Targeting $\text{S100P}$ Inhibits Colon Cancer Growth and Metastasis by Lentivirus-Mediated RNA Interference and Proteomic Analysis

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$\text{S100P}$ was recently found to be overexpressed in a variety of cancers and is considered a potential target for cancer therapy, but the functional role or mechanism of action of $\text{S100P}$ in colon cancer is not fully understood. In the present study, we knocked down the gene expression of $\text{S100P}$ in colon cancer cells using lentivirus-mediated RNA interference. This step resulted in significant inhibition of cancer cell growth, migration and invasion in vitro and tumor growth and liver metastasis in vivo. Moreover, $\text{S100P}$ downstream target proteins were identified by proteomic analysis in colon cancer DLD-1 cells with deletion of $\text{S100P}$. Knockdown of $\text{S100P}$ led to downregulation of thioredoxin 1 and $\beta$-tubulin and upregulation of Rho guanosine diphosphate (GDP) dissociation inhibitor $\alpha$ (RhoGDIA), all potential therapeutic targets in cancer. Taken together, these findings suggest that $\text{S100P}$ plays an important role in colon tumorigenesis and metastasis, and the comprehensive and comparative analyses of proteins associated with $\text{S100P}$ could contribute to understanding the downstream signal cascade of $\text{S100P}$, leading to tumorigenesis and metastasis.

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INTRODUCTION

Colorectal cancer is one of the most common malignancies worldwide. Despite the improvement in its prognosis and therapy in the last few decades, nearly one-half of colorectal cancers relapse from metastasis after curative surgery (1). It is essential to develop new targets and therapeutic approaches, and therapeutic target development requires identification of novel functional molecules, their mechanisms of action and strategies for intervention (2). $\text{S100P}$ is a 95-amino acid residue protein that is a member of the $\text{S100}$ family. The protein was first purified from placenta with a restricted cellular distribution (3). $\text{S100P}$ proteins consist of $\text{Ca}^{2+}$ binding proteins of the elongation factor (EF)-hand type that mediate $\text{Ca}^{2+}$-dependent signal transduction pathways involved in the regulation of a number of cellular processes such as cell cycle progression and differentiation (4). Overexpression of $\text{S100P}$ was observed in various cancers, including pancreatic (5), breast (6), colon (7), prostate (8) and lung carcinomas (9). $\text{S100P}$ was also reported to correlate with tumor growth and metastasis in breast (6,10,11), colon (12) and pancreatic (3) cancer. $\text{S100P}$ ectopic expression in a non-metastatic rat mammary cell line caused a notable increase in local muscle invasion and a significant induction of metastasis in tumor-bearing animals (10). Gene transfer or extracellular addition of $\text{S100P}$ increased tumor growth and metastasis, and silencing of $\text{S100P}$ resulted in the reduction of the tumor growth and secondary metastatic volume in models of pancreatic cancer (3). Extracellular $\text{S100P}$ interacts with the receptor of advanced glycation end products (RAGE), which is associated with various cancers and stimulates Erk and nuclear factor (NF)-$\kappa$B activity (13). Overexpression of $\text{S100P}$ in pancreatic cancer Panc-1 cells protects cancer cells against cell death induced by chemotherapeutic 5-fluorouracil (5-FU) (3) and leads to increased $\text{S100A6}$ and cathepsin D expression, both of which are involved in cellular invasion (14). $\text{S100P}$ also appears to function to increase gastric cancer growth and invasion of the cancer cells (15). Despite recent advances in understanding the biology of $\text{S100P}$, the functional role or mechanism of action of $\text{S100P}$ in colon cancer is poorly understood. Fuentes et al. (12) showed that $\text{S100P}$ is specifically expressed in human colon cancer tissue but not in nor-
mal colon tissue, and exogenous S100P increases colon cancer SW480 cell proliferation and cell migration in vitro and up-regulates Erk phosphorylation and NFκB activation. Thus, it appears that S100P is a potential therapeutic target for colon cancer.

In the present study, we investigated the functional role and molecular mechanisms of S100P activity in colon cancer. We found that knockdown of S100P expression by lentiviral vector-mediated RNA interference (RNAi) inhibited colon cancer cell growth, migration and invasion in vitro, as well as tumor growth and liver metastasis in vivo. Furthermore, thioredoxin 1 (trx-1), β-tubulin and Rho GDP dissociation inhibitor α (RhoGDIA) were identified as S100P downstream target proteins in colon cancer cells by proteomic analysis.

MATERIALS AND METHODS

Cell Culture

Colon cancer cell lines DLD-1 and SW620 were used for the present study. All cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C in a humidified 5% CO2 incubator.

Lentiviral shRNA Vector Construction, Production and Transduction

Lenti-shRNA vector construction was done as previously described (16). Briefly, we synthesized DNA fragments containing GAC AGC ACA as the loop for short-hairpin RNA (shRNA) and cloned the shRNA targeting human S100P (5′-AAC TCA CTG AAG TCC ACC TGG GCA TCT CC-3′) into human U6 promoter–containing pBluescript SK(+) plasmid (pU6) after annealing. Then we subcloned the U6-shRNA cassettes into the lentiviral vector (16). A lentivirus carrying shRNA-targeting firefly luciferase (shLuc: 5′-TGC GCT GGT TGG GCA TCT CC-3′) was used as the control. Lentiviral packaging and transduction were carried out as previously described (16).

Reverse Transcription and Quantitative Real-time Polymerase Chain Reaction

Total RNA was isolated using Trizol (Invitrogen), and cDNA synthesis was performed using a Superscript First-Strand Synthesis Kit (Promega, Madison, WI, USA) (17). The quantification of mRNA levels was carried out using SYBR® Green PCR Master Mix (Applied Biosystems, Warrington, UK; PCR, polymerase chain reaction) and an ABI 7500 Real-Time PCR System (Applied Biosystems). The following forward and reverse primers were used: S100P, 5′-ATG ACG GAA CTA GAG ACA GCC ATG GGC-3′ and 5′-GGA ATC TGT GAC ATC TCC AGG GCA TCA-3′ (12); GAPDH, 5′-CCA GCC GGG CCC CAT GCC TC-3′ and 5′-ATG AGC CCC AGC CTT CTC CAT-3′. The relative expression of S100P was normalized to that of GAPDH, an endogenous housekeeping gene.

Western Blot

The SDS-PAGE and Western blot analysis was performed as described (17). The primary antibodies used were polyclonal antibodies against S100P, β-tubulin, thioredoxin, RhoGDIA and actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Cell Proliferation and Colony Formation Assays

Cell proliferation was measured using a methylthiazoletetrazolium (MTT) assay (17). For colony formation assay, 5,000 cells in complete DMEM were seeded onto 10-cm culture dish and allowed to grow for 14 d to form colonies, which were then stained with coomassie blue. The rate of colony formation was calculated with the following equation: colony formation rate = (number of colonies/number of seeded cells) × 100%.

Culture of Colon Cancer Cells in Three-Dimensional Collagen Gels

A total of 5,000 cells were collected and resuspended in a 2-mL solution
containing complete DMEM, rat tail collagen I (BD Biosciences, Franklin Lakes, NJ, USA), to support the growth of cells in a three-dimensional matrix (18). The solution was added into six-well plates and incubated for 30 min at 37°C until a homogenous gel was formed. The culture medium was added to the space surrounding collagen I gel embedded with cells and replenished every 2 d. After 14 d, results were observed and photographed. The number of spheroids was counted at 20 random fields with magnification of 40×.

Wound Healing and Cell Invasion Assays

A wound-healing assay was performed as described (14). Assayed cells (1 × 10⁶) were seeded on a six-well plate and cultured for 24 h. A scratch was made on the cell monolayer with a 200-μL pipette tip. The photographs were taken immediately and at 72 h after wounding. A 48-well Boyden chamber and polycarbonate membrane precoated with matrigel (BD Biosciences) with 10-μm pores (Neuro Probe, Gaithersburg, MD, USA) was used to evaluate the cancer cell invasion ability (19). DMEM with 10% fetal bovine serum was added to the lower compartment as a chemoattractant. DMEM with 10% fetal bovine serum was added to the lower compartment as a chemoattractant. DLD-1 or SW620 (4 × 10⁴) suspended in 50 μL DMEM with 0.5% BSA was loaded onto the upper compartment of each chamber. After incubation in a humidified atmosphere of 5% CO₂ at 37°C for 13 h (DLD-1 cells) or 38 h (SW620 cells), the cells on the upper surface of the membrane were gently scraped off, and the cells on the bottom surface of the membrane were fixed with 4% paraformaldehyde and stained with hematoxylin. Images were captured using a microscope at a ×100 magnification, and invasion cells were counted in 10 random selected fields. All experiments were done in triplicate.

Tumor Growth and Liver Metastasis Model in Nude Mice

Six-week-old male athymic nude mice were purchased from the Animal House, Chinese University of Hong Kong. All experiments were approved by the Animal Experimental Ethics Committee of the Chinese University of Hong Kong. Nude mice were injected subcutaneously with 1 × 10⁶ DLD-1 cells transduced with lenti-shLuc or lenti-shS100P. The volumes of tumors were monitored at the indicated times and calculated according to the formula: 0.5 × length × width². In vivo liver metastatic capability of colorectal cancer cells was evaluated in athymic nude mice (n = 10/group) as described previously (20). Briefly, a small left ab-
dominal incision was made under sterile conditions and spleen was exteriorized. Control or S100P knockdown DLD-1 cells ($1 \times 10^6$) in 0.1 mL PBS were injected into the spleen by means of a sterile tuberculin syringe and a 27-gauge needle. Ten minutes after the injection, a splenectomy was performed. The abdomen was closed with nylon sutures. After 6 wks, the animals were euthanized and liver metastases were examined.

Proteomic Analysis

The procedures were carried out as described previously (16,19). Briefly, for the first dimension, immobilized pH gradient (IPG) strips (13 cm, pH 4–7, nonlinear [NL]) were used according to the manufacturer’s instructions. The second dimension was run in 12.5% uniform sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel for protein separation. Thereafter, the protein spots were visualized by silver staining. All gel images were digitalized using a scanner (GS-800 calibrated densitometer; Bio-Rad, Hercules, CA, USA) with the Quantity One software (Bio-Rad). All images were analyzed using PDQuest (version 8.0, Bio-Rad) for spot detection and quantification. Spots of interest were selected and excised from the gels for identification. Protein identification was achieved by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) using a 4700 Proteomics Analyzer (TOF/TOF) (Applied Biosystems). Peptide mass mapping was carried out using the program MASCOT (Matrix Science, London, UK) against an NCBInr database with a GPS explorer software (Applied Biosystems). Tryptic autolytic fragments and notable contamination were excluded from the peak lists before the database search.

Statistical Analysis

Data are expressed as mean ± SD in this study. Statistical analysis was performed by using the independent samples t test (SPSS Inc., Chicago, IL, USA).

RESULTS

Efficient Knockdown of S100P Expression by Lentiviral Vector–Mediated RNAi in Colon Cancer Cells

To examine the effect of blocking S100P expression by lentiviral vector–mediated RNAi, real-time PCR and Western blot analysis were performed on DLD-1 and SW620 colon cancer cells. As shown in Figure 1A, S100P mRNA expression was reduced by 90% after lenti-shS100P infection in DLD-1 cells, but control lenti-shLuc did not alter S100P expression. The protein expression diminished to barely detectable levels in the DLD-1 cells infected with lenti-shS100P compared with the cells infected with the control lenti-shLuc or the uninfected blank cells (Figure 1C). Similar data were achieved in SW620 cells after lenti-shS100P infection (Figure 1B).

RNAi-Mediated Knockdown of S100P Suppressed Colon Cancer Cell Proliferation In Vitro

As revealed by MTT assay, S100P knockdown significantly reduced cell proliferation of colon cancer DLD-1 and SW620 cells (Figure 2A, B). As shown in Figure 2C and D, DLD-1 and SW620 cells infected with lenti-shS100P showed significant reduction in the colony formation. No apparent difference was found between the parental cells and the control cells (which were infected with lenti-shLuc). Moreover, Figure 3A and B showed the images of colon cancer DLD-1 and SW620 cell growth in a three-dimensional culture model. There was a clear decrease in both the size and number of spheroids (Figure 3A and Figure 4D) in cells with lenti-shS100P compared with the control cells. These results indicate a positive correlation between
the expression of S100P and the rate of colon cancer cell growth.

**Knockdown of S100P Inhibited Colon Cancer Cell Migration and Invasion In Vitro**

The effect of S100P on colon cancer cell migration and invasion was investigated by using a monolayer wound healing and chamber assays. As shown in Figure 4A, DLD-1–shS100P cells after a wound scratch significantly inhibited the ratio of wound sealing compared with the control DLD-1–shLuc cells. In addition, knockdown of S100P inhibited colon cancer cell invasion through matrigel-coated membranes (Figure 4B).

**RNAi Targeting S100P Inhibited Tumor Growth and Liver Metastasis of Colon Cancer In Vivo**

Tumor formation was compared between DLD-1–shLuc and DLD-1–shS100P cells in nude mice. Cells were inoculated in nude mice, and tumor growth was measured after 6 wks. As shown in Figure 5A and B, tumor formation was observed in all mice inoculated with DLD-1–shLuc or DLD-1–shS100P cells, while knockdown of S100P in DLD-1 cells showed a decrease in the size of tumors when compared with its control counterpart (Figure 5A). Moreover, Figure 5C shows the macroscopic appearance of liver metastasis after intrasplenic injection of colon cancer DLD-1 cells into the athymic nude mice spleen. Some macroscopic nodules (that is, metastasis) were found at the liver surface 6 wks after injection with DLD-1–shLuc cells and in 5 of 10 mice with macroscopic nodules (50% liver metastasis), whereas no macroscopic nodule was found at the liver surface after injection with DLD-1–shS100P cells in spleen.

**Proteomic Analysis of S100P Downstream Target Proteins in Colon Cancer Cells**

Image analysis using the PDQuest two-dimensional software identified 30 protein spots that displayed differential expression among DLD-1 transduced with lenti-shLuc and lenti-shS100P (Figure 6). Subsequently, each spot was analyzed and 24 S100P-associated proteins were identified by MALDI-TOF MS (Table 1). These proteins were all differentially expressed at least two-fold (either increased or decreased) when the comparison between the control and the S100P knockdown colon cancer DLD-1 cells was made. Of these, 15 were upregulated and 9 were downregulated. Among the 24 proteins, 3 with the most significant differential expression identified above were selected and further analyzed by Western blotting (Figure 6A, B). Consistent with two-dimensional gel electrophoresis (2-DE), and MALDI-TOF MS results, thioredoxin 1 and β-tubulin protein were significantly downregulated upon S100P-knockdown cancer cells. Additionally, higher expression protein level of RhoGDIA was found in the S100P-knockdown cells.

**DISCUSSION**

Recently, S100P proteins have become a major interest in cancer because they are overexpressed in a variety of tumors and their putative involvement in the metastatic process (2–4,21). An earlier report indicated that exogenous S100P increased colon cancer SW480 cell proliferation and cell migration in vitro (12). However, the role of S100P in tumorigenesis and metastasis of colon cancer in vivo and its molecular mechanisms have never been elucidated. In the current study, we suppressed S100P expression in colon cancer cells using lentiviral vector–mediated RNAi to determine its exact role in colon tumorigenesis and metastasis. Our group developed an efficient and convenient lenti-shRNA system that provided shRNA and fluorescent marker protein coexpression and allowed for easily identified transduced cells (16). Lentiviral infection has advan-
Lentivirus can infect both dividing and nondividing cells with high efficiency, achieve long-term stable expression of the transgene and have low immunogenicity. Lenti-shS100P was used to effectively knock down the expression of S100P in colon cancer cells. We observed a significant inhibition of cell growth by MTT, colony formation assay and the three-dimensional culture model in vitro when the expression of S100P was suppressed by RNAi in DLD-1 and SW620 cells. Also, knockdown of S100P drastically inhibited colon cancer cell migration and invasion in vitro, which is similar to its suggested role in pancreatic cancer (3). Furthermore, we used an in vivo animal model to explore the exact role of S100P in colon cancer tumorigenesis and liver metastasis. We observed a marked inhibition of colon cancer tumorigenicity and liver metastasis through knockdown of S100P expression in a nude mice model in vivo. These results indicated that S100P played a crucial role in the tumorigenesis and metastasis of colon cancer.

It is known that S100 proteins affect cell function by both intracellular and extracellular mechanisms (22). S100 proteins are also implicated in promoting cancer progression through specific roles in cell survival and apoptosis pathways (22). Recent studies have demonstrated that the actions of S100P are mediated by activation of RAGE in pancreatic and colon cancer cells (2,12,13). In the present study, proteomic analysis was used to identify S100P-associated downstream proteins in colon cancer cells. Among 24 differentially expressed spots, down-regulation of two proteins (trx-1 and β-tubulin) and upregulation of RhoGDIA were confirmed to be valid after Western blot analysis of DLD-1 cells with deletion of S100P. Trx-1 is a ubiquitously expressed small redox protein that is a key regulator of cellular redox balance. Trx-1 acts as an antioxidant, growth-promoting, antiapoptotic and inflammation-modulating protein that provides reduc-

**Figure 5.** RNAi targeting S100P inhibited tumor growth and liver metastasis of colon cancer in vivo. (A) Knockdown of S100P inhibited tumor growth in vivo. *P < 0.05; **P < 0.01. (B) Photographs of nude mice at 6 wks after inoculation with DLD-1 cells (n = 5). (C) Macroscopic appearances of liver metastasis. Suppression of S100P reduced liver metastases of colon cancer cells in the intrasplenic injection mouse model (n = 10). Metastatic tumor nodules were identified as whitish and patchy areas (indicated by arrows). shLuc, DLD-1 colon cancer cells transduced with lenti-shLuc; shS100P, DLD-1 colon cancer cells transduced with lenti-shS100P.

**Figure 6.** Differentially expressed protein spots among DLD-1 transduced with lenti-shLuc or lenti-shS100P (A) Selected regions of 2-DE gels illustrate differentially expressed proteins among DLD-1 transduced with lenti-shLuc or lenti-shS100P. Enlarged images of interest spots were shown. (B) Confirmation of differential expression of thioredoxin 1, β-tubulin and RhoGDIA. Western blot analysis showed that thioredoxin 1, β-tubulin and RhoGDIA proteins were differentially expressed among DLD-1 transduced with lenti-shLuc or lenti-shS100P. Actin was used as an internal control.
ing equivalents and a transcriptional regulator (23). Trx-1 expression was reported to be increased in several human cancers, including lung (24,25), liver (26), pancreatic (27), colorectal (28,29) and gastric carcinoma (30). Increased trx-1 levels are associated with increased proliferation of tumor cells, inhibition of apoptosis, aggressive tumor growth and decreased patient survival (23). In human colorectal cancer, trx-1 overexpression is related to a poor prognosis in patients with liver metastases (28,29).

Tubulin is the basic subunit of microtubules and the α, β tubulin dimer assembles forming microtubules. Microtubules are important cellular targets for anticancer therapy because of their key role in cell division, intracellular transport, maintenance of cell shape and cellular motility (31). Microtubule-targeting drugs such as taxanes and vinca alkaloids have been used successfully to treat a variety of cancers in the clinic (32). Tubulin as an antitumor target continues to attract the attention of significant drug discovery and development (33).

Table 1. Differentially expressed proteins between control and S100P knockdown colon cancer cells were identified by MALdi-TOF MS.

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* Molecular mass/isoelectric point (M/pI).

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DISCLOSURE
The authors declare that they have no competing interests as defined by Molecular Medicine, or other interests that might be perceived to influence the results and discussion reported in this paper.

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