SYMPOSIUM: MOLECULAR GENETICS IN SARCOMA

S100A6 Expression and Function in Human Osteosarcoma

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Abstract There is a critical need to identify markers that can accurately identify existing or predict future metastatic disease in patients with osteosarcoma since the majority of patients present with undetectable micrometastatic disease. We previously reported S100A6 is overexpressed in human osteosarcoma and increased expression of S100A6 by immunohistochemistry correlated with decreased clinical metastasis. We have established 11 primary cultures from biopsies of patients with osteosarcoma and ten of the 11 primary cultures have increased expression of S100A6 relative to normal human osteoblasts. To further explore possible mechanisms for metastasis suppression previously reported, we used in this report siRNA-mediated knockdown of S100A6 in four commonly used human osteosarcoma lines, then examined their cell adhesion, migration, and invasion properties. Knockdown of S100A6 expression inhibited cell adhesion and promoted cell migration and invasion in these lines. Conversely, S100A6 overexpression enhanced cell adhesion and inhibited cell invasion. Our data demonstrate S100A6 is commonly overexpressed in human osteosarcoma. S100A6 may inhibit osteosarcoma metastasis by promoting cell adhesion and inhibiting cell motility and invasion. Thus, S100A6 may be considered a potential marker for human osteosarcoma with prognostic value for identifying patients without metastases.

Introduction

Osteosarcoma is the most common primary malignancy of bone, with a peak incidence in the second decade of life [33, 48]. Although 80% of patients have metastatic or micrometastatic disease at the time of diagnosis, less than 15% of these patients are clinically detectable because most of the patients have micrometastatic disease [20, 24, 47, 50]. Because we cannot distinguish between patients who have metastatic disease from those who do not, nearly all patients receive both chemotherapy and surgical resection. Therefore, there is a clinical need to identify genetic markers that can accurately predict the presence or absence of metastasis, thus sparing the 20% of patients who will never develop metastasis the need for chemotherapy. Currently, the genetic event(s) leading to the development of human osteosarcoma are not known; this may reflect the heterogeneity of this malignancy. Mutations of Rb and p53 have been identified in a subset of patients with osteosarcoma, yet there is no consensus on genetic alteration [8, 15, 36, 39, 49]. Therefore, identification of markers specific for human osteosarcoma remains an ongoing process.

We recently identified S100A6 as a potential prognostic marker of human osteosarcoma [29]. Over 80% of the

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paraffin-embedded samples had overexpression of S100A6. Increased expression of S100A6 was associated with decreased clinical metastasis at diagnosis and at followup. S100A6 is a member of the S100 protein family that includes over 20 members, and several members of the S100 protein family have been linked to human cancers [2, 4, 7, 19, 22, 25, 30]. For example, S100A4 has been associated with metastasis in several human cancers, including colon, breast, esophageal, and non-small cell lung cancers [21, 34, 38, 46]. Several S100 proteins are commonly used clinically as immunohistochemical markers to identify and classify a number of human tumors [2, 4, 19].

S100A6 is an intracellular protein that is overexpressed in several human tumors [10, 19]. Proteomic profiling of musculoskeletal soft tissue sarcomas by mass spectrometry demonstrates increased expression of S100A6 [18]. Although little is known about the exact functional role of S100A6, it does interact with the actin cytoskeleton through tropomyosin [5, 14]. The actin microfilament system attaches to the adherens junctions and is involved in cell contractility and adhesion-dependent signaling with the extracellular matrix [37]. S100A6 also interacts indirectly with chaperone proteins such as Hsp70 in the nucleus [42]. Molecular chaperones such as the heat shock proteins play pivotal roles in regulating cellular processes such as protein processing, DNA replication, and transcription. These functions, particularly the cell-cell and cell-matrix interactions, may play an important role in cancer metastasis. In order for a tumor cell to metastasize, it must (a) overcome local adhesive forces, (b) migrate, (c) invade the microvasculature, (d) survive in the vasculature, (e) extravasate at the secondary site, (f) proliferate and (g) recruit new blood vessels [43, 51].

Based on our previous immunohistochemical findings on tumor samples [29] and the reported cellular functions of S100A6, we hypothesized that: (1) S100A6 is highly expressed in these primary cultures and commercially available cell lines; (2) siRNA mediated knockdown of S100A6 inhibits cell adhesion; and (3) siRNA mediated knockdown of S100A6 promotes cell motility and invasion.

Materials and Methods

The objectives of this investigation were to (1) establish primary cultures of osteosarcoma from patient samples; (2) explore expression of S100A6 in primary osteosarcoma samples and commercially available cell lines; (3) identify siRNA target sequences and determine knockdown efficiency; (4) investigate the effects of siRNA mediated knockdown of endogenous S100A6 on cell adhesion; and (5) examine the effects of knockdown of S100A6 on cell

motility and invasion. We established 11 primary cultures from patients and determined the expression level of S100A6 by qRT-PCR in these samples and four commercial lines (TE85, MNNG/HOS, 143B, and MG63) in triplicate. We identified efficient siRNA target sites for S100A6 and assessed the ability for osteosarcoma cells to adhere to a Type I collagen matrix when endogenous S100A6 expression was knocked down. Adhesion experiments were performed in triplicate and performed in three batches. Next, we determined the ability for TE85, MNNG/HOS, 143B, and MG63 osteosarcoma cells to migrate across a wound created on a monolayer of cells when endogenous S100A6 expression was knocked down. Finally, we examined the effects of S100A6 overexpression and knockdown on osteosarcoma cell invasion. Again, at least two sets of experiments were performed for each cell line in the migration and invasion assays.

Cell lines (TE85, MNNG/HOS, 143B, MG63, HEK293, C3H10T1/2, and hFOB) were from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in the recommended media (complete Modified Eagle Medium [MEM] or Dulbecco's Modified Eagle Medium [DMEM]) containing 10% fetal bovine serum (FBS; HyClone, Logan, UT), 100 units of penicillin, and 100 μg of streptomycin. Unless otherwise indicated, all chemicals were purchased from Sigma-Aldrich (St Louis, MO) or Fisher (Pittsburgh, PA). We chose to use the ATCC osteosarcoma cell lines (TE85, MNNG/HOS, 143B, and MG63) because these are well characterized and commercially available lines.

Human osteosarcoma samples (n = 23) from biopsy or resection cases performed at the University of Chicago between 2004 and 2006 were immediately collected for primary cultures. The use and selection of human tumor specimens followed the guidelines approved by the Institutional Review Board. Attempts were made to identify patients who had not been exposed to chemotherapy. Patients who had biopsies performed at an outside institution were not included because the first fresh tissue obtainable would have been the postneoadjuvant chemotherapy resection sample. The purpose of our banking efforts is to establish a resource for future evaluation of potential markers. Informed consent was obtained from the patient or legal guardian. To ensure we had representative tumor tissue, all open biopsies performed in the operating room included a frozen section. The tumor samples taken were divided in half (one for frozen section and the other for our tissue banking purposes). The presence of tumor cells was verified by histology. Eleven primary cultures were successfully established. Eight of the 11 samples had frozen sections performed to ensure we had tumor tissue. One sample was a core needle biopsy in which a frozen section was not performed for practical patient care issues.

The remaining two samples were resection specimens in which those samples for permanent sections were divided and half sent for tissue banking. The frozen and permanent sections verified the samples were tumor samples. Only one sample came from a patient who was not chemotherapy naïve. The sample was obtained from a resection of a liver metastasis in a patient who received chemotherapy at the time of initial treatment, 6 years and 10 months prior to the resection of the liver metastasis. Again, the tumor was harvested free of liver tissue and half sent for our tissue banking and the other half sent for histology. Patient age, location of tumor, initial stage, and current status were recorded for each patient (Table 1). All collected samples were minced, treated with collagenase (0.2 mg/mL) at 37°C, and plated in MEM with 20% FBS (HyClone), penicillin, and streptomycin. Cells that were expanded successfully and to a maximum of two passages were frozen down in media containing 10% dimethyl sulfoxide and stored at −70°C. We expanded primary cultures that were successfully established, and which subsequently survived a freeze-thaw cycle.

For RNA isolation from the cells and quantitative polymerase chain reaction (qPCR), subconfluent cells were seeded in culture flasks in complete medium supplemented with 1% fetal calf serum. After the indicated treatments, total RNA was isolated using TRIZOL reagents (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. We performed the qPCR as described previously [26]. Briefly, 10 μg of total RNA were used to generate cDNA templates by reverse transcription with hexamer and Superscript II reverse transcriptase (Invitrogen). The qPCR primers were 18-mers designed by using the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The qPCR primers were GGGAGGGTGACAAGCACA and TCCGGTCCAAGTCTTCCA. Duplicate reactions were carried out and all samples were normalized to GAPDH. As a control, we used C3H10T1/2 mesenchymal stem cells (MSCs) because the C3H10T1/2 cells have relatively high expression of S100A6.

Given the known interaction of S100A6 with the cytoskeleton, we next investigated the effects of S100A6 overexpression and siRNA-mediated knockdown on cell adhesion to a Type I collagen substrate in vitro. We therefore first sought to knock down the endogenous expression S100A6 in the commercially available osteosarcoma cell lines (TE85, MNNG/HOS, 143B, and MG63). Based on our previous finding that decreased S100A6 expression is associated with decreased clinical metastasis, our objective was to determine the functional effects of S100A6 knockdown on phenotypes that are important for metastasis in vitro. We first determined the optimal sites on the human S100A6 gene for efficient RNAi-mediated knockdown.

To maximize siRNA efficiency and minimize any off-target effects, we used the Applied Biosystems (Austin, TX) software available at www.ambion.com/techlib/resources/RNAi/index.html to select optimal target sequences to human S100A6. The selected target sites were screened for their knockdown efficiency using our recently developed pSOS-HUS system [27] (Fig. 1A). This vector consists of a chimeric sequence between GFP and the S100A6 target gene driven by an hEF1 promoter as well as H1 and U6 dual promoters driving the expression of the siRNA cassette. Thus, on introducing pSOS-based siRNA plasmids into mammalian cells, the siRNA fragments generated form an RNA duplex with the target chimeric sequence (Fig. 1B). Cells were transfected using LipofectAMINE (Invitrogen, Carlsbad, CA) according to the manufacture's protocol and efficiency visualized by fluorescent imaging. Using this system, the optimal siRNA target sites to S100A6 were: AGAAGGAGCTCACCATTGG and AGCTCACCATTGGCTCGAA. The adenoviruses expressing siRNAs to both

Table 1. Clinical data on primary cultures

Sample	Age	Location	Initial stage	Current status
1	22	tibia	IIB	NED
2	16	Femur	IIB	NED
3	7	humerus	IIB	NED
4	12	femur	IIB	NED
5	13	femur	IV	Dead
6	14	tibia	IIB	NED
7	16	femur	IIA	NED
8	16	liver metastasis (primary: femur)	IIB	Dead; sample from liver metastasis at 82 months
9	43	arm (extraskeletal)	IIA	Dead; metastasis at 21 months
10	17	humerus	IIB	NED
11	18	femur	IIB	NED

NED = no evidence of disease.

Fig. 1A–B A schematic of siRNA knockdown strategy is illustrated. Potential target sites for siRNA-mediated knockdown were characterized using our pSOS-HUS system. (**A**) Vector map showing the siRNA cassettes expression driven by a dual promoter system and a chimeric transcript between GFP and S100A6 is shown. (**B**) On introduction into mammalian cells, the siRNAs produced will form a duplex with the fusion transcript and be degraded by the cell's double stranded RNase activity.
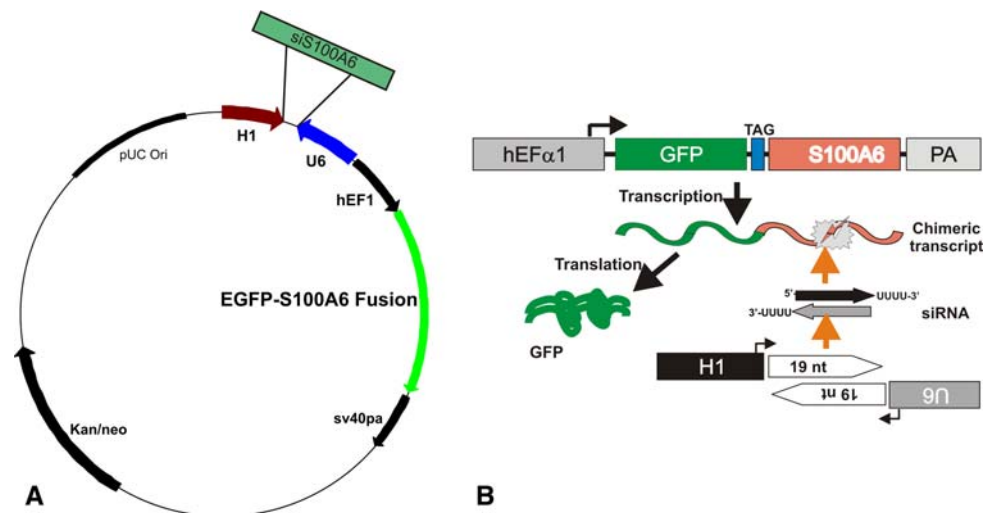
target sequences were pooled to maximize knockdown efficiency in the subsequent experiments.

For our transient transfections, subconfluent cells were transfected with S100 expression vectors using Lipofect-AMINE (Invitrogen, Carlsbad, CA). At 24 hrs after transfection, the cells were collected and lysed in Laemmli sample buffer. Cleared total cell lysate was denatured by boiling and loaded onto a 12% SDS-polyacrylamide gel (approx.10 μg total proteins per lane). After electrophoretic separation, proteins were transferred to an Immobilon-P membrane (Millipore, Bedford, MA) via electroblotting. The membrane was blocked with 5% nonfat milk in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween-20) at room temperature for one hour and probed with an anti-S100A6 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 60 minutes, followed by a 30-minute incubation with an anti-mouse secondary antibody conjugated with horseradish peroxidase (Pierce, Rockford, IL). The presence of S100A6 protein was detected by using the SuperSignal West Pico Chemiluminescent Substrate kit (Pierce Biotechnology, Rockford, IL) and recorded using the Kodak 440CF ImageStation (Kodak, Rochester, NY).

We generated recombinant adenovirus expressing S100A6 or siS100A6 using the AdEasy (Stratagene, La Jolla, CA) technology as described previously [17]. Briefly, the coding sequences for human S100A6 or the selected siRNA cassettes were cloned into pAdTrack-CMV or pSES [27], respectively, and subsequently used to generate adenoviral recombinants. These were the S100A6 or siS100A6 adenoviruses, respectively. Recombinant adenoviruses were produced and amplified in packaging HEK293 cells. An analogous adenovirus containing a GFP (AdGFP) or RFP (AdRFP) tag was used as a control [17]. Each tested cell line was infected with adenovirus expression S100A6, siS100A6, or GFP/RFP controls. All PCR-amplified fragments and cloning junctions were verified by DNA sequencing. The knockdown efficiency of the siS100A6 adenoviral vectors were tested in the four commercially available cell lines (TE85, 143B, MNNG/HOS, and MG63). The total quantified S100A6 message for the control infections was compared to the siS100A6 infections to determine the percentage of knockdown.

We performed the cell adhesion assay as previously reported [28, 35]. Briefly, 96-well plates were coated with 10 μg/mL of rat tail Type I collagen (BD Biosciences, Bedford, MA). We chose to use Type I collagen because it is one of the most predominant matrix proteins in bone. The wells were blocked with 1% bovine serum albumin (BSA) and 1×10^4 cells in 0.1% BSA were added to the wells in triplicate. At 20 minutes, the wells were washed to remove unattached cells and fixed in formalin. The cells were stained with 1% methylene blue in 0.01 M borate buffer, pH 8.5, and washed. The methylene blue was extracted with ethanol and 0.1 M HCl and absorbance was read at 630 nm using a microplate photometer. We created a standard curve with known number of cells per well. The assay was repeated in at least three batches.

The cell migration experiments were carried out as previously described [26]. Each of the cell lines (TE85, MNNG/HOS, 143B, MG63) was plated into 12-well plates and allowed to attach with complete media with 10% fetal calf serum (FCS) for 3 hours. Cells were washed with serum-free media, cultured with 1% FCS, and infected with AdS100A6, AdsiS100A6, AdRFP, or AdGFP adenovirus at a comparable titer. Twenty hours after infection, the monolayer of cells was wounded using micropipette tips. Bright field and fluorescence images were taken at 0, 10, 20, and 30 hours after wounding to document cell migration across the wound. The results were repeated in at least two batches of experiments.

Cell invasion was assessed using a Matrigel invasion assay. Briefly, Transwell (Corning Costar, Corning, NY)

inserts containing polycarbonate membranes with 8 μM pores were coated with Matrigel (BD Biosciences, Bedford, MA) at 20 μg per well at room temperature overnight. The membrane was rehydrated with serum free media for 2 hours. For each of the 24-well inserts, 1×10^5 cells in serum free media and 0.1% Bovine Serum Albumin (BSA) were plated onto the upper chamber. The bottom chamber was filled with complete media and 10% Fetal Calf Serum (FCS) as a chemoattractant. The cells were allowed to invade for 24 hours. Noninvading cells were removed from the upper chamber by using moist cotton tipped swabs. The membranes containing invading cells on the undersurface of the membrane were fixed with 100% methanol and stained with hematoxylin. Ten random high power fields (hpf) were counted per insert. The assay was performed in triplicate and in two batches.

To compare expression (quantitative RT-PCR) from the cell lines or primary cultures with normal osteoblasts we used a non-parametric Sign test. We compared the relative adhesion of cells with S100A6 overexpression or knockdown against the GFP vector control using a t-test. Finally, we compared the number of invaded cells per hpf in the three treatment groups (overexpression, knockdown, or GFP vector control) using a t-test. Analyses were performed using STATA version 7 (Stata Corporation, College Station, TX).

Results

Ten of the 11 primary cultures we established had at least a twofold increased expression of S100A6 relative to human osteoblasts (Fig. 2). Three samples had greater than 10 times the expression level. Sample #8 had S100A6 expression slightly greater than osteoblasts. Likewise, three of the four commercially available osteosarcoma cell lines (TE85, 143B, MG63) had high expression of S100A6, whereas one of the lines (MNNG/HOS) had an expression level slightly above the level of normal osteoblasts. In total, 13 out of 15 osteosarcoma cell lines/primary cultures had a greater than twofold increase (p = 0.004) in S100A6 expression compared to osteoblasts. As expected, 8 of 11 patients had AJCC Stage IIB disease at presentation. One patient presented with Stage IV disease while two developed metastasis at 21 and 82 months after the initial presentation. Three patients died from their disease.

The loss of GFP expression demonstrates the silencing efficiency of the siRNA sequence to the target gene containing a chimeric sequence between GFP and the S100A6 in the pSOS-HUS vector (Fig. 3A). Knockdown was also confirmed by real-time RT-PCR and was specific and effective. S100A6 siRNAs resulted in knockdown efficiencies ranging from 37% to 51% against endogenous S100A6 (Fig. 3B) in the four cell lines. Other members of the S100 protein family, including S100A2, S100A3, S100A4, and S100A5, demonstrated no knockdown by the S100A6 siRNA fragments (data not shown). Western blot analysis (Fig. 3C) demonstrates knockdown was detectable even with very high expression of S100A6 (via transient transfections in HEK293 cells) and with endogenous S100A6 in 143B cells, which has the highest S100A6 expression among the four lines.

Overexpression of S100A6 enhanced cell adhesion in all the cell lines tested (Fig. 4). Specifically, overexpression of

Fig. 2 The S100A6 expression in osteosarcoma cell lines and primary osteosarcoma cultures is shown. Commercially available osteosarcoma cell lines (TE85, MNNG/HOS, 143B, MG63) as well as 11 primary cultures were examined for S100A6 expression. C3H10T1/2 mesenchymal stem cells (MSCs) were used as controls. S100A6 is highly expressed in the majority of the primary cultures and well-established lines compared with normal human osteoblasts.
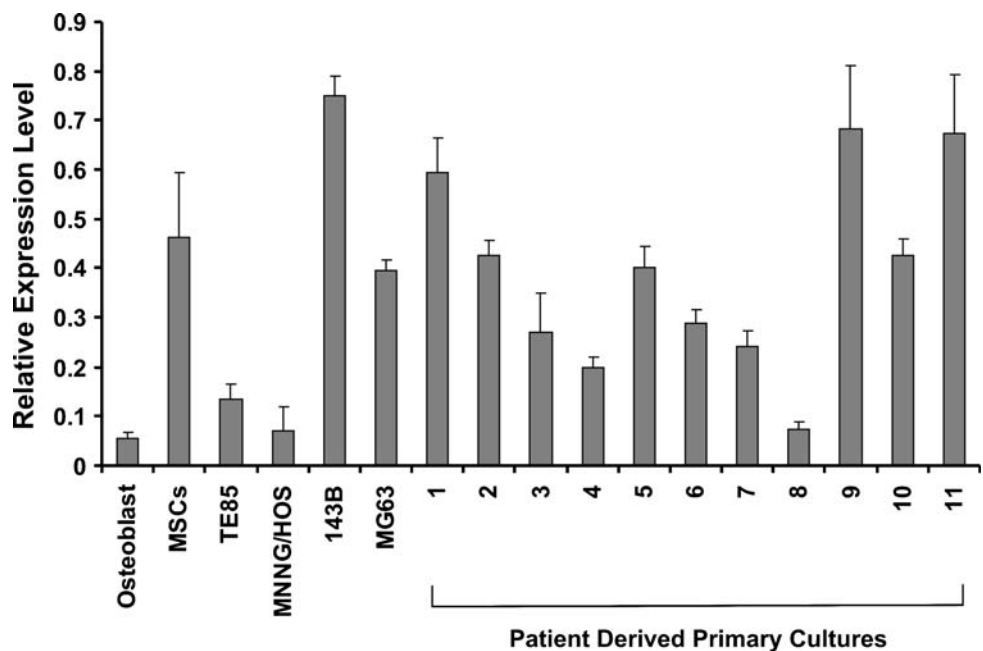
Fig. 3A–C The characterization of siRNA-mediated knockdown of S100A6 expression is shown. The efficacy of the siRNA sequences were examined in human osteosarcoma cells. (**A**) The loss of GFP signal signifies the efficiency of siRNA-mediated knockdown by the siRNA sequences against the chimeric message of S100A6 and GFP from the pSOS-HUS system. (**B**) The percentage S100A6 mRNA knockdown determined by qRT-PCR is shown for each of the cell lines infected with the Ad-siS100A6 adenovirus. (**C**) A Western blot analysis of S100A6 knockdown is shown. HEK293 cells transiently transfected with a GFP control vector, co-transfected with S100A6 and the siS100A6 vectors, or transfected with the S100A6 vector only are also shown. Knockdown of endogenous S100A6 by the AdsiS100A6 adenovirus in 143B cells is also shown.
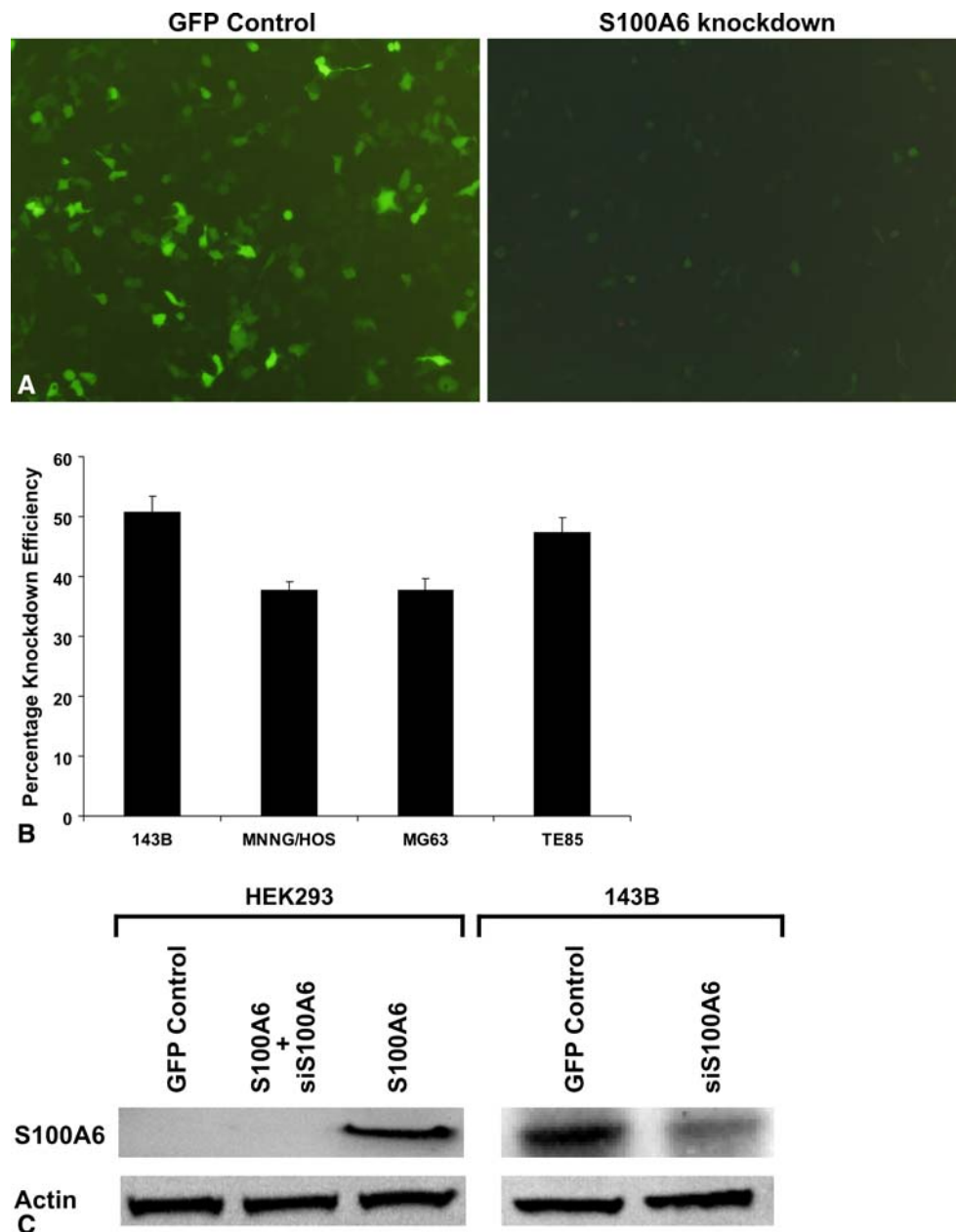
S100A6 increased the relative cell-matrix adhesion by 59.7%, 30%, 54.8%, and 27.9% in the TE85, MNNG/HOS, 143B, and MG63 cells, respectively, with p values of 0.01, 0.05, 0.01, and 0.01, respectively. In concordance with our overexpression data, when endogenous S100A6 was knocked down by siRNA, relative cell-matrix adhesion decreased in all four cell lines examined. Specifically, the decrease in the relative adhesion was 14.4%, 15.8%, 16.8%, and 34.3% in the TE85, MNNG/HOS, 143B, and MG63 cell lines, respectively, with p values of 0.01, 0.05, 0.04, and 0.04, respectively.

S100A6 knockdown resulted in increased cell motility (Fig. 5). The TE85 and 143B cell lines are shown as representative examples because these represent the least and most tumorigenic and metastatic cell lines in this group of cells [28]. Similar results were seen in the MNNG/HOS and MG63 cell lines (data not shown). Overall, knockdown of S100A6 resulted in increased cell motility. These findings are consistent with our finding that S100A6 promotes cell adhesion. Similar to our motility assay, the cell invasion assay demonstrated S100A6 overexpression inhibited cell invasion while S100A6 knockdown resulted in increased cell invasion (Fig. 6A–B). Representative images from MG63 cells are shown (Fig. 6A). Overexpression of S100A6 resulted in a 40%, 53%, 43%, and 67% decrease in invasion compared to the GFP controls in the TE85, MNNG/HOS, 143B, and MG63 lines, respectively (p value 0.05, 0.018, 0.001, and < 0.001 respectively). Knockdown
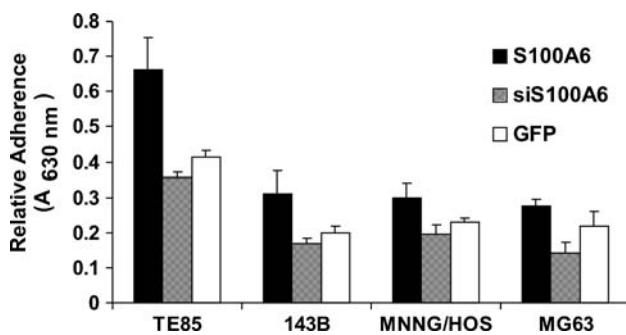
Fig. 4 This figure illustrates the effect of S100A6 on osteosarcoma cell adhesion. Target sites with optimal knockdown efficiency were used to generate AdsiS100A6 adenoviruses. Osteosarcoma cells were transduced with AdsiS100A6, AdS100A6, or AdGFP. Overexpression of S100A6 enhanced cell adhesion, whereas knockdown S100A6 inhibited adhesion. Experiments were performed in triplicate.

of S100A6 resulted in a 95%, 163%, 72%, and 46% increase in invasion compared to the GFP controls in the TE85, MNNG/HOS, 143B, and MG63 lines, respectively (p value 0.036, < 0.001, 0.001, and < 0.001 respectively).

Discussion

One of the clinical challenges in the treatment of osteosarcoma patients is to identify the 20% of patients who never develop metastases. If we are able to identify this group of patients, then we may spare these patients of the morbidity associated with systemic chemotherapy treatment. The objective of this investigation was to examine and characterize a potential marker, S100A6, which may have prognostic value in osteosarcoma patients. Based on our previously published results and other reports on the cellular function of S100A6, we hypothesized (1) primary cultures and commercially available cell lines from osteosarcoma patients would have high expression of S100A6, and (2) S100A6 functions to promote cell adhesion, inhibit cell migration, and inhibit cell invasion in osteosarcoma cells. All these phenotypes are important for cancer metastasis.

Although our data suggest a relationship between metastasis and S100A6 in osteosarcoma, we note some
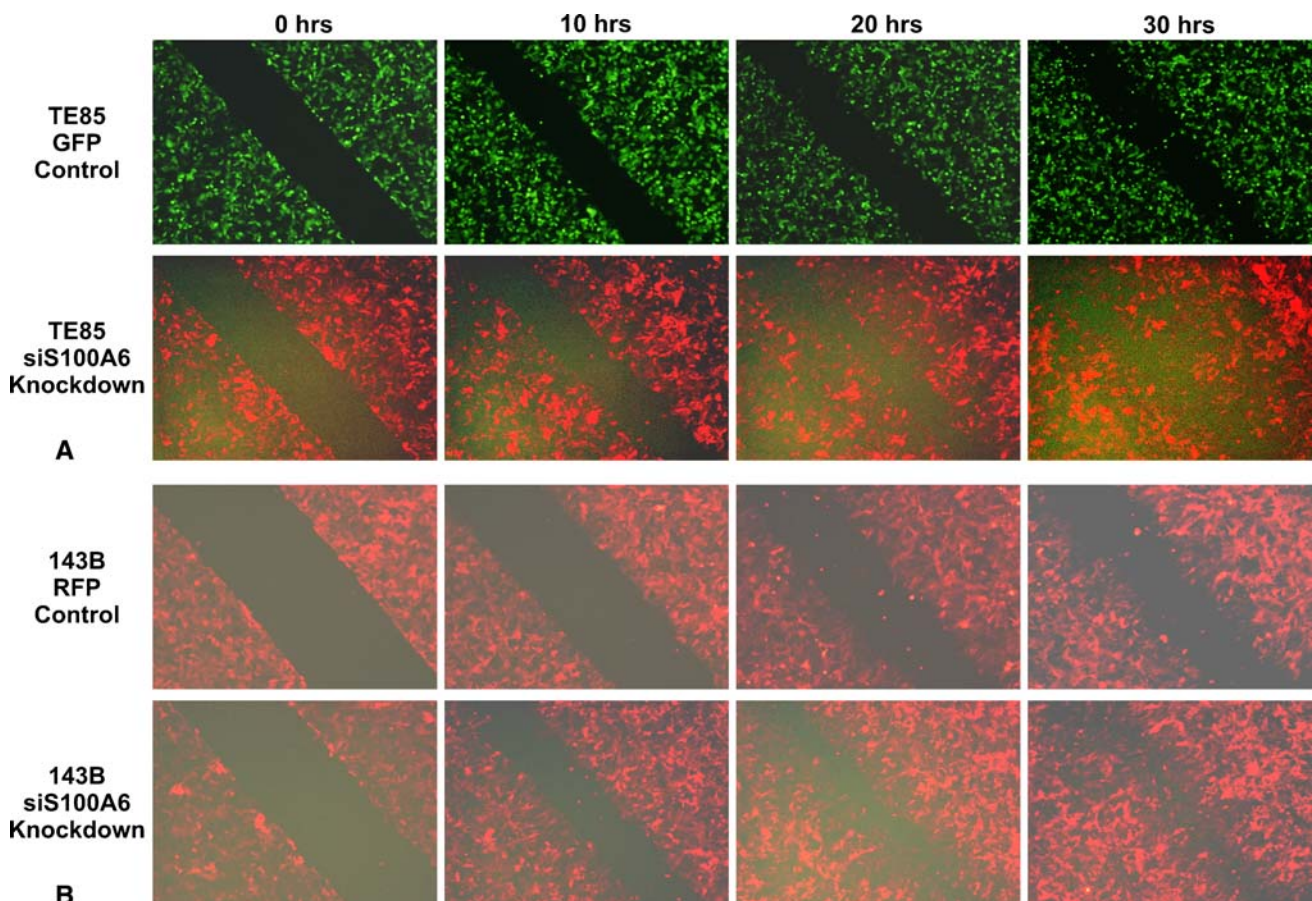

Fig. 5A–B This figure shows the effect of S100A6 on osteosarcoma cell motility. Osteosarcoma cells (TE85, MNNG/HOS, MG63, 143B) were transduced with AdGFP/AdRFP or AdsiS100A6. The monolayers of cells were wounded using a pipette tip. The images were taken at 0, 10, 20, and 30 hours to examine the migration of the cells to close the wound. Migration of TE85 (**A**) and 143B (**B**) osteosarcoma cells is shown as an example. Knockdown of endogenous S100A6 promoted cell motility as demonstrated by the more robust closure of the wound in the siS100A6 groups compared to the GFP or RFP controls. The assay was repeated at least twice.

Fig. 6A–B The effect of S100A6 on cell invasion is shown. Osteosarcoma cells (TE85, MNNG/HOS, 143B, and MG63) were transduced with AdGFP, AdS100A6, or Ad-siS100A6. Cells were allowed to invade through a membrane coated with Matrigel matrix proteins and counted. (**A**) Representative images of the invaded cells (MG63) are shown (100x mag). (**B**) A graph of the number of cell counted per high power field is depicted for all the cell lines examined. The assay was repeated twice.
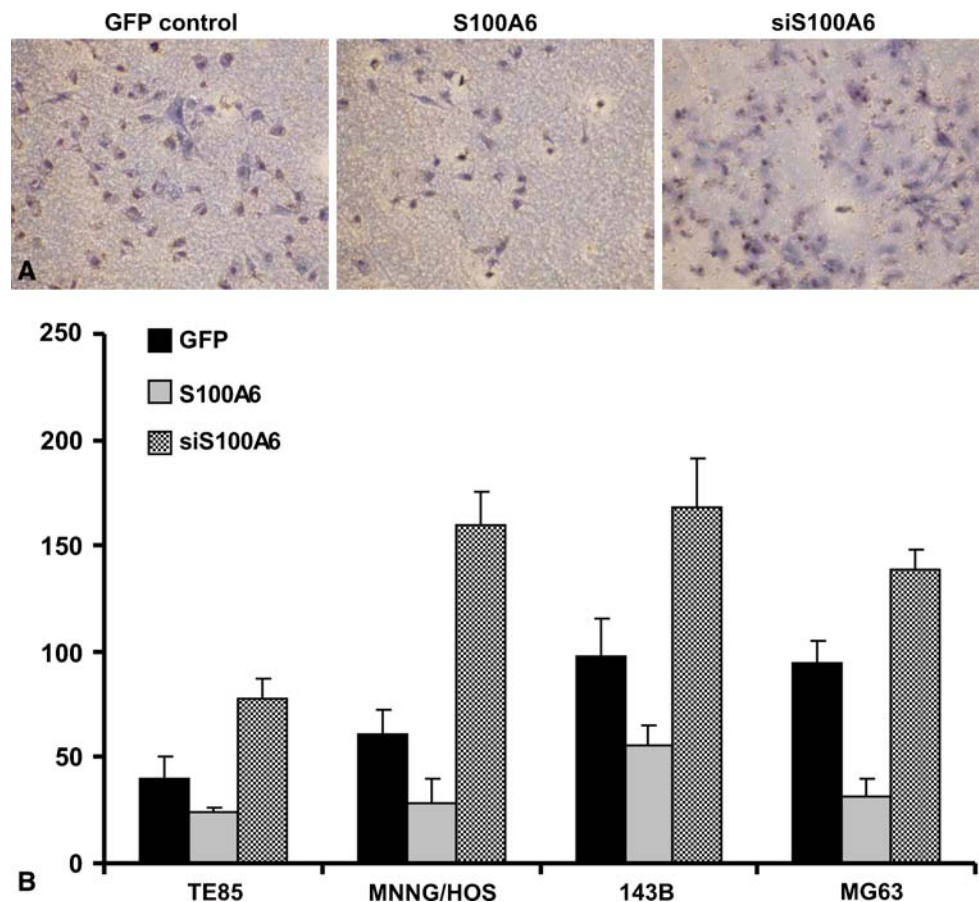
limitations. One of our main objectives in this study was to further understand the basic mechanism in which S100A6 may modulate osteosarcoma metastasis. We were able to examine only 11 patient samples, since these were the only primary cultures that survived our banking process. Because of the limited number of patients, we cannot draw clear conclusions regarding the relationship between S100A6 and metastasis in this cohort. We had only one specimen derived from a metastatic lesion. When we reviewed our bank of primary cultures to be expanded and RNA collected, we had 23 samples. However, many samples could not be expanded when they were thawed. Therefore, we were unable to expand and collect RNA of all of the samples that were frozen down initially for our qRT-PCR analysis. Furthermore, there was also a subset of patients whose primary cultures could not be expanded to be frozen down when the samples were first harvested at the index procedures. As a result, we had only 11 primary cultures that were successfully frozen down, thawed, and expanded. As with any attempt at primary cultures, it is possible our established cultures may have contamination with fibroblasts. As described, we made every attempt to ensure we had representative tumor samples in our banking process. While it is possible there may be some stromal/fibroblast cells, we believe the majority of the cells in the

samples are tumor cells. Nevertheless, to minimize expansion of stromal/fibroblast cells, we minimized the number of passages of our primary cultures. Certainly, we can isolate single cells and expand these into cell lines. However, this would eliminate the heterogeneity of a tumor population. Furthermore, this would require multiple passages that may select for phenotypes that may not necessarily represent the initial tumor population. Finally, there is only one commercially available human osteoblast line, which we used as a reference for our RT-PCR analysis. Nevertheless, the relative low expression of S100A6 in this normal osteoblast line correlates with our previous immunohistochemical analysis of osteosarcoma samples in which the surrounding normal bone had minimal immunoreactivity [29]. Furthermore, this is a commonly used line to represent human osteoblasts in the literature.

We established primary cultures from patient specimens and examined these primary cultures as well as four commercially available human osteosarcoma cell lines for endogenous S100A6 expression. Relative to human osteoblasts the majority of our primary cultures and the commercially available lines had increased S100A6 expression. There is little literature on S100A6 and osteosarcoma. Nevertheless, our finding of increased expression of S100A6 is consistent with the findings by

Muramatsu et al. [32] who examined a number of human tumors and found S100A6 expression was consistently elevated in their few osteosarcoma samples. Similarly, S100A6 is overexpressed in several other human tumors such as melanoma, squamous cell carcinoma, malignant fibrous histiocytoma (MFH), and carcinomas of the thyroid, breast, and colon [10, 19]. Among the commercially available lines, we found different levels of S100A6 expression. In particular, we found a wide difference in S100A6 expression in two highly tumorigenic lines, 143B and MNNG/HOS. This difference may be due to how these lines were derived. Both the 143B and MNNG/HOS cell lines were transformed by k-ras and the MNNG mutagen, respectively. The tumorigenicity of these two lines is most likely reflective of the effects of the different transforming agents. These two agents can alter the expression of a large number of genes affecting multiple downstream pathways. Therefore, these two agents may directly or indirectly alter S100A6 expression at different magnitudes, which can explain the different S100A6 expression levels. Specifically, ras directly upregulates the expression of S100A6 [6]. Therefore, the relatively high expression of S100A6 in these cell lines may reflect the downstream effects of ras transformation.

We found knockdown of S100A6 inhibited cell adhesion and promoted cell motility and invasion. Cell adhesion, cell motility, and invasion are important initial steps in the metastatic cascade [16, 40, 43, 51]. Our findings on the potential role of S100A6 on cell adhesion and cell motility are supported by other reports [14, 31] of the interaction of S100A6 with the cytoskeleton. S100A6 interacts with the actin cytoskeleton through interactions with tropomyosin and caldesmon [14, 31]. Dynamic polymerization and depolymerization of the actin filament system at the lamellipodium of a cell are critical for migration [41]. Although it has yet to be investigated, the interaction of S100A6 with the actin microfilament in this subcellular compartment may play a role in motility. Furthermore, localization studies demonstrate S100A6 is primarily localized on the plasma membrane and to some extent on the nuclear membrane in a calcium-dependent manner [44].

In addition to tropomyosin and caldesmon, S100A6 also interacts with several members of the Annexin family, including Annexin II [23], and the specific binding sites have been mapped [45]. Annexin II is a membrane-associated protein that regulates actin-associated protein complexes and is important for membrane trafficking, cell migration, and invasion [9, 11, 12]. Colocalization studies demonstrate the S100A6-Annexin II complex is primarily found on the cytoplasmic side of the plasma membrane [3]. Furthermore, Annexin II has been identified to localize to the lamellipodia of migrating/invading cells where actin polymerization is enriched [1]. Interestingly, increased Annexin II expression has been correlated with decreased metastasis in patients with osteosarcoma [13]. Specifically, patient samples derived from resected pulmonary metastatic nodules had decreased Annexin II expression compared with primary osteosarcoma tumors. When Annexin II was overexpressed in osteosarcoma cells, there was a decrease in metastasis in an experimental metastasis (eg, tail vein injection) mouse model [13]. These results are similar to our findings with S100A6. Taken together, our data as well as others support the hypothesis that interactions between S100A6 and the actin microfilament system through actin-associated proteins may regulate cell adhesion and migration.

Our data suggest increased S100A6 expression is a common event in human osteosarcoma. Furthermore, overexpression of S100A6 in osteosarcoma cells in vitro enhances cell adhesion and decreases invasion, whereas knockdown of endogenous S100A6 inhibits cell adhesion. Similarly, knockdown of endogenous S100A6 promotes cell motility and invasion. Taken together, these findings suggest S100A6 may play an important role in osteosarcoma metastasis and may be considered a prognostic marker.

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