FCM

To identify the induction of apoptosis, treated cells underwent propidium iodide (PI) staining and fluorescence-activated cell sorting (FACS) as to the manufacture's instruction. In brief, the

S100A4 cDNA or pcDNA3.1 transfected BCPAP and ML-1 cells and S100A4 siRNA transfected DRO cells were plated at a density of 1×10^5 cells/ml for 72 hours. Cells were collected by gentle trypsinization, washed in phosphate-buffered saline (PBS), pelleted by centrifugation and fixed in 70% ethanol. Immediately prior to staining, cells were washed twice in PBS and re-suspended in PBS containing RNAse A (20 µg/ml). Cells were stained with propidium iodide (final concentration 10 µg/ml) for 10 min at room temperature. Samples were analyzed by FACS (FL-3 channel) using a Beckman Coulter Counter Epics XL flow cytometer (Beckman Coulter, Miami, FL, USA). For each sample, 50,000 events were collected and stored for subsequent analysis using EXPO software (version 2.0; Applied Cytometry Systems, Sheffield, UK). The percentage of cells in the sub-G0 phase was quantitated as an estimate of cells undergoing apoptosis.

In vitro Apoptosis Detection by TUNEL

The S100A4 cDNA or pcDNA3.1 transfected BCPAP and ML-1 cells and S100A4 siRNA transfected DRO cells (1×10^4) were cultured on chamber slides for 24 h. Apoptosis of the cells was evaluated on the basis of the TUNEL assay according to the manufacturer's instructions. All assays were performed in quadruplicate.

VEGF Assay

The culture medium of the the S100A4 cDNA or pcDNA3.1 transfected BCPAP and ML-1 cells, S100A4 cDNA/MMP-9 siRNA or/VEGF siRNA transfected BCPAP and ML-1 cells and S100A4 siRNA transfected DRO cells grown in six-well plates was collected. After collection, the medium was spun at 800 × g for 3 minutes at 4°C to remove cell debris. The supernatant was either frozen at –20°C for VEGF assay later or assayed immediately using commercially available ELISA kits (R&D Systems, Inc., Minneapolis, MN, USA).

MMP-9 Activity Assay

The culture medium of the the S100A4 cDNA or pcDNA3.1 transfected BCPAP and ML-1 cells, S100A4 cDNA/MMP-9 siRNA or/VEGF siRNA transfected BCPAP and ML-1 cells and S100A4 siRNA transfected DRO cells grown in six-well plates and incubated at 37°C. After 24 hours,the complete medium was removed and the cells were washed with serumfree medium. The cells were then incubated in serum-free medium for 48 hours. MMP-9 activity in the medium and cell lysate was detected by using Fluorokine E Human MMP-9 Activity Assay kit (R&D Systems) according to the protocol of the manufacturer.

Western Blot Analysis

Cells were lysed in lysis buffer [50 mmol/L Tris (pH 7.5), 100 mmol/L NaCl, 1 mmol/L EDTA, 0.5% NP40, 0.5% Triton X-100, 2.5 mmol/L sodium orthovanadate, 10 uL/mL protease inhibitor cocktail, and 1 mmol/L PMSF] by incubating for 20 minutes at 4°C. The protein concentration was determined using the Bio-Rad assay system. Total proteins were fractionated using SDS-PAGE and transferred onto nitrocellulose membrane (Hercules, CA, USA). The membranes were blocked with 5% nonfat dried milk or bovine serum albumin in 1×TBS buffer containing 0.1% Tween 20 and then incubated with appropriate primary antibodies: S100A4 (1:100), MMP-9 (1:150), VEGF (1:100) and anti- -actin. Horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-goat IgG was used as the secondary antibody and the protein bands were detected using the enhanced chemiluminesence detection system. Normalization of the results was done by running parallel Western blots using actin as control. The optical density was quantified using an AlphaEase FC Version4 analysis software (Alphalmager HP, Alpha Innotech, San Leandro, CA, USA) and the results are presented as the mean of three independent experiments with error bars representing SD.

Statistical Analysis

Data presented are of three independent replicates, and the paired Student's *t*-test was used to assess statistical significance. A probability (*p*-value) ≤ 0.05 was considered statistically significant.

Results

Effect of siRNA on S100A4 Expression on Thyroid Cancer Cells

S100A4 expression in DRO, BCPAP and ML-1 cells was detected by Western blot. As shown in Figure 1A, higher S100A4 expression level was found in DRO cell, and lower S100A4 expression level was found in BCPAP and ML-1 cells. In the present study, DRO cells was used to transfect S100A4 siRNA to knockdown of

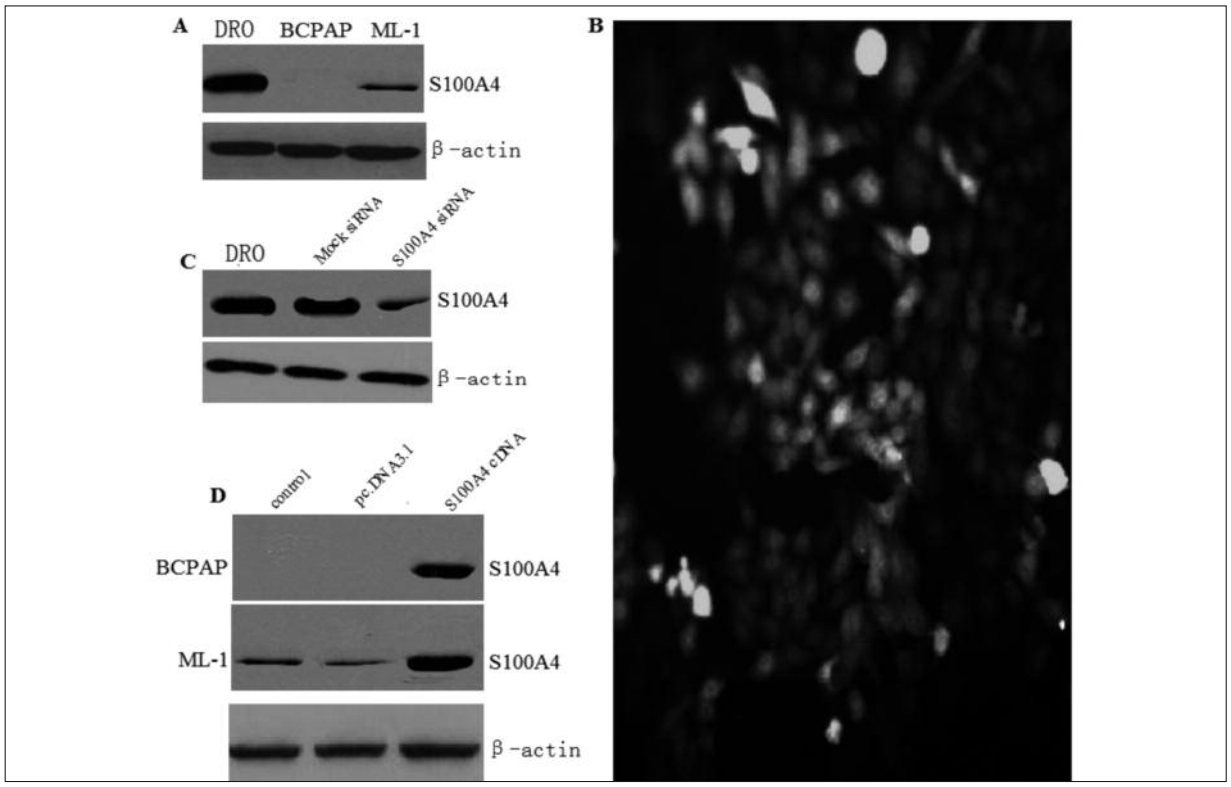

Figure 1. S100A4 gene knockdown by siRNA transfection or overexpressed by S100A4 cDNA transfection in thyroid cancer cells. ***A,*** Representative images showing expression of S100A4 protein in DRO, BCPAP and ML-1 cells as analyzed by Western blot. ***B,*** Photomicrographs showing transfection of fluorescein-labeled siRNA in DRO cells. (Magnification: ×100.) ***C,*** Representative images showing expression of S100A4 protein in nonsilencing siRNA control and S100A4-siRNA-transfected DRO cells as analyzed by Western blot. ***D,*** Effect of S100A4 overexpression by transfection of S100A4-pcDNA3.1 plasmid on BCPAP and ML-1 cells. Representative images showing expression of S100A4 in vector (pcDNA3.1) and pcDNA3.1-S100A4 transfected cells as analyzed by Western blot analysis. Equal loading of protein was confirmed by stripping the blots and reprobing with β-actin antibody.

S100A4, and BCPAP and ML-1 cells was used to transfect S100A4 cDNA to overexpress S100A4.

As shown in Figure 1 B, high (70%) transfection efficiency of siRNA was observed in DRO cells. As determined by Western blot analysis, cells transfected with S100A4 siRNA displayed significant reduction in the expression levels of S100A4 protein (Figure 1 C). Nonsilencing siRNA did not exhibit any effect on protein levels of S100A4 (Figure 1 C). These data confirmed the suppression effect of siRNA and established the efficiency of siRNA transfection. Because S100A4 expression was observed to be very low 48 h after transfection, we selected this time point for further studies.

Effect of S100A4 Overexpression on Thyroid Cancer Cells

BCPAP and ML-1 cells transfected with pc.DNA3.1-S100A4 plasmid displayed a significant increase in the expression levels of S100A4 as compared with vector control (Figure 1 D). The overexpression of S100A4 was confirmed by performing western blot analysis.

S100A4 Promotes Migration and Invasion of Thyroid Cancer Cells

As we reported in Figure 1 A, BCPAP and ML-1 cells expresses lower endogenous S100A4 compared with DRO cell lines. Thus, we used BCPAP and ML-1 to generate S100A4 stable cell lines. *In vitro* cell migration and invasion assays showed that enhanced S100A4 expression promoted both the migration and invasion abilities of BCPAP and ML-1 cells. The migration ability of S100A4/BCPAP cells increased 4-fold and invasion ability increased ~3.4-fold compared with pcDNA3/BCPAP control cells (Figure 2 A). Similarly, the migration ability of S100A4/ML-1 cells increased 3.1-fold and invasion ability increased 2.8-fold compared with pcDNA3/ML-1 control cells (Figure 2 B).

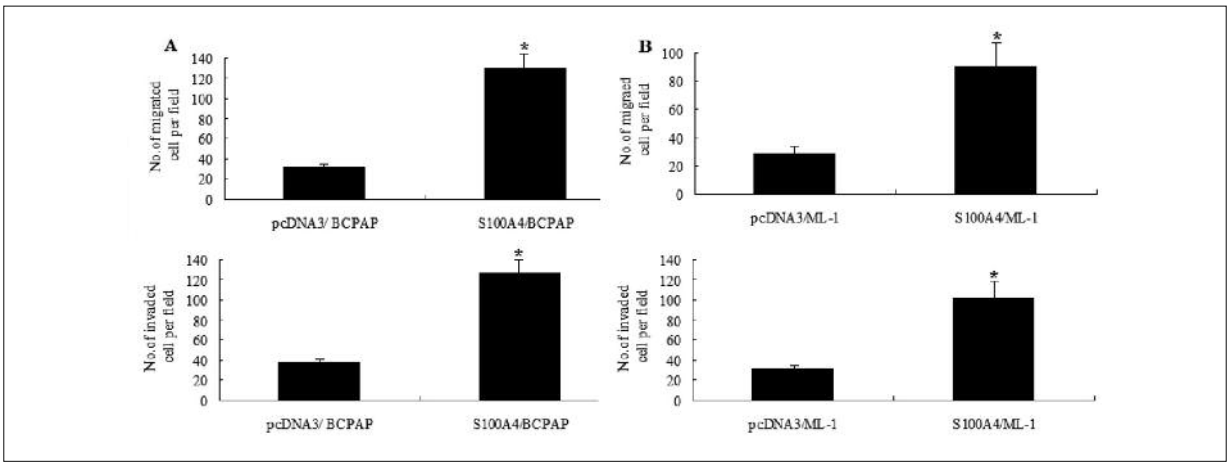

Figure 2. S100A4 overexpression induces *in vitro* cell invasion and migration. ***A,*** Migration and invasion assays in BCPAP cells. ***B,*** Migration and invasion assays in ML-1 cells. Magnification, ×100. Columns, mean obtained from three individual experiments; bars, SD. $p < 0.05$ (*t* test).

Effect of S100A4 Gene Knockdown Inhibits Migration and Invasion of DRO cells

In vitro cell migration and invasion assays showed that knockdown of S100A4 expression inhibited both the migration and invasion abilities of DRO cells. The migration ability of S100A4 siRNA/DRO cells decreased 4-fold (Figure 3 A) and invasion ability decreased 3.4-fold compared with siRNA control cells (Figure 3 B).

Knockdown of S100A4 decreased MMP-9 expression and its activity in thyroid cancer cells. To explore whether the invasiveness of transfected cells was associated with MMP-9 induction, Western blotting were conducted to detect the alteration in the expression of MMP-9. We found that MMP-9 protein levels was dramatically decreased in the S100A4 siRNA-transfected DRO cells (Figure 4A). Next, we examined whether the down-regulation of S100A4 could lead to a decrease in MMP-9 activity. There was a 3.2-fold decrease in the activity of MMP-9 (Figure 4C). Furthermore, we found that overexpression of S100A4 increased the expression and activity of MMP-9 in BCPAP and ML-1 cells (Figure 4B).

Knockdown of S100A4 Decreased VEGF Expression and its Activity in Thyroid Cancer Cells

We further investigated whether S100A4 expression has any effect on VEGF induction. Western blotting were done to detect the expression of VEGF. We found that VEGF protein was dramatically reduced in the S100A4 siRNA-transfected DRO cells (Figure 5A). Most importantly, we also found that the downregulation of S100A4 could lead to a decrease in VEGF level (Figure 5C). Our results also showed that overexpression of S100A4 increased the expression and the level of VEGF in BCPAP and ML-1 cells (Figure 5 B and C).

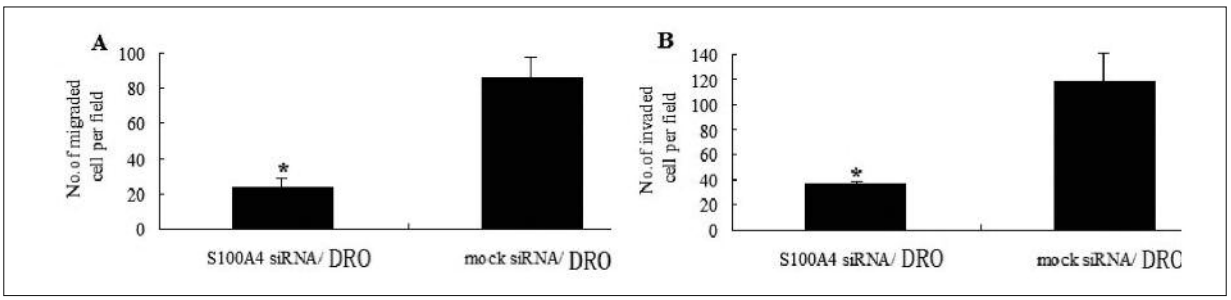

Figure 3. Knockdown of S100A4 expression reduces *in vitro* cell invasion and migration. ***A,*** Migration assays in DRO cells. ***B,*** Migration and invasion assays in DRO cells. Magnification, ×100. Columns, mean obtained from three individual experiments; bars, SD. $p < 0.05$ (*t* test).

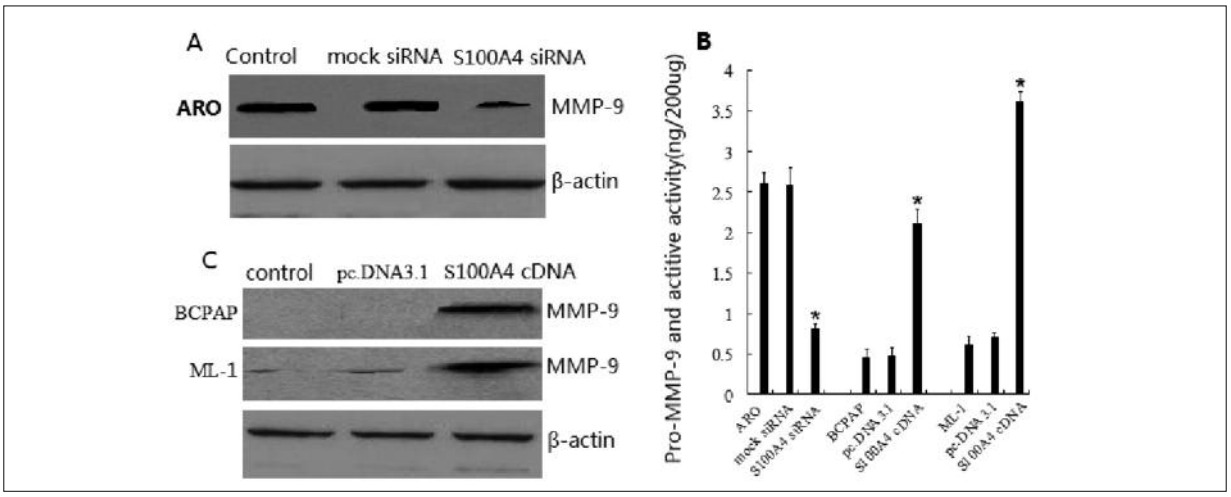

Figure 4. MMP-9 expression was up-regulated by S100A4 cDNA transfection and down-regulated by S100A4 siRNA transfection. ***A,*** Western blot analysis of MMP-9 protein expression in S100A4 siRNA-transfected DRO. ***B,*** Western blot analysis of MMP-9 protein expression in S100A4 cDNA-transfected BCPAP and ML-1 cells. ***C,*** MMP-9 activity assay showing that MMP-9 was up-regulated by S100A4 cDNA transfection and down-regulated by S100A4 siRNA transfection. columns, mean from three separate experiments; bars, SD ($p < 0.05$).

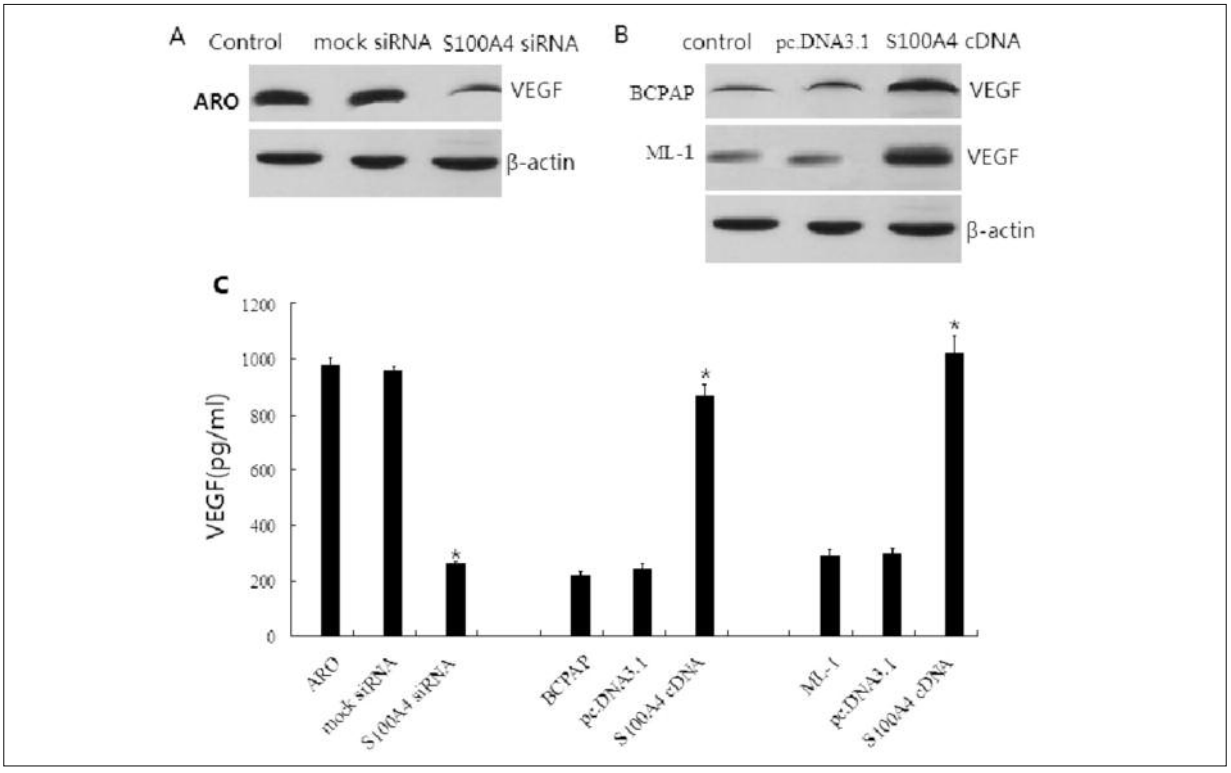

Figure 5. VEGF expression was up-regulated by cDNA transfection and down-regulated by S100A4 siRNA transfection. ***A,*** Western blot analysis of VEGF protein expression in siRNA-transfected DRO cells. ***B,*** Western blot analysis of VEGF protein expression in S100A4 cDNA-transfected DRO cells. ***C,*** VEGF assay showing that VEGF level in culture medium was up-regulated by cDNA transfection and down-regulated by S100A4 siRNA transfection. columns, mean from three separate experiments; bars, SD (*$p < 0.05$).

Down-Regulation of MMP-9 and VEGF Decreased Cancer Cell Invasion

To further confirm the role of MMP-9 and VEGF in BCPAP and ML-1 cell invasion, the S100A4 cDNA-transfected BCPAP and ML-1 cell (stable transfectant) were transfected with human MMP-9 siRNA or VEGF siRNA. Down-regulation of MMP-9 or VEGF by siRNA transfection showed low expression of MMP-9 or VEGF protein as confirmed by Western blot analysis, respectively (Figure 6A). To further test whether the S100A4 cDNA -induced invasion of BCPAP and ML-1 cells is mediated through MMP-9 and VEGF, we examine the invasive potential of MMP-9 siRNA or VEGF siRNA-transfected BCPAP and ML-1 cells that were stably transfected with S100A4 cDNA. As illustrated in Figure 6B-C, both MMP-9 siRNA and VEGF siRNA-transfected cells showed a low level of penetration through the Matrigel-coated membrane compared with the control siRNA-transfected cells. The invasion ability decreased 6-fold and 1.5-fold in the invaded BCPAP cells transfected with MMP-9 siRNA and VEGF siRNA compared with that of control siRNA, respectively (data not shown). The invasion ability decreased 3.6-fold and 1.2-fold in the invaded ML-1 cells transfected with MMP-9 siRNA and VEGF siRNA compared with that of control siRNA, respectively (data not shown). The results above shown S100A4 promotes invasion mainly by MMP-9 upregulation.

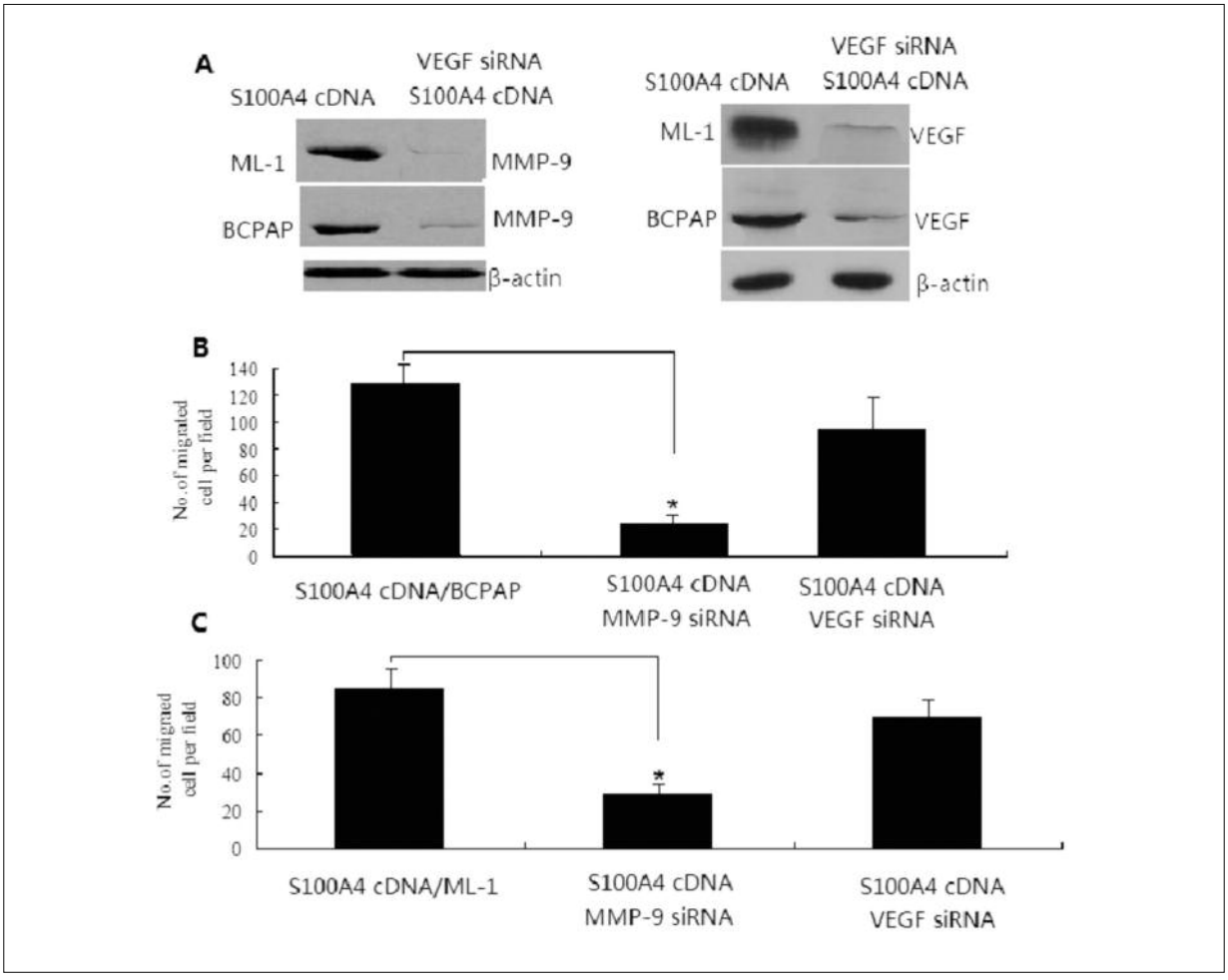

Figure 6. Down-regulation of MMP-9 or VEGF by siRNA transfection decreased tumor cell invasion. ***A,*** Down-regulation of MMP-9 or VEGF by siRNA transfection showed low-expression of MMP-9 or VEGF protein in S100A4 cDNA-transfected BCPAP and ML-1 cells as confirmed by Western blot analysis. ***B, C,*** Invasion assay showing that MMP-9 or VEGF siRNA-transfected cells resulted low penetration of cells through the Matrigel-coated membrane. columns, mean from three separate experiments; bars, SD (*$p < 0.01$).

S100A4 Overexpression Promoted AngioGenesis and in vivo Growth of Thyroid Cancer Cells

To analyze the effect of S100A4 gene overexpression in BCPAP cells in affecting angiogenesis, the cells alone or expressing GFP or S100A4 were used for the angiogenesis assay *in vivo*. BCPAP xenografts induced angiogenesis on the CAM, and overexpression of S100A4 significantly increased tumor-induced angiogenesis while the tumor size was similar 4 days after the implantation (Figure 7A). This result suggested that S100A4 expression initially promoted angiogenesis before affecting tumor growth. To test the effect of S100A4 on tumor growth, the tumor sizes were analyzed 9 days after the implantation of BCPAP cells on the CAM cells. Over-expression of S100A4 greatly promoted the tumor growth when it was analyzed 9 days after the implantation (Figure 7B). Western blot assay of the tumor sections showed that S100A4 overexpression in BCPAP cells increased the expression of VEGF in tumor sections (Figure 7C), further confirming the crucial role of S100A4 in regulating the expression of VEGF during the tumor angiogenesis. Furthermore, the results (Figure 7D) showed that HUVEC treated with conditioned media from mock- and S100A4-transfected BCPAP cells were able to form capillary-like structures. It shows that HUVEC treated with conditioned media from S100A4-transfected BCPAP cells showed more capillary-like networks (54.6%), as compared with the controls. S100A4 overexpression has the same results on ML-1 cell angiogenesis (data not shown).

Down-Regulation of S100A4 Expression Inhibited Angiogenesis of Thyroid Cancer DRO Cells

To analyze the effect of S100A4 gene silencing in DRO cells in affecting angiogenesis, the cells alone or S100A4 gene silencing were used for the angiogenesis assay *in vivo*. DRO xenografts induced angiogenesis on the CAM, and S100A4 gene silencing significantly inhibited tumor-induced angiogenesis 4 days after the implantation (Figure 8A). Western blot assay of the tumor sections showed that S100A4 gene si-

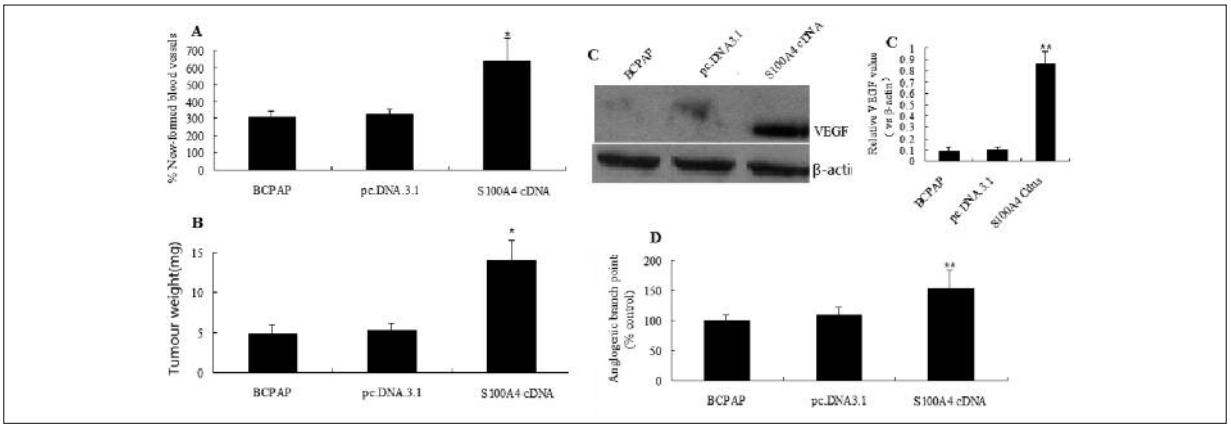

Figure 7. Over-expression of S100A4 promoted angiogenesis and growth of tumor. The BCPAP cells were stablely transfected with pc.DNA3.1-S100A4. ***A,*** BCPAP and BCPAP/pc.DNA3.1-S100A4 cells were harvested 24 h after the infection, and re-suspended in the serum-free medium. The cells were mixed with Matrigel at 1:1 ratio, and 30 µl of the mixture (3×10^6 cells) was implanted onto the CAM of a 9 day old chicken embryo. The CAM was cut off 4 days after the implantation, and the number of blood vessels on the CAM was counted under microscope. At least five unit areas of each CAM were counted under the tumor. The results are mean and SD from two experiments (n = 8). *Indicates the significant difference when compared to the pc.DNA3.1 control ($p < 0.05$). ***B,*** BCPAP and BCPAP/pc.DNA3.1-S100A4 cells were implanted onto the CAM as above. The tumors were harvested and weighed 9 days after the implantation. The data indicate the mean ± SD (n = 8) of three independent experiments. *Indicates the significant difference when compared to the pc.DNA3.1 control ($p < 0.05$). ***C,*** BCPAP and BCPAP/pc.DNA3.1-S100A4 cells were mixed and implanted onto the CAM of a 9 day old chicken embryo as above. After 9 days of incubation, tumor tissues were grounded by liquid nitrogen. Tissue lysates were analyzed by immunoblotting for VEGF expression. Relative VEGF levels were from three replicate experiments. *Indicates the significant difference when compared to the vector control ($p < 0.05$). ***D,*** HUVEC endothelial cells treated with conditional medium from BCPAP and BCPAP/pc.DNA3.1-S100A4 cells by *in vitro* angiogenesis assay. The conditioned medium of BCPAP and BCPAP/pc.DNA3.1-S100A4 cells was collected following filtering of medium. HUVEC endothelial cells seeded in eight-chamber slides were cultured with the above medium for 72 h until the formation of capillary network was observed. In the end of the experiment, angiogenesis was assessed by H&E staining and photographed under a microscope columns, mean from three separate experiments; bars, SD ($p < 0.01$).

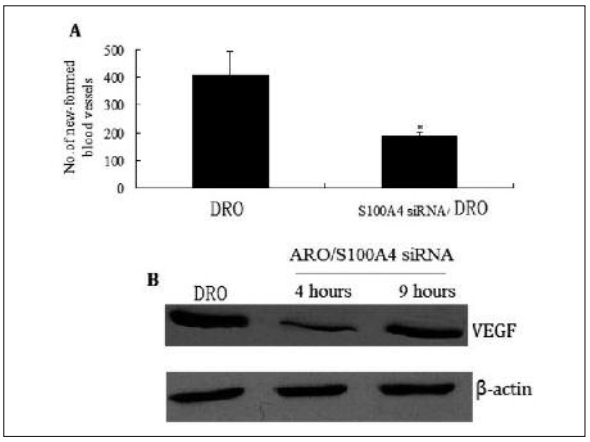

Figure 8. Down-regulation of S100A4 expression inhibited angiogenesis of thyroid cancer DRO cells. The DRO cells were transient transfected with S100A4 siRNA for 48 hours. ***A,*** DRO and DRO/S100A4 siRNA cells re-suspended in the serum-free medium. The cells were mixed with Matrigel at 1:1 ratio, and 30 µl of the mixture (3×10^6 cells) was implanted onto the CAM of a 9 day old chicken embryo. The CAM was cut off 4 days after the implantation, and the number of blood vessels on the CAM was counted under microscope. At least five unit areas of each CAM were counted under the tumor. The results are mean and SD from two experiments (n = 8). *Indicates the significant difference when compared to the DRO control ($p < 0.05$). ***B,*** DRO and DRO/S100A4 siRNA cells were mixed and implanted onto the CAM of a 9 day old chicken embryo as above. After 4 days of incubation, tumor tissues were grounded by liquid nitrogen. Tissue lysates were analyzed by immunoblotting for VEGF expression. Relative VEGF levels were from three replicate experiments. *Indicates the significant difference when compared to the DRO control ($p < 0.05$).

lencing in DRO cells decreased the expression of VEGF in tumor sections 4 days after the implantation (Figure 8B), further confirming the crucial role of S100A4 in regulating the expression of VEGF during the tumor angiogenesis. Furthermore, the results showed that HUVEC treated with conditioned media from S100A4 gene silencing DRO cells did not form capillary-like structures (data not shown). However, S100A4 gene silencing did not greatly inhibit the tumor growth when it was analyzed 9 days after the implantation. Furthermore, VEGF and S100A4 in tumor sections did not greatly decrease compared to the DRO cells control (data not shown). That transient transfection only lasts a short term to inhibit target gene may be the results.

Down-Regulation of S100A4 Expression by siRNA Promotes DRO cell Growth Inhibition and Apoptosis

The effect of S100A4 protein reduction on DRO cell proliferation was examined. Cell proliferation was measured by BrdU uptake after S100A4 knockdown in DRO cells. As shown in Figure 9A, cell proliferation in DRO/S100A4-siRNA cells was reduced by 42.7 ± 5.4% compared with that in DRO/mock siRNA cells.

We examined the growth inhibitory effects of down-regulation of S100A4 expression using the MTT assay in DRO cells. Significant cell growth inhibition was shown in DRO/S100A4-siRNA cells compared with that in DRO/mock siRNA cells (Figure 9B).

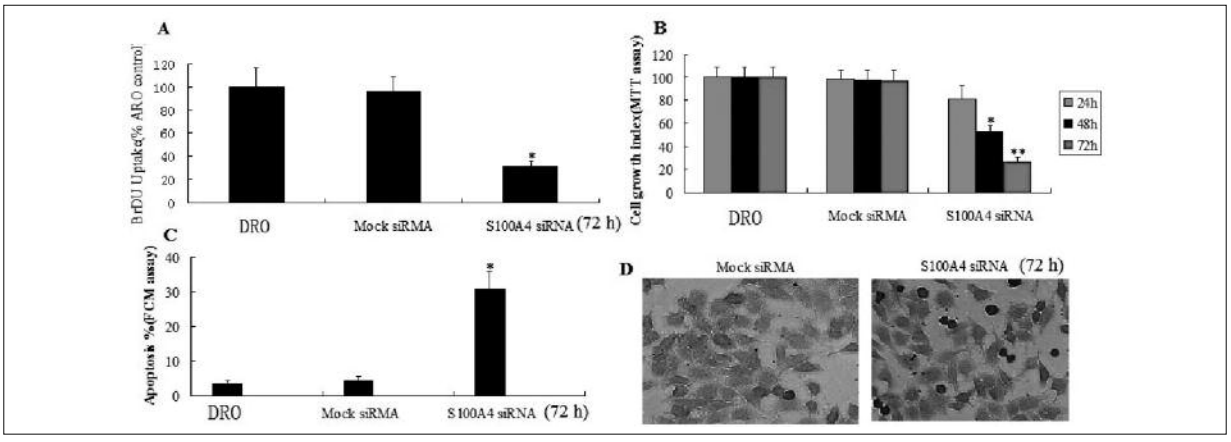

Figure 9. Effect of down-regulation of S100A4 expression on DRO cell growth and apoptosis , BrdU uptake in DRO/S100A4-siRNA cells compared with that in DRO/mock-siRNA cells after 72 h incubation at 37°C. Data are presented as mean ± sem of three experiments. ***B,*** Inhibition of cancer cell growth by S100A4 siRNA as measured by MTT assay. C, Induction of DRO cell apoptosis by S100A4 siRNA as measured by FCM. D, Induction of DRO cell apoptosis by S100A4 siRNA as measured by TUNEL. Points, mean of three separate experiments having six determinations per experiment for each experimental condition; bars, SD. *$p < 0.05$; **$p < 0.01$, relative to control.

Next, we examined whether the inhibition of cell growth was also accompanied by the induction of apoptosis induced by S100A4-siRNA. FCM assay demonstrated S100A4-siRNA transfection for 72 hours induced significant apoptosis in DRO cells (Figure 9C). TUNEL assay has the same result as FCM assay, the TUNEL-positive cells in S100A4 siRNA DRO cells was 8.4 ± 1.2, which is higher compared with that in DRO/mock siRNA cells (0.78 ± 0.2) (Figure 9 D). These results provided convincing data showing that S100A4-siRNA could induce apoptosis in DRO cells.

Discussion

S100A4 plays important roles in human cancers, including thyroid cancer [8,29-32]. It has been shown to enhance tumor growth and inhibition of apoptosis, motility, migration, invasiveness, angiogenesis, tumor spreading and metastasis[41]. However, the precise role and mechanism of S100A4 for tumor cell proliferation, invasion, and angiogenesis remains unclear.

The ability to migrate is a prerequisite for a cancer cell to escape the primary tumor and enter the circulation. However, to travel through the surrounding stroma,these locally invading cells must be able to proteolytically degrade extracellular matrix components. Matrix metalloproteinases (MMPs) play an essential role in this process, and several experimental strategies have established an intimate connection between S100A4 and certain members of the MMP family. Down-regulation of S100A4 expression in osteosarcoma cells led to reduced expression of MMP-2 and MT1-MMP, with a subsequent reduction in MMP-2 activity and a reduced ability to migrate through Matrigel-coated filters[42]. The invasive capability of human prostate cancer cells is also stimulated by S100A4, at least partly through transcriptional activation of MMP-9[43]. In osteosarcoma cell lines[44]. MMP-2 or other MMPs were unable to detect any induction upon treatment with extracellular S100A4, suggesting that intracellular functions of S100A4 are involved, at least in certain cell systems. S100A4 is associated with thyroid tumor invasion and metastasis[29-32], but the impact of S100A4 expression on MMP activation was not investigated in thyroid cancer.

In this study, we found down-regulation of S100A4 reduced both VEGF expression and MMP-9 protein expression, but also inactivated the pro-MMP-9 protein to its active form. Furthermore, down-regulation of S100A4 by siRNA decreased cell invasion but S100A4 overexpression by cDNA transfection increased tumor cell invasion. To further test whether the S100A4-induced invasion of thyroid cancer cells is mediated through MMP-9, we used Matrigel invasion chamber assay to examine the invasive potential of MMP-9 siRNA or VEGF siRNA-transfected thyroid cancer cells that were stably transfected with S100A4.We found only MMP-9 siRNA-transfected cells showed a low level of penetration through the Matrigel-coated membrane compared with the control siRNA-transfected cells. Although VEGF siRNA-transfected cells showed a low level of penetration through the Matrigel-coated membrane,there was no statistical significance compared to the control siRNA. The results above shown S100A4-induced invasion of thyroid cancer cells was mainly through MMP-9 pathway.

Based on observations in transgenic mice, S100A4 has been identified as a potent stimulator of angiogenesis[45-46]. The mechanisms that participate in this process are only starting to become unraveled. Augmented MMP-13 expression in endothelial cells was associated with

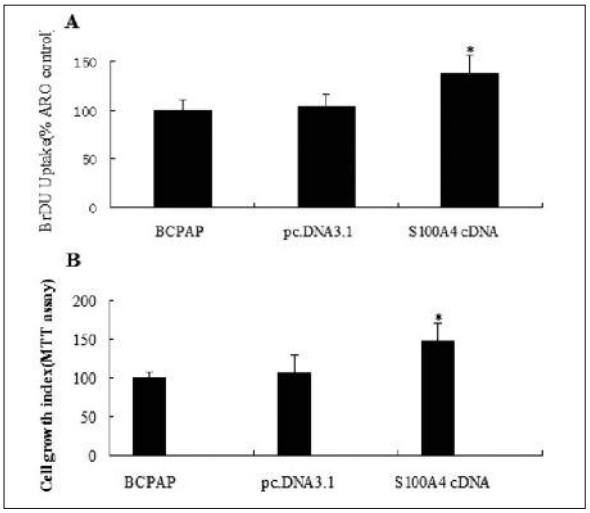

Figure 10. Effect of S100A4 overexpression on BCPAP cell growth ***A,*** BrdU uptake in BCPAP/S100A4 cells compared with that in BCPAP/pc.DNA3.1 cells after 72 h incubation at 37°C. Data are presented as mean ± sem of three experiments. ***B,*** Promotion of cancer cell growth by BCPAP/S100A4 as measured by MTT assay. Points, mean of three separate experiments having six determinations per experiment for each experimental condition; bars, SD. *$p <$ 0.05, relative to control.

S100A4-mediated stimulation of capillary-like growth in three-dimensional Matrigel cultures *in vitro*[47], and through interaction with annexin II, extracellular S100A4 accelerated tissue plasminogen activator-mediated conversion of plasminogen to plasmin, resulting in capillary-like tube formation of human cerebromicrovascular endothelial cells[48]. Interestingly, S100A4-stimulated plasmin activation may also contribute to the observed activation of MMP-2 and MMP-13. However, it seems unlikely that annexin II binding or plasminogen conversion would result in increased transcription of several MMPs.

In this study, we have shown down-regulation of S100A4 by siRNA decreased cell angiogenesis but S100A4 overexpression increased cell angiogenesis. The vascular endothelial growth factor (VEGF) is one of the most potent endothelial cell mitogens and plays a crucial role in both angiogenesis and lymphogenesis[34]. In the present study, we found VEGF was induced in S100A4-overexpressed tissue and cells and VEGF was inhibited in S100A4 siRNA transfected tissue and cells.We therefore concluded S100A4-induced angiogenesis of thyroid cancer cells was in part through VEGF pathway.

S100A4 has been implicated in the proliferation, apoptosis, cell cycle progression of many malignant tumors. Hua et al[40] has reported S100A4 inhibition by RNAi lead to reduced proliferation and increased apoptosis of gastric cancer cell line BGC823 by decreasing the expression of both NF-kappaB p65 and phospho (Ser32)-I-kappaB-alpha. Tabata et al[50] has found knockdown of S100A4 strongly suppressed cell growth, induced G2 arrest and eventual apoptosis by inducing expression of the tumor suppressor genes PRDM2 and VASH1. In human anaplastic thyroid carcinoma cells (DRO), knockdown of S100A4 by RNA interference inhibited the growth and increased the sensibility of DRO to paclitaxel after S100A4 knockdown[33]. It was demonstrated using S100A4 knockout mice that S100A4 might cooperate with p53 to induce apoptosis also *in vivo*[51]. S100A4-/- mice are viable and phenotypically normal in the postnatal period, but 10% of the animals develop tumors at age 10 to 14 months. The Authors speculated that this could be explained by loss of p53 tumor suppressor activity due to impaired interaction with S100A4.

In the present study,we found down-regulation of S100A4 by siRNA promotes apoptosis and inhibits growth but S100A4 overexpression by cDNA transfection promotes tomour growth. Thus, S100A4 may act both as a tumor suppressor. Because proliferation and apoptosis was also regulated by both VEGF and MMP-9 protein expression, we therefore concluded S100A4 promotes apoptosis and inhibits growth by VEGF and MMP-9 signal.

Conclusions

We presented experimental evidence which strongly supports the antitumor and antimetastatic and angiogenesis effects of S100A4 sliencing in thyroid cancer.Downregulation of S100A4 could be an effective approach for the inactivation of its target genes MMP-9 and VEGF expression, resulting in the inhibition of invasion, metastasis and angiogenesis, which could be useful for devising novel preventive and therapeutic strategies for thyroid cancer.

Acknowledgements

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Competing Interests

The Authors declare that they have no competing interests.

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