Original Paper

The Calcium-Binding Protein S100A6 Accelerates Human Osteosarcoma Growth by Promoting Cell Proliferation and Inhibiting Osteogenic Differentiation

Yasha Li^{a,b} Eric R. Wagner^b Zhengjian Yan^{b,c} Zhonliang Wang^{a,b} Gaurav Luther^b
Wei Jiang^b Jixing Ye^{b,d} Qiang Wei^{b,c} Jing Wang^{b,c} Lianggong Zhao^{b,e} Shun Lu^{b,f}
Xin Wang^{b,g} Maryam K. Mohammed^b Shengli Tang^{b,h} Hao Liu^{b,c} Jiaming Fan^{b,c}
Fugui Zhang^{b,c} Yulong Zou^{b,c} Dongzhe Song^{b,g} Junyi Liao^{b,c} Rex C. Haydon^b
Hue H. Luu^b Tong-Chuan He^{a,b,c}

^aStem Cell Biology and Therapy Laboratory, Ministry of Education Key Laboratory of Child Development and Disorders, The Children's Hospital of Chongqing Medical University, Chongqing, China; ^bMolecular Oncology Laboratory, Department of Orthopaedic Surgery and Rehabilitation Medicine, The University of Chicago Medical Center, Chicago, USA; ^cMinistry of Education Key Laboratory of Diagnostic Medicine, and the Affiliated Hospitals of Chongqing Medical University, Chongqing, China; ^dSchool of Bioengineering, Chongqing University, Chongqing, China; ^eDepartment of Orthopaedic Surgery, the Second Affiliated Hospital of Lanzhou University, Lanzhou, China; ^fDepartment of Orthopaedic Surgery, Shandong Provincial Hospital and Shandong University School of Medicine, Jinan, China; ^gDepartment of Surgery, West China Hospital of Sichuan University, Chengdu, China; ^hDepartment of Surgery, Zhongnan Hospital of Wuhan University, Wuhan, China

Key Words

Osteosarcoma • Bone tumor • S100A6 • Apoptosis • S100 proteins • BMP9 • Osteoblastic differentiation • Mesenchymal stem cells

Abstract

Background/Aims: Although osteosarcoma (OS) is the most common primary malignancy of bone, its molecular pathogenesis remains to be fully understood. We previously found the calcium-binding protein S100A6 was expressed in ~80% of the analyzed OS primary and/or metastatic tumor samples. Here, we investigate the role of S100A6 in OS growth and progression. **Methods:** S100A6 expression was assessed by qPCR and Western blotting. Overexpression or knockdown of S100A6 was carried out to determine S100A6's effect on proliferation, cell cycle, apoptosis, tumor growth, and osteogenic differentiation. **Results:** S100A6 expression was readily detected in human OS cell lines. Exogenous S100A6 expression promoted cell proliferation *in vitro* and tumor growth in an orthotopic xenograft model of human OS. S100A6 overexpression reduced the numbers of OS cells in G1 phase and increased viable cells under serum starvation condition. Conversely, silencing S100A6 expression induced the production of cleaved caspase 3, and increased early stage apoptosis. S100A6

T.-C. He, MD, PhD and Hue H. Luu, MD

Molecular Oncology Laboratory, The University of Chicago Medical Center, 5841 South Maryland Avenue, MC 3079, Chicago, IL 60637, (USA); and Department of Orthopaedic Surgery, The University of Chicago Medical Center, 5841 South Maryland Avenue, MC3079, Chicago, IL 60637, (USA); E-Mail tche@bsd.uchicago.edu and hluu@bsd.uchicago.edu

knockdown increased osteogenic differentiation activity of mesenchymal stem cells, while S100A6 overexpression inhibited osteogenic differentiation. BMP9-induced bone formation was augmented by S100A6 knockdown. **Conclusion:** Our findings strongly suggest that S100A6 may promote OS cell proliferation and OS tumor growth at least in part by facilitating cell cycle progression, preventing apoptosis, and inhibiting osteogenic differentiation. Thus, it is conceivable that targeting S100A6 may be exploited as a novel anti-OS therapy.

© 2015 The Author(s)
Published by S. Karger AG, Basel

Introduction

Osteosarcoma (OS) is the most common primary bone malignancy, with a high rate of overt or microscopic pulmonary metastases at the time of diagnosis [1-7]. Even with improved chemotherapy and surgical techniques, many OS patients fail to achieve long-term disease-free survival due to the development of resistance to therapeutic interventions [1, 8-17]. Although numerous molecular and genetic alterations have been associated with OS development [2, 3, 6, 7, 18-21], the step-wise molecular pathogenesis underlying OS development and progression remains unclear. We previously demonstrated that Wnt/-catenin, IGF signaling, nuclear receptor signaling, and lysophosphatidic acid acyltransferase β (LPAAT β) may be involved in OS development and/or metastasis [22-26]. Furthermore, we have shown that OS may be caused by differentiation defects, as osteogenic BMPs fail to induce osteogenic differentiation and promote OS tumor growth [6, 7, 27, 28]. Thus, understanding the molecular mechanisms underlying bone and skeletal development may provide clues to the molecular pathogenesis of OS [20, 28-33].

We previously found that S100A6 was highly expressed in approximately 80% of primary and/or metastatic tumor samples derived from OS patients, as well as in the patient-derived human OS cell lines, when compared to normal osteoprogenitors and osteoblasts [34, 35]. S100A6 is a member of the S100 family of proteins, consisting of over 20 low molecular weight EF-hand calcium binding proteins located on human chromosome 1 [36-39]. Several S100 proteins, including S100A2, S100A4, S100A7, and S100B, have been shown to be upregulated or otherwise differentially expressed in human tumors [37, 39-42]. S100A6 was shown to be overexpressed in several types of human tumors [37, 43-53]. Although S100A6 has been shown to interact with cytoskeletal elements, heat-shock response proteins, and with regulatory elements of the ubiquitin and apoptotic cascades [40, 54, 55], the functional role of S100A6 in OS tumorigenesis and progression remains unclear.

Here, we investigated the role of S100A6 in human OS tumorigenesis. S100A6 expression was readily detected in human OS cell lines. Exogenous expression of S100A6 promoted cell proliferation *in vitro* and OS tumor growth in an orthotopic xenograft model of human OS. Mechanistically, S100A6 overexpression significantly reduced the population of OS cells in G1 phase and increased the population of viable cells under serum starvation conditions. Conversely, silencing S100A6 expression induced the late apoptotic marker cleaved caspase 3 and increased the size of the cell population undergoing early stage apoptosis. Silencing S100A6 expression increased the osteogenic differentiation activity of mesenchymal stem cells (MSCs), while S100A6 overexpression inhibited osteogenic differentiation. Accordingly, S100A6 knockdown effectively augmented bone formation induced by BMP9. Taken together, these findings strongly suggest that overexpression of S100A6 may promote OS tumor growth and OS cell proliferation. It is conceivable that targeting S100A6 may lead to the development of novel anti-OS therapies.

Materials and Methods

Tissue culture and chemicals

Human OS cell lines MG63, 143B, MNG/HOS, and TE85, as well as HEK293 and C3H10T_{1/2}, were purchased from ATCC (Manassas, VA). We also used a highly tumorigenic and metastatic OS subline MG63.2, as previously reported [24, 56]. The iMEFs are mouse MSCs reversibly immortalized by retroviral

or *piggyBac* vectors as previously reported [57-59]. Cell lines were maintained in conditions as described elsewhere [60, 61]. Unless otherwise indicated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or Thermo-Fisher Scientific (Pittsburgh, PA).

Construction and generation of recombinant adenoviral vectors expressing S100A6, siS100A6, RFP, GFP, and BMP9

Recombinant adenoviruses expressing human S100A6 and human BMP9 were generated using AdEasy technology [62-64]. The siRNA knockdown oligonucleotide cassettes for human S100A6 were designed by using the *siDESIGN* website and subcloned into shuttle vector pSES or pAdTrace-OK and pAdEasy system, as previously described [65-67]. The two siRNA target sites of human S100A6 coding region are 5'-AGA AGG AGC TCA CCA TTGG-3' and 5'-AGC TCA CCA TTG GCT CGAA-3'. DNA sequencing confirmed the siRNA cassettes. It is noteworthy that the first siRNA also targets the coding region of mouse S100A6. The resultant recombinant adenovirus plasmids were used to package and amplify recombinant adenoviruses in HEK293 or the recently engineered 293pTP cells [68]. Adenoviruses expressing S100A6 (i.e., AdS100S6) and siS100A6 (i.e., AdR-siS100A6) co-expressed GFP and RFP, respectively, as markers for monitoring gene expression and virus infection efficiency. An analogous adenovirus expressing RFP (i.e., AdRFP) or GFP (i.e., AdGFP) was used as a mock control [69-71]. For all adenovirus-mediated infections, polybrene (8 µg/ml) was added to increase the efficiency of adenoviral infection [72].

RNA isolation and quantitative PCR (TqPCR)

Total RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA) and subjected to reverse transcription reaction with hexamer and M-MuLV Reverse Transcriptase (New England Biolabs, Ipswich, MA). The cDNA products were diluted 10- to 100-fold and used as PCR templates. Gene-specific PCR primers were designed by using the Primer3 Plus program (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>) to amplify genes of interest (approximately 150-250bp). The quantitative real-time-PCR analysis of the cDNA samples was carried out using our recently optimized TqPCR protocol [73]. Briefly, the SYBR Green qPCR reactions (Bio-Rad Laboratories) were set up according to manufacturer's instructions. The cycling program was modified by incorporating 4 cycles of touchdown steps prior to the regular cycling program [73]. The PCR products were confirmed by resolving PCR products on 1.5% agarose gels. All samples were normalized using GAPDH as a reference gene. Primer sequences are available upon request.

Cell proliferation assay (Trypan Blue staining)

Trypan blue cell proliferation assays were performed as previously described [74, 75]. Subconfluent tumor cells were infected with AdS100A6, AdR-siS100A6, or control adenoviruses and re-plated at 72 hours in 2% FBS complete media. At the indicated time points, cells were collected by trypsinization and viable cells were counted in the presence of trypan blue [23]. Each assay was done in triplicate and repeated at least twice.

Crystal violet cell viability assay

Crystal violet proliferation assays were performed as previously described [76]. Subconfluent OS cells were plated in 12-well tissue culture plates and infected with AdS100A6, AdR-siS100A6, or control adenoviruses. The cells were maintained for 4 days in 2% FBS complete media. At the end of day 5, the cells were stained with crystal violet to visualize cell viability. Quantitative measurements occurred by suspending crystal violet stain in 10% acetic acid and absorbance measured by spectrometry at A_{570} [76]. Each assay was done in triplicate and repeated at least twice.

Western blotting analysis

Subconfluent 143B cells were infected with Ad-S100A6, AdR-siS100A6, or AdRFP adenovirus. At 72h after infection, the cells were collected and lysed in Laemmli sample buffer. Western blot analysis was performed using an anti-human S100A6 mouse monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), as previously described [35]. The presence of S100A6 protein was detected by using SuperSignal West Pico Chemiluminescent Substrate kit (Pierce Biotechnology, Rockford, IL) and recorded using the Kodak 440CF ImageStation (Kodak, Rochester, NY). The β -actin levels were also determined to assess the sample loading.

Immunostaining

Immunocytochemical staining was carried out as described [75, 77, 78]. Briefly, subconfluent cells were infected with AdS100A6, AdR-siS100A6, or control adenoviruses. After 72 hours, the cells were fixed with 10% formalin, blocked with bovine serum, avidin and biotin blocking solution, and probed with an anti-cleaved caspase-3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Following incubation with secondary antibody conjugated to horseradish peroxidase (Pierce, Rockford, IL), the staining was visualized under light microscopy.

Flow cytometry analysis

For cell cycle analysis, subconfluent cells were infected with AdS100A6, AdR-siS100A6, or control adenoviruses for 24h. The cells were trypsinized and re-plated at subconfluent conditions in 6-well cell culture plates. Cells were harvested at 48h, fixed with 70% ethanol, and stained with propidium iodide. Cells were FACS analyzed using BD FACSCanto.

For apoptosis assays, subconfluent cells were infected with AdS100A6, Ad-siS100A6, or AdRFP control adenoviruses. After 24h, the cells were trypsinized and re-plated at subconfluent conditions in 6-well cell culture plates containing 1% FBS complete DMEM. Cells were harvested after 3 days, washed with PBS, stained with propidium iodide and Annexin V-FITC (BD Biosciences, San Jose, CA), and analyzed by FACS using BD FACSCanto.

Alkaline phosphatase assay

Quantitative ALP activity was assessed with a modified assay using the Great Escape SEAP Chemiluminescence assay kit (BD Clontech, Mountain View, CA) and qualitatively with histochemical staining assay (using a mixture of 0.1 mg/ml naphthol AS-MX phosphate and 0.6 mg/ml Fast Blue BB salt), as previously described [79-81]. Qualitative ALP activity assay was assessed with histochemical staining assay as described [82-84]. Each assay condition was performed in triplicate and the results were repeated in at least three independent experiments.

Matrix mineralization assay (Alizarin Red S staining)

Subconfluent C3H10T_{1/2} and iMEF cells were plated in 12-well cell culture plates and infected with AdS100A6, AdR-siS100A6, or control adenoviruses. These cells were cultured in 10% FBS complete DMEM in the presence of ascorbic acid (50 µg/mL) and β-glycerophosphate (10 mM). After 14 days, the mineralized matrix nodules were stained with calcium precipitate using Alizarin Red S staining as described [85, 86]. Briefly, cells were fixed with 0.05% (v/v) glutaraldehyde at room temperature for 10 min and washed with distilled water; fixed cells were incubated with 0.4% Alizarin Red S (Sigma-Aldrich, St. Louis, MO) for 5 min, followed by extensive washing with distilled water. The staining of calcium mineral deposits was recorded under bright field microscopy.

Mouse orthotopic xenograft tumor model

All animal work was conducted according to the protocol approved by the Institutional Animal Care and Use Committee of The University of Chicago (protocol #71328). Stable 143B-Luc cells were established by using a *piggyBac* transposon vector expressing firefly luciferase [59]. Stable 143B-Luc cells were infected with AdS100A6, AdR-siS100A6, or control adenoviruses. After 36h, the cells were harvested, suspended in PBS, injected (1 × 10⁶/0.1ml PBS/injection) subperiosteally and into the proximal posterior tibial compartment of athymic nude mice (female, Harlan Sprague Dawley, 4-6 wk old, 6 mice per group), as previously described [76, 87]. Tumor volume and doubling time were calculated using the Schwartz Formula as previously reported [74, 88, 89]. At 4 wk after implantation, primary tumors were harvested for histologic evaluation and µCT imaging, as previously described [56, 75].

Xenogen bioluminescence imaging

Animals were anesthetized with isoflurane, injected intraperitoneally with D-Luciferin sodium salt (Gold Biotechnology, St Louis, MO) at 100mg/kg in 0.1 ml sterile PBS and imaged with the Xenogen IVIS 200 imaging system. Pseudoinages were obtained by superimposing the emitted light over gray-scale photographs of study animals. Quantitative analysis was done with Xenogen's Living Image V2.50.1 as described [75, 76, 87, 90].

Subcutaneous stem cell implantation

The iMEF cells were infected with AdBMP9 and AdS100A6, AdR-siS100A6, or control adenoviruses. At 24h post-infection, the cells were harvested with trypsin, resuspended in PBS, and injected into the flanks of athymic nude mice (3×10^6 cells/injection). After 4 weeks, the implantation site masses were recovered for μ CT imaging, as previously described [75, 91]. After the imaging, the data was analyzed via the Amira software (Visage Imaging, San Diego, CA).

Histologic evaluation

Retrieved tissues were fixed, decalcified in 10% buffered formalin, and embedded in paraffin. Serial sections of the embedded specimens were stained with hematoxylin and eosin (H & E) as previously described [75].

Statistical analysis

A two-tailed Student's t-test was used to compare the S100A6 and siS100A6 groups to the controls in the calculations for the tumor doubling time, cell proliferation, crystal violet staining, Xenogen bioluminescence imaging, alkaline phosphatase assays, and cell cycle analysis. Depicted error bars represent 95% standard error. A $p < 0.05$ was defined as statistically significant.

Results

Endogenous expression of S100A6 is high in human osteosarcoma cell lines

While several members of the S100 protein family, such as S100A2, S100A4, S100A6, S100A7, and S100B, have been found to be overexpressed or differentially expressed in tumor samples compared to control cell lines [37, 40], their expression pattern in OS has not been previously described. Thus, we first examined the endogenous expression of the five S100 proteins in five human OS cell lines (MG63, MG63.2, 143B, MNNG/HOS, TE85) using

Fig. 1. Endogenous S100 expression, and the over-expression or knockdown of S100A6 in human osteosarcoma cell lines. (a) Endogenous expression of S100A2, S100A4, S100A6, S100A7 and S100B in five human OS cell lines analyzed. Total RNA was isolated from the OS cells, and quantitative TqPCR was performed using human GAPDH as the reference gene (a). (b) S100A6 overexpression and knockdown demonstrated in osteosarcoma cells. Subconfluent cells were infected with Ad-S100A6, AdR-siS100A6, or Ad-RFP control adenovirus. At 72h after infection, total RNA was isolated and the expression of S100A6 was analyzed using TqPCR with a pair of human

S100A6 specific primers and GAPDH as the reference gene (b). All PCR reactions were done in triplicate. “***”, $p < 0.01$. (c) Western blotting analysis. Subconfluent 143B cells were infected with AdS100A6, AdR-siS100A6, or AdRFP control adenovirus. At 72h after infection, total cell lysate was prepared and subjected to Western blotting analysis using an S100A6 antibody as described [35]. A β -actin antibody was used to assess equal loading of the samples.

Fig. 2. Overexpression of S100A6 accelerates human OS cell proliferation. (a) & (b) Cell proliferation assays via trypan blue cell counting. Subconfluent cells 143B (a) and MG63 (b) cells were seeded in 12-well cell culture plates and infected with AdS100A6, AdR-siS100A6, or AdRFP control adenoviruses. At the indicated time points after infection, cells were collected, stained with trypan blue and counted under a microscope. Each sample point was done in triplicate. Doubling times were calculated using the Schwartz formula. “*” indicates $p < 0.05$ compared to the RFP control groups. (c) Crystal violet staining of viable cells. Subconfluent cells MG63 and 143B cells were seeded in 12-well cell culture plates and infected and cultured for 4 days. Cell were stained with crystal violet and quantitatively measured by spectrometry (A_{570}). “*” indicates $p < 0.01$ compared to the control groups. Each assay condition was performed in triplicate. Representative results are shown.

quantitative RT-PCR (TqPCR) analysis. As shown in Fig. 1a, the expression of endogenous S100A6 was consistently present across all OS cell lines, while there was variable expression of the other four S100 proteins under the same amplification conditions.

Exogenous overexpression of S100A6 promotes human OS cell proliferation

Overexpression and silencing of S100A6 in human OS cells, respectively, were achieved using the recombinant adenoviruses AdS100A6 and AdR-siS100A6. Both viruses were constructed and generated using the AdEasy system. The AdS100A6, AdR-siS100A6, and control adenoviruses were titrated to ensure equal infection efficiency among the human OS cell lines. As demonstrated by TqPCR with GAPDH standardization, the recombinant adenoviruses were able to overexpress or silence S100A6 expression in both 143B and MG63 cells (Fig. 1b), which was further confirmed by Western blotting analysis using S100A6 antibody (Fig. 1c).

Cell proliferation assay demonstrated that S100A6 overexpression led to an increase in the cell proliferation rate of 143B OS cells. Cells transfected with AdS100A6 demonstrated a statistically significant increase in cell count when compared to controls at day 3 and day 5 after plating ($p < 0.05$) (Fig. 2a), as well as a decrease in cell population doubling times ($p < 0.02$). Specifically, the doubling times for the control, S100A6, and siS100A6 groups

Fig. 3. S100A6 profoundly promotes human OS tumor growth in an orthotopic tumor model. (a) & (b) Representative gross and bioluminescent images for each treatment group in 143B cells. 1.5×10^6 143B cells transduced with AdS100A6, AdR-siS100A6, or AdRFP control adenoviruses, were injected into the subperiosteal region and posterior compartment of the proximal posterior tibia in athymic nude mice. Xenogen bioluminescence images are shown at 1, 2, and 3 weeks (a). The mice were sacrificed after 4 weeks, and the primary tumors were harvested (a). Xenogen bioluminescent measurements for each group were quantitative analyzed using Xenogen living image software (b). “*” indicates $p < 0.05$ compared to the control groups. (c) The tumor growth curve. The tumor masses were measured every 3-4 days. Doubling times were calculated using the Schwartz formula. “*” indicates $p < 0.05$ compared to the control groups. (d) μ CT imaging of tibia integrity. The tibias for each treatment group were imaged by using μ CT and the 3D reconstruction was performed with Amira software. Note the bone destruction of the tibia and fibula in the S100A6 group (indicated by the arrow). Representative images are shown.

were 22.41 ± 0.59 , 20.91 ± 0.43 , and 23.58 ± 0.59 hrs, respectively. A similar increase in proliferation rate was observed when S100A6 was overexpressed in MG63 cells (Fig. 2b, $p < 0.01$). Doubling times for the MG63 cells transduced with control, S100A6, and siS100A6 adenoviruses were 25.38 ± 0.76 , 23.13 ± 0.58 , and 26.36 ± 0.57 hrs, respectively, reflecting a decrease in the cell proliferation rate ($p < 0.005$). Although adenoviral-mediated knockdown of S100A6 appeared to decrease the overall cell proliferation in both cell lines, the results were not statistically significant when compared to the control ($p > 0.05$).

The ability of S100A6 overexpression to promote OS cell proliferation was further demonstrated by crystal violet staining assay. When subconfluent 143B and MG63 cells were transduced with AdS100A6, AdR-siS100A6 or AdRFP for 4 days, viable cell staining with crystal violet revealed that S100A6 overexpression increased the density of viable cells, while knockdown of S100A6 expression led to a decrease in cell density compared to control group cell viability. Relative absorbance values for 143B cells transduced with control, S100A6, and siS100A6 adenoviruses were 62.5 ± 8.7 , 94.75 ± 11.0 , and 52.25 ± 9.12 , respectively ($p < 0.005$), while the relative absorbances for the MG63 cells transduced with control, S100A6, and siS100A6 adenoviruses were 66.75 ± 7.4 , 94.75 ± 8.8 , and 54 ± 7.13 ,

Fig. 4. Histologic evaluation of the orthotopic OS tumors. The harvested tumor samples were fixed in 10% formalin, decalcified, and paraffin embedded. H&E staining was performed, and representative images from each group are shown. The outline of the tibia is indicated by the dashed line at 40 x. The boxes indicate the region shown in the higher magnification images. Note the tumor growth and bone destruction of the posterior proximal tibia (arrows) in the S100A6 group and to a lesser extent in the control groups. The bone marrow space in the S100A6 group was filled with tumor (asterisks). This is in contrast to the normal marrow in the control and siS100A6 groups. B, bone cortex of the posterior tibia; M, marrow; and T, tumor.

respectively ($p < 0.01$) (Fig. 2c). These results strongly indicate that S100A6 can promote cell proliferation in OS.

Overexpression of S100A6 promotes primary tumor growth in an orthotopic model of human OS

We sought to test the effect of S100A6 overexpression or silencing on OS tumor growth *in vivo*. Using our previously established orthotopic xenograft tumor model of human OS [27, 56, 74], we prepared firefly luciferase-tagged 143B cells infected with AdS100A6, AdR-siS100A6, or AdRFP and injected an equal number of the transduced OS cells into the subperiosteal region of the proximal posterior tibia in athymic nude mice. Tumor growth was monitored via Xenogen bioluminescence imaging and bi-weekly caliper measurements. At the end of week 4, the mice were sacrificed and the limbs with injection sites were harvested for evaluation by MicroCT and histology. Across all time points, we found that OS cells transduced with AdS100A6 showed higher Xenogen signals and/or larger tumor masses compared to those transduced with AdRFP or AdR-siS100A6 (Fig. 3a). There was a statistically significant increase in the average bioluminescence signal by the third week ($p < 0.05$) (Fig. 3b).

Measured tumor volumes were consistent with corresponding bioluminescence signals, and increased significantly in the S100A6 group starting on day 14 ($p < 0.03$). Although S100A6 knockdown seemingly decreased growth of the primary tumor, this result only approached statistical significance ($p > 0.06$). S100A6 overexpression decreased the tumor doubling time by over 23% compared to that of the RFP control group's (Fig. 3c). Specifically, the doubling times for the RFP control, S100A6, and siS100A6 groups were 7.92 ± 0.81 , 6.02 ± 0.73 , and 8.79 ± 0.45 days, respectively. In addition to promoting primary tumor growth, S100A6 overexpression showed readily detectable osteolytic destruction of the retrieved proximal tibias and fibulas (Fig. 3d). Histologic staining demonstrated osteolytic lesions of the posterior tibial bone cortex and tumor cell infiltration into the bone marrow cavity in the S100A6 overexpression group. Bone destruction was less apparent in the control and siS100A6 treated group (Fig. 4). These results suggest that S100A6 can effectively promote OS tumor growth in an orthotopic murine model of human osteosarcoma.

Fig. 5. S100A6 promote cell cycle progression and inhibits apoptosis. (a) Cell cycle analysis by flow cytometry. Subconfluent 143B cells were infected with AdS100A6, AdR-siS100A6, or AdRFP adenoviruses for 48h, collected, fixed, stained with propidium iodide, and subjected to flow cytometry. Percent of the analyzed cells in the G1 was graphed and shown. “*” indicates $p < 0.05$ compared to the control groups. (b) Immunohistochemical staining for cleaved caspase 3. Subconfluent 143B cells were infected with AdS100A6, AdR-siS100A6, or control AdRFP adenoviruses. At 3 days after infection, immunohistochemical staining with an anti-cleaved caspase-3 antibody was performed. Representative images are shown. Control IgG or without primary antibody staining were used as negative controls (data not shown). Representative results are shown. (c) & (d) Apoptosis analysis. Subconfluent MG63 (c-i) and 143B (c-ii) cells were seeded in 6-well plates and infected with AdS100A6, AdR-siS100A6, or AdRFP adenoviruses in 1% FBS DMEM medium for 3 days. Cells were collected, fixed and stained with Annexin V-FITC (BD Bioscience) and propidium iodide, and then subjected to FACS analysis to sort out living/normal versus early stages of apoptosis. The percentages of cells in each stage were calculated and graphed (d). FACS analysis was done in triplicate for each assay condition. Representative results are shown. “*” indicates $p < 0.01$ compared to the control groups.

S100A6 promotes cell cycle progression and inhibits apoptosis in human OS cells

To understand the possible mechanism underlying proliferation and tumor growth, we examined the effect of S100A6 expression on cell cycle progression in human OS cell lines. Subconfluent 143B cells were transduced with AdS100A6, AdR-siS100A6, or AdRFP for 48h and subjected to flow cytometry. Consistent with the aforementioned results of cell proliferation assays, a significantly lower percentage (e.g., 24.98%) of cells were found to be in G1 phase following transduction with AdS100A6 when compared to the control (33.3%) and AdsiS100A6 transduction group (36.8%) ($p < 0.05$) (Fig. 5a).

We further examined the effect of S100A6 expression on apoptosis in human OS cell lines. When the OS cells were transduced with AdS100A6, AdR-siS100A6, and AdRFP and stained with an antibody against cleaved caspase 3, increased expression was readily detected in OS cells transduced with siS100A6; caspase 3 expression was less apparent in the control and S100A6 groups (Fig. 5b).

The effect of S100A6 on expression of pro-apoptotic genes in human OS cells was investigated using FACS analysis. S100A6 overexpression decreased the number of cells undergoing early-stage apoptosis compared to transduction with RFP; silencing S100A6 increased the number of cells undergoing early-stage apoptosis (Fig. 5c). Quantitatively, the percentage of early apoptotic cells decreased from 48.9% in the control group to

Fig. 6. S100A6 inhibits osteogenic differentiation of mesenchymal stem cells. (a) & (b). Alkaline phosphatase (ALP) activity assay. Subconfluent iMEFs (a) and C3H10T_{1/2} (b) cells were infected with AdS100A6, AdR-siS100A6, or AdRFP control adenoviruses. ALP activity was measured at the indicated time points. “*” indicates $p < 0.05$ compared to the control groups. Each assay condition was performed in triplicate. (c) ALP activity histochemical staining. Subconfluent iMEFs and C3H10T_{1/2} cells were transduced with AdS100A6, AdR-siS100A6, or AdRFP control adenoviruses. At 7 days after infection, cells were stained histochemically for ALP activity. Each assay condition was performed in triplicate. Representative results are shown. (d) Alizarin Red S staining. Subconfluent iMEFs and C3H10T_{1/2} were infected with AdS100A6, AdR-siS100A6, or AdGFP control adenoviruses, and cultured in mineralization medium for 14 days. Cells were fixed and stained with Alizarin Red solution. Representative images of cell culture dishes are shown. (e) Silencing S100A6 augments BMP9-induced ectopic bone formation. Subconfluent iMEFs co-infected with AdBMP9 and AdGFP or Ad-siS100A6 were subcutaneously injected into the flanks of athymic nude mice ($n = 4/\text{group}$). At 4 weeks after implantation, the mice were euthanized and ectopic bone masses were retrieved for μCT imaging. Quantitative analysis was done using Amira imaging software.

17.78% in the S100A6 overexpression group ($p < 0.01$) (Fig. 5d). Knockdown of S100A6 expression increased the number of the cells in the early apoptotic phase from 48.9% to 61.7% compared to the control ($p < 0.01$) (Fig. 5d). Consequently, overexpression of S100A6 significantly increased the living cell populations in both MG63 and 143B lines (Fig. 5d) ($p < 0.01$). Taken together, these results demonstrate that S100A6 overexpression promotes the OS cell survival while silencing S100A6 expression increases OS cells' susceptibility to apoptosis.

Knockdown of S100A6 promotes osteogenic differentiation of MSCs

Given our prior work demonstrating that human OS development is at least in part caused by defects in the process of osteogenic differentiation [6, 7, 27, 92], we tested

the effect of S100A6 on osteogenic differentiation of MSCs. By transducing cells from the commonly-used MSC lines iMEFs and C3H10T_{1/2} with AdS100A6, AdR-siS100A6, or AdRFP, we found that S100A6 knockdown effectively induced alkaline phosphatase (ALP) activity, while S100A6 overexpression seemingly inhibited ALP activity, although not to a statistically significant level when compared to the control ($p > 0.05$) (Fig. 6a & 6b). However, data from histochemical staining of ALP activity, demonstrated a statistically significant increase in ALP positive staining among both iMEFs and C3H10T_{1/2} cells transduced with AdsiS100A6. By comparison, overexpression of S100A6 reduced the positive staining of ALP activity in both MSC cell lines (Fig. 6c). These results strongly suggest that silencing S100A6 expression may promote mesenchymal progenitor cells towards an osteogenic lineage – a process which may be inhibited by S100A6 overexpression.

We further examined the effect of S100A6 on the late stages of osteogenic differentiation and BMP9-mediated bone formation. To analyze the effects of S100A6 overexpression and knockdown on matrix mineralization of MSCs, iMEFs and C3H10T_{1/2} cells with transduced with AdS100A6, AdR-siS100A6, or AdRFP and grown in mineralization medium for 14 days. Positive mineralization nodules stained with Alizarin Red S were observed in the S100A6 knockdown group, whereas S100A6 overexpression inhibited mineralized matrix formation *in vitro* (Fig. 6d).

We have previously demonstrated BMP9 to be one of the most potent osteogenic BMPs [28, 29, 32, 63, 64, 79, 93]. In pilot studies, it was demonstrated that S100A6 knockdown induces ALP activity, but not to a degree sufficient for induction of ectopic bone formation *in vivo*. Here, we tested the effect of S100A6 expression on BMP9-mediated bone formation. When subconfluent iMEFs co-infected with AdBMP9, AdGFP or AdsiS100A6 were subcutaneously injected into the flanks of athymic nude mice for 4 weeks, MicroCT imaging analysis indicated that the ectopic bone masses formed were significantly larger in the S100A6 knockdown group compared to those formed in the BMP9 + GFP group (Fig. 6e). It is worth noting that S100A6 overexpression did not significantly affect BMP9-induced ectopic bone formation (data not shown). Quantitative analysis using the Amira imaging software revealed that silencing of S100A6 enhanced the bone mass volume of the BMP9-induced ectopic bone formation from 1.67 ± 0.58 to 4.96 ± 1.28 mm³ ($p < 0.001$) (data not shown). Taken together, the above *in vitro* and *in vivo* data strongly suggest that silencing S100A6 expression may facilitate osteogenic differentiation of MSCs.

Discussion

Several members of the S100 protein family have previously been implicated in tumorigenesis across a range of tumor types [39-54]. Our own group has previously demonstrated that S100A6 expression was upregulated in OS compared to mesenchymal progenitor cells [35]. However, the functional role of S100A6 in tumorigenesis remains poorly characterized. Here, we chose to analyze the role of S100A6 in regulating the tumorigenic phenotypes associated with human OS to elucidate a possible role for S100A6 in development of OS.

Results of adenovirus-mediated overexpression or knockdown of S100A6 in human OS cell lines demonstrated that S100A6 accelerates a tumorigenic phenotype by promoting cell proliferation and primary tumor growth. This effect is mediated at least in part by S100A6's ability to facilitate cell cycle progression and to promote a pro-survival, anti-apoptotic phenotype in OS cells. Conversely, knockdown of endogenous S100A6 enhances the expression of apoptotic markers and induces cells to progress along the osteoblastic differentiation cascade. We further demonstrated that knockdown of endogenous S100A6 not only leads to an increase in early and late osteogenic markers, but also augments BMP9-induced ectopic bone formation.

The potential link between cancer and members of the S100 protein family, namely S100A2, S100A4, S100A6, S100A7, and S100B, stems from altered levels of expression across

multiple tumor types [37, 39]. Upon comparing the expression of S100 proteins in human OS cell lines, we found significant upregulation of S100A6 across all cell lines. We have previously demonstrated that endogenous expression of S100A6 is increased in patient-derived OS cell lines relative to normal osteoblasts [34, 35]. Interestingly, in a small set of mostly high-grade clinical OS samples, although increased S100A6 staining was detected in more than 80% of examined samples; increased expression levels were correlated clinically with decreased metastasis [34, 35]. In our current investigation, we did not observe pulmonary metastases despite significantly increased tumor growth with overexpression of S100A6 (data not shown). It is possible that S100A6 may have two distinct and independent functions regulating tumor growth and phenotypes for metastasis. Alternatively, the discrepancy may be due to a small patient sample size or due to a limited range of disease severity across samples.

In concordance with our current and previous findings, Muramatsu et al. have also reported increased S100A6 levels in OS tumor specimens [94]. S100A6 expression has been well studied in tumorigenesis; increased endogenous S100A6 expression has been noted across a variety of tumor types [40]. The increased expression levels also seem to directly correlate with tumor size and aggressive phenotypes [40]. These reports are consistent with our *in vitro* and *in vivo* findings, where overexpression of S100A6 leads to development of a more proliferative phenotype and a larger primary tumor. Similar results have been reported from expression analyses of gastric cancer and melanoma, where increased S100A6 levels were correlated with larger, more aggressive tumors [95, 96]. Although the difference in size of the siRNA mediated knockdown tumors did not reach statistical significance in our study, there was a noticeable decrease when compared to the controls that may be secondary to incomplete knockdown of S100A6 in the tumor cells. Nonetheless, our results strongly suggest that expression of S100A6 may directly correlate with the aggressive nature of tumors.

In its native form, binding of dimerized S100A6 to calcium induces a conformational change that facilitates binding to target proteins [39, 42, 54]. S100A6 expression is upregulated by many hormones, growth factors, and stress response mechanisms [39, 40, 42]. S100A6 deficiency has been shown to significantly inhibit the proliferation of fibroblasts, osteoblasts, and pancreatic tumor and duct cells [40, 55, 97]. It has been suggested that S100A6 controls cell proliferation by targeting cell cycle regulatory proteins. It has been reported that S100A6 binds p53 in a calcium dependent manner through its tetramerization and N-terminal domains, similar to other S100 proteins [54, 55]. Furthermore, S100A6 was shown to interfere with the phosphorylation of p53, preventing the cell from undergoing apoptosis [98]. Multiple cytogenetic abnormalities have been described in OS, including mutations in the tumor suppressor *p53* and in multiple oncogenes, including *Rb* and *Myc* [6, 7, 18]. Therefore, the ability of S100A6 to bind and modulate the pathways associated with p53, Rb, or any other oncogene, may explain the tumorigenic phenotypes we observed upon S100A6 overexpression in OS cells.

The role of S100A6 in osteogenic differentiation may also provide a potential clue to its pro-survival phenotype in OS cells. Osteogenic differentiation requires a delicate balance between proliferation and differentiation of mesenchymal progenitor cells [93, 99-101]. We have demonstrated that disruptions in this balance may lead to and/or accelerate OS development and progression [6, 7, 27, 92]. Thus, augmentation of proliferation or inhibition of apoptosis would promote the tumorigenic transformation in OS development. Accordingly, we found that S100A6 knockdown in mesenchymal progenitor cells promotes differentiation and decreases cellular proliferation.

In summary, our results indicate that S100A6 is an important regulator in OS tumorigenesis. The expression level of S100A6 is upregulated in human OS. Overexpression of S100A6 results in a more tumorigenic phenotype in OS, promotes cell proliferation and tumor growth. These effects may be caused by the ability of S100A6 to regulate the cell cycle, apoptosis, and osteogenic differentiation. It is, thus, conceivable that targeting S100A6 may lead to the development of novel anti-OS therapies.

Acknowledgments

The reported work was supported in part by research grants from the National Institutes of Health (AT004418, AR50142, AR054381 to TCH, RCH and HHL), and the 973 Program of Ministry of Science and Technology (MOST) of China (#2011CB707900 to TCH). MKM was a recipient of Howard Hughes Medical Institute Medical Research Fellowship. This work was also supported in part by The University of Chicago Core Facility Subsidy grant from the National Center for Advancing Translational Sciences (NCATS) of the National Institutes of Health through Grant UL1 TR000430. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Disclosure Statement

The authors declare no competing financial interests.

References

- Isakoff MS, Bielack SS, Meltzer P, Gorlick R: Osteosarcoma: Current treatment and a collaborative pathway to success. *J Clin Oncol* 2015;33:3029-3035.
- Morrow JJ, Khanna C: Osteosarcoma genetics and epigenetics: Emerging biology and candidate therapies. *Crit Rev Oncog* 2015;20:173-197.
- Kansara M, Teng MW, Smyth MJ, Thomas DM: Translational biology of osteosarcoma. *Nat Rev Cancer* 2014;14:722-735.
- Moore DD, Luu HH: Osteosarcoma. *Cancer Treat Res* 2014;162:65-92.
- Davis AM, Bell RS, Goodwin PJ: Prognostic factors in osteosarcoma: A critical review. *J Clin Oncol* 1994;12:423-431.
- Tang N, Song WX, Luo J, Haydon RC, He TC: Osteosarcoma development and stem cell differentiation. *Clin Orthop Relat Res* 2008;466:2114-2130.
- Wagner ER, Luther G, Zhu G, Luo Q, Shi Q, Kim SH, Gao JL, Huang E, Gao Y, Yang K, Wang L, Teven C, Luo X, Liu X, Li M, Hu N, Su Y, Bi Y, He BC, Tang N, Luo J, Chen L, Zuo G, Rames R, Haydon RC, Luu HH, He TC: Defective osteogenic differentiation in the development of osteosarcoma. *Sarcoma* 2011;2011:325238.
- Meyers PA, Gorlick R: Osteosarcoma. *Pediatr Clin North Am* 1997;44:973-989.
- Chou AJ, Gorlick R: Chemotherapy resistance in osteosarcoma: Current challenges and future directions. *Expert Rev Anticancer Ther* 2006;6:1075-1085.
- Sleijfer S, Gelderblom H: Current clinical trials for advanced osteosarcoma and soft tissue sarcoma. *Curr Opin Oncol* 2014;26:434-439.
- Botter SM, Neri D, Fuchs B: Recent advances in osteosarcoma. *Curr Opin Pharmacol* 2014;16:15-23.
- Zhou W, Hao M, Du X, Chen K, Wang G, Yang J: Advances in targeted therapy for osteosarcoma. *Discov Med* 2014;17:301-307.
- Luetke A, Meyers PA, Lewis I, Juergens H: Osteosarcoma treatment - where do we stand? A state of the art review. *Cancer Treat Rev* 2014;40:523-532.
- Yamamoto N, Tsuchiya H: Chemotherapy for osteosarcoma - where does it come from? What is it? Where is it going? *Expert Opin Pharmacother* 2013;14:2183-2193.
- Yang J, Zhang W: New molecular insights into osteosarcoma targeted therapy. *Curr Opin Oncol* 2013;25:398-406.
- Ando K, Heymann MF, Stresing V, Mori K, Redini F, Heymann D: Current therapeutic strategies and novel approaches in osteosarcoma. *Cancers (Basel)* 2013;5:591-616.
- Nataraj V, Batra A, Rastogi S, Khan SA, Sharma MC, Vishnubhatla S, Bakhshi S: Developing a prognostic model for patients with localized osteosarcoma treated with uniform chemotherapy protocol without high dose methotrexate: A single-center experience of 237 patients. *J Surg Oncol* 2015;112:662-668.
- Sandberg AA, Bridge JA: Updates on the cytogenetics and molecular genetics of bone and soft tissue tumors: Osteosarcoma and related tumors. *Cancer Genet Cytogenet* 2003;145:1-30.

- 19 Helman LJ, Meltzer P: Mechanisms of sarcoma development. *Nat Rev Cancer* 2003;3:685-694.
- 20 Green JT, Mills AM: Osteogenic tumors of bone. *Semin Diagn Pathol* 2014;31:21-29.
- 21 Kuijjer ML, Hogendoorn PC, Cleton-Jansen AM: Genome-wide analyses on high-grade osteosarcoma: Making sense of a genomically most unstable tumor. *Int J Cancer* 2013;133:2512-2521.
- 22 Haydon RC, Zhou L, Feng T, Breyer B, Cheng H, Jiang W, Ishikawa A, Peabody T, Montag A, Simon MA, He TC: Nuclear receptor agonists as potential differentiation therapy agents for human osteosarcoma. *Clin Cancer Res* 2002;8:1288-1294.
- 23 He BC, Chen L, Zuo GW, Zhang W, Bi Y, Huang J, Wang Y, Jiang W, Luo Q, Shi Q, Zhang BQ, Liu B, Lei X, Luo J, Luo X, Wagner ER, Kim SH, He CJ, Hu Y, Shen J, Zhou Q, Rastegar F, Deng ZL, Luu HH, He TC, Haydon RC: Synergistic antitumor effect of the activated ppargamma and retinoid receptors on human osteosarcoma. *Clin Cancer Res* 2010;16:2235-2245.
- 24 Su Y, Wagner ER, Luo Q, Huang J, Chen L, He BC, Zuo GW, Shi Q, Zhang BQ, Zhu G, Bi Y, Luo J, Luo X, Kim SH, Shen J, Rastegar F, Huang E, Gao Y, Gao JL, Yang K, Wietholt C, Li M, Qin J, Haydon RC, He TC, Luu HH: Insulin-like growth factor binding protein 5 suppresses tumor growth and metastasis of human osteosarcoma. *Oncogene* 2011;30:3907-3917.
- 25 Rastegar F, Gao JL, Shenaq D, Luo Q, Shi Q, Kim SH, Jiang W, Wagner ER, Huang E, Gao Y, Shen J, Yang K, He BC, Chen L, Zuo GW, Luo J, Luo X, Bi Y, Liu X, Li M, Hu N, Wang L, Luther G, Luu HH, Haydon RC, He TC: Lysophosphatidic acid acyltransferase beta (lpaatbeta) promotes the tumor growth of human osteosarcoma. *PLoS One* 2010;5:e14182.
- 26 Denduluri SK, Olumuyiwa Idowu O, Wang Z, Liao Z, Yan Z, Mohammed MK, Ye J, Wei Q, Wang J, Zhao L, Luu HH: Insulin-like growth factor (igf) signaling in tumorigenesis and the development of cancer drug resistance. *Genes Dis* 2015;2:13-25.
- 27 Luo X, Chen J, Song WX, Tang N, Luo J, Deng ZL, Sharff KA, He G, Bi Y, He BC, Bennett E, Huang J, Kang Q, Jiang W, Su Y, Zhu GH, Yin H, He Y, Wang Y, Souris JS, Chen L, Zuo GW, Montag AG, Reid RR, Haydon RC, Luu HH, He TC: Osteogenic bmps promote tumor growth of human osteosarcomas that harbor differentiation defects. *Lab Invest* 2008;88:1264-1277.
- 28 Wang J, Zhang H, Zhang W, Huang E, Wang N, Wu N, Wen S, Chen X, Liao Z, Deng F, Yin L, Zhang J, Zhang Q, Yan Z, Liu W, Zhang Z, Ye J, Deng Y, Luu HH, Haydon RC, He TC, Deng F: Bone morphogenetic protein-9 (bmp9) effectively induces osteo/odontoblastic differentiation of the reversibly immortalized stem cells of dental apical papilla. *Stem Cells Dev* 2014;23:1405-1416.
- 29 Luther G, Wagner ER, Zhu G, Kang Q, Luo Q, Lamplot J, Bi Y, Luo X, Luo J, Teven C, Shi Q, Kim SH, Gao JL, Huang E, Yang K, Rames R, Liu X, Li M, Hu N, Liu H, Su Y, Chen L, He BC, Zuo GW, Deng ZL, Reid RR, Luu HH, Haydon RC, He TC: Bmp-9 induced osteogenic differentiation of mesenchymal stem cells: Molecular mechanism and therapeutic potential. *Curr Gene Ther* 2011;11:229-240.
- 30 Jo A, Denduluri SK, Zhang B, Wang Z, Yin L, Yan Z, Kang R, Shi LL, Mok J, Lee MJ, Haydon RC: The versatile functions of sox9 in development, stem cells, and human diseases. *Genes Dis* 2014;1:149-161.
- 31 Teven CM, Farina EM, Rivas J, Reid RR: Fibroblast growth factor (fgf) signaling in development and skeletal diseases. *Genes Dis* 2014;1:199-213.
- 32 Lamplot JD, Qin J, Nan G, Wang J, Liu X, Yin L, Tomal J, Li R, Shui W, Zhang H, Kim SH, Zhang W, Zhang J, Kong Y, Denduluri S, Rogers MR, Pratt A, Haydon RC, Luu HH, Angeles J, Shi LL, He TC: Bmp9 signaling in stem cell differentiation and osteogenesis. *Am J Stem Cells* 2013;2:1-21.
- 33 Basu-Roy U, Basilico C, Mansukhani A: Perspectives on cancer stem cells in osteosarcoma. *Cancer Lett* 2013;338:158-167.
- 34 Luu HH, Zhou L, Haydon RC, Deyrup AT, Montag AG, Huo D, Heck R, Heizmann CW, Peabody TD, Simon MA, He TC: Increased expression of s100a6 is associated with decreased metastasis and inhibition of cell migration and anchorage independent growth in human osteosarcoma. *Cancer Lett* 2005;229:135-148.
- 35 Luo X, Sharff KA, Chen J, He TC, Luu HH: S100a6 expression and function in human osteosarcoma. *Clin Orthop Relat Res* 2008;466:2060-2070.
- 36 Schafer BW, Heizmann CW: The s100 family of ef-hand calcium-binding proteins: Functions and pathology. *Trends Biochem Sci* 1996;21:134-140.
- 37 Emberley ED, Murphy LC, Watson PH: S100 proteins and their influence on pro-survival pathways in cancer. *Biochem Cell Biol* 2004;82:508-515.
- 38 Santamaria-Kisiel L, Rintala-Dempsey AC, Shaw GS: Calcium-dependent and -independent interactions of the s100 protein family. *Biochem J* 2006;396:201-214.

- 39 Bresnick AR, Weber DJ, Zimmer DB: S100 proteins in cancer. *Nat Rev Cancer* 2015;15:96-109.
- 40 Lesniak W, Slomnicki LP, Filipek A: S100a6 - new facts and features. *Biochem Biophys Res Commun* 2009;390:1087-1092.
- 41 Ji YF, Huang H, Jiang F, Ni RZ, Xiao MB: S100 family signaling network and related proteins in pancreatic cancer (review). *Int J Mol Med* 2014;33:769-776.
- 42 Gross SR, Sin CG, Barraclough R, Rudland PS: Joining s100 proteins and migration: For better or for worse, in sickness and in health. *Cell Mol Life Sci* 2014;71:1551-1579.
- 43 Wang XH, Zhang LH, Zhong XY, Xing XF, Liu YQ, Niu ZJ, Peng Y, Du H, Zhang GG, Hu Y, Liu N, Zhu YB, Ge SH, Zhao W, Lu AP, Li JY, Ji JF: S100a6 overexpression is associated with poor prognosis and is epigenetically up-regulated in gastric cancer. *Am J Pathol* 2010;177:586-597.
- 44 Hua Z, Chen J, Sun B, Zhao G, Zhang Y, Fong Y, Jia Z, Yao L: Specific expression of osteopontin and s100a6 in hepatocellular carcinoma. *Surgery* 2011;149:783-791.
- 45 Kilanczyk E, Graczyk A, Ostrowska H, Kasacka I, Lesniak W, Filipek A: S100a6 is transcriptionally regulated by beta-catenin and interacts with a novel target, lamin a/c, in colorectal cancer cells. *Cell Calcium* 2012;51:470-477.
- 46 Sato Y, Harada K, Sasaki M, Nakanuma Y: Clinicopathological significance of s100 protein expression in cholangiocarcinoma. *J Gastroenterol Hepatol* 2013;28:1422-1429.
- 47 Li Z, Tang M, Ling B, Liu S, Zheng Y, Nie C, Yuan Z, Zhou L, Guo G, Tong A, Wei Y: Increased expression of s100a6 promotes cell proliferation and migration in human hepatocellular carcinoma. *J Mol Med (Berl)* 2014;92:291-303.
- 48 Duan L, Wu R, Zou Z, Wang H, Ye L, Li H, Yuan S, Li X, Zha H, Sun H, Zhang Y, Chen X, Zhou L: S100a6 stimulates proliferation and migration of colorectal carcinoma cells through activation of the mapk pathways. *Int J Oncol* 2014;44:781-790.
- 49 Zhang J, Zhang K, Jiang X: S100a6 as a potential serum prognostic biomarker and therapeutic target in gastric cancer. *Dig Dis Sci* 2014;59:2136-2144.
- 50 Chen X, Liu X, Lang H, Zhang S, Luo Y, Zhang J: S100 calcium-binding protein a6 promotes epithelial-mesenchymal transition through beta-catenin in pancreatic cancer cell line. *PLoS One* 2015;10:e0121319.
- 51 Lyu X, Li H, Ma X, Li X, Gao Y, Ni D, Shen D, Gu L, Wang B, Zhang Y, Zhang X: High-level s100a6 promotes metastasis and predicts the outcome of t1-t2 stage in clear cell renal cell carcinoma. *Cell Biochem Biophys* 2015;71:279-290.
- 52 Lyu XJ, Li HZ, Ma X, Li XT, Gao Y, Ni D, Shen DL, Gu LY, Wang BJ, Zhang Y, Zhang X: Elevated s100a6 (calcylin) enhances tumorigenesis and suppresses cxcl14-induced apoptosis in clear cell renal cell carcinoma. *Oncotarget* 2015;6:6656-6669.
- 53 Wang T, Liang Y, Thakur A, Zhang S, Yang T, Chen T, Gao L, Chen M, Ren H: Diagnostic significance of s100a2 and s100a6 levels in sera of patients with non-small cell lung cancer. *Tumour Biol* DOI:10.1007/s13277-015-4057-z.
- 54 Fernandez-Fernandez MR, Rutherford TJ, Fersht AR: Members of the s100 family bind p53 in two distinct ways. *Protein Sci* 2008;17:1663-1670.
- 55 Slomnicki LP, Nawrot B, Lesniak W: S100a6 binds p53 and affects its activity. *Int J Biochem Cell Biol* 2009;41:784-790.
- 56 Su Y, Luo X, He BC, Wang Y, Chen L, Zuo GW, Liu B, Bi Y, Huang J, Zhu GH, He Y, Kang Q, Luo J, Shen J, Chen J, Jin X, Haydon RC, He TC, Luu HH: Establishment and characterization of a new highly metastatic human osteosarcoma cell line. *Clin Exp Metastasis* 2009;26:599-610.
- 57 Huang E, Bi Y, Jiang W, Luo X, Yang K, Gao JL, Gao Y, Luo Q, Shi Q, Kim SH, Liu X, Li M, Hu N, Liu H, Cui J, Zhang W, Li R, Chen X, Shen J, Kong Y, Zhang J, Wang J, Luo J, He BC, Wang H, Reid RR, Luu HH, Haydon RC, Yang L, He TC: Conditionally immortalized mouse embryonic fibroblasts retain proliferative activity without compromising multipotent differentiation potential. *PLoS One* 2012;7:e32428.
- 58 Wang N, Zhang W, Cui J, Zhang H, Chen X, Li R, Wu N, Chen X, Wen S, Zhang J, Yin L, Deng F, Liao Z, Zhang Z, Zhang Q, Yan Z, Liu W, Ye J, Deng Y, Wang Z, Qiao M, Luu HH, Haydon RC, Shi LL, Liang H, He TC: The piggybac transposon-mediated expression of sv40 t antigen efficiently immortalizes mouse embryonic fibroblasts (mefs). *PLoS One* 2014;9:e97316.
- 59 Chen X, Cui J, Yan Z, Zhang H, Chen X, Wang N, Shah P, Deng F, Zhao C, Geng N, Li M, Denduluri SK, Haydon RC, Luu HH, Reid RR, He TC: Sustained high level transgene expression in mammalian cells mediated by the optimized piggybac transposon system. *Genes Dis* 2015;2:96-105.

- 60 Chen X, Luther G, Zhang W, Nan G, Wagner ER, Liao Z, Wu N, Zhang H, Wang N, Wen S, He Y, Deng F, Zhang J, Wu D, Zhang B, Haydon RC, Zhou L, Luu HH, He TC: The e-f hand calcium-binding protein s100a4 regulates the proliferation, survival and differentiation potential of human osteosarcoma cells. *Cell Physiol Biochem* 2013;32:1083-1096.
- 61 Zhang H, Wang J, Deng F, Huang E, Yan Z, Wang Z, Deng Y, Zhang Q, Zhang Z, Ye J, Qiao M, Li R, Wang J, Wei Q, Zhou G, Luu HH, Haydon RC, He TC, Deng F: Canonical wnt signaling acts synergistically on bmp9-induced osteo/odontoblastic differentiation of stem cells of dental apical papilla (scaps). *Biomaterials* 2015;39:145-154.
- 62 He TC, Zhou S, da Costa LT, Yu J, Kinzler KW, Vogelstein B: A simplified system for generating recombinant adenoviruses. *Proc Natl Acad Sci U S A* 1998;95:2509-2514.
- 63 Cheng H, Jiang W, Phillips FM, Haydon RC, Peng Y, Zhou L, Luu HH, An N, Breyer B, Vanichakarn P, Szatkowski JP, Park JY, He TC: Osteogenic activity of the fourteen types of human bone morphogenetic proteins (bmps). *J Bone Joint Surg Am* 2003;85-A:1544-1552.
- 64 Kang Q, Sun MH, Cheng H, Peng Y, Montag AG, Deyrup AT, Jiang W, Luu HH, Luo J, Szatkowski JP, Vanichakarn P, Park JY, Li Y, Haydon RC, He TC: Characterization of the distinct orthotopic bone-forming activity of 14 bmps using recombinant adenovirus-mediated gene delivery. *Gene Ther* 2004;11:1312-1320.
- 65 Luo Q, Kang Q, Song WX, Luu HH, Luo X, An N, Luo J, Deng ZL, Jiang W, Yin H, Chen J, Sharff KA, Tang N, Bennett E, Haydon RC, He TC: Selection and validation of optimal sirna target sites for rna-mediated gene silencing. *Gene* 2007;395:160-169.
- 66 Luo J, Deng ZL, Luo X, Tang N, Song WX, Chen J, Sharff KA, Luu HH, Haydon RC, Kinzler KW, Vogelstein B, He TC: A protocol for rapid generation of recombinant adenoviruses using the adeasy system. *Nat Protoc* 2007;2:1236-1247.
- 67 Deng F, Chen X, Liao Z, Yan Z, Wang Z, Deng Y, Zhang Q, Zhang Z, Ye J, Qiao M, Li R, Denduluri S, Wang J, Wei Q, Li M, Geng N, Zhao L, Zhou G, Zhang P, Luu HH, Haydon RC, Reid RR, Yang T, He TC: A simplified and versatile system for the simultaneous expression of multiple sirnas in mammalian cells using gibson DNA assembly. *PLoS One* 2014;9:e113064.
- 68 Wu N, Zhang H, Deng F, Li R, Zhang W, Chen X, Wen S, Wang N, Zhang J, Yin L, Liao Z, Zhang Z, Zhang Q, Yan Z, Liu W, Wu D, Ye J, Deng Y, Yang K, Luu HH, Haydon RC, He TC: Overexpression of ad5 precursor terminal protein accelerates recombinant adenovirus packaging and amplification in hek-293 packaging cells. *Gene Ther* 2014;21:629-637.
- 69 Liu X, Qin J, Luo Q, Bi Y, Zhu G, Jiang W, Kim SH, Li M, Su Y, Nan G, Cui J, Zhang W, Li R, Chen X, Kong Y, Zhang J, Wang J, Rogers MR, Zhang H, Shui W, Zhao C, Wang N, Liang X, Wu N, He Y, Luu HH, Haydon RC, Shi LL, Li T, He TC, Li M: Cross-talk between egf and bmp9 signalling pathways regulates the osteogenic differentiation of mesenchymal stem cells. *J Cell Mol Med* 2013;17:1160-1172.
- 70 Wen S, Zhang H, Li Y, Wang N, Zhang W, Yang K, Wu N, Chen X, Deng F, Liao Z, Zhang J, Zhang Q, Yan Z, Liu W, Zhang Z, Ye J, Deng Y, Zhou G, Luu HH, Haydon RC, Shi LL, He TC, Wei G: Characterization of constitutive promoters for piggybac transposon-mediated stable transgene expression in mesenchymal stem cells (mscs). *PLoS One* 2014;9:e94397.
- 71 Wang N, Zhang H, Zhang BQ, Liu W, Zhang Z, Qiao M, Zhang H, Deng F, Wu N, Chen X, Wen S, Zhang J, Liao Z, Zhang Q, Yan Z, Yin L, Ye J, Deng Y, Luu HH, Haydon RC, Liang H, He TC: Adenovirus-mediated efficient gene transfer into cultured three-dimensional organoids. *PLoS One* 2014;9:e93608.
- 72 Zhao C, Wu N, Deng F, Zhang H, Wang N, Zhang W, Chen X, Wen S, Zhang J, Yin L, Liao Z, Zhang Z, Zhang Q, Yan Z, Liu W, Wu D, Ye J, Deng Y, Zhou G, Luu HH, Haydon RC, Si W, He TC: Adenovirus-mediated gene transfer in mesenchymal stem cells can be significantly enhanced by the cationic polymer polybrene. *PLoS One* 2014;9:e92908.
- 73 Zhang Q, Wang J, Deng F, Yan Z, Xia Y, Wang Z, Ye J, Deng Y, Zhang Z, Qiao M, Li R, Denduluri SK, Wei Q, Zhao L, Lu S, Wang X, Tang S, Liu H, Luu HH, Haydon RC, He TC, Jiang L: Tqpcr: A touchdown qpcr assay with significantly improved detection sensitivity and amplification efficiency of sybr green qpcr. *PLoS One* 2015;10:e0132666.
- 74 Luu HH, Kang Q, Park JK, Si W, Luo Q, Jiang W, Yin H, Montag AG, Simon MA, Peabody TD, Haydon RC, Rinker-Schaeffer CW, He TC: An orthotopic model of human osteosarcoma growth and spontaneous pulmonary metastasis. *Clin Exp Metastasis* 2005;22:319-329.

- 75 Li R, Zhang W, Cui J, Shui W, Yin L, Wang Y, Zhang H, Wang N, Wu N, Nan G, Chen X, Wen S, Deng F, Zhou G, Liao Z, Zhang J, Zhang Q, Yan Z, Liu W, Zhang Z, Ye J, Deng Y, Luu HH, Haydon RC, He TC, Deng ZL: Targeting bmp9-promoted human osteosarcoma growth by inactivation of notch signaling. *Curr Cancer Drug Targets* 2014;14:274-285.
- 76 He BC, Gao JL, Zhang BQ, Luo Q, Shi Q, Kim SH, Huang E, Gao Y, Yang K, Wagner ER, Wang L, Tang N, Luo J, Liu X, Li M, Bi Y, Shen J, Luther G, Hu N, Zhou Q, Luu HH, Haydon RC, Zhao Y, He TC: Tetrandrine inhibits wnt/beta-catenin signaling and suppresses tumor growth of human colorectal cancer. *Mol Pharmacol* 2011;79:211-219.
- 77 Bi Y, He Y, Huang J, Su Y, Zhu GH, Wang Y, Qiao M, Zhang BQ, Zhang H, Wang Z, Liu W, Cui J, Kang Q, Zhang Z, Deng Y, Li R, Zhang Q, Yang K, Luu HH, Haydon RC, He TC, Tang N: Functional characteristics of reversibly immortalized hepatic progenitor cells derived from mouse embryonic liver. *Cell Physiol Biochem* 2014;34:1318-1338.
- 78 Gao Y, Huang E, Zhang H, Wang J, Wu N, Chen X, Wang N, Wen S, Nan G, Deng F, Liao Z, Wu D, Zhang B, Zhang J, Haydon RC, Luu HH, Shi LL, He TC: Crosstalk between wnt/beta-catenin and estrogen receptor signaling synergistically promotes osteogenic differentiation of mesenchymal progenitor cells. *PLoS One* 2013;8:e82436.
- 79 Kang Q, Song WX, Luo Q, Tang N, Luo J, Luo X, Chen J, Bi Y, He BC, Park JK, Jiang W, Tang Y, Huang J, Su Y, Zhu GH, He Y, Yin H, Hu Z, Wang Y, Chen L, Zuo GW, Pan X, Shen J, Vokes T, Reid RR, Haydon RC, Luu HH, He TC: A comprehensive analysis of the dual roles of bmps in regulating adipogenic and osteogenic differentiation of mesenchymal progenitor cells. *Stem Cells Dev* 2009;18:545-559.
- 80 Luo Q, Kang Q, Si W, Jiang W, Park JK, Peng Y, Li X, Luu HH, Luo J, Montag AG, Haydon RC, He TC: Connective tissue growth factor (ctgf) is regulated by wnt and bone morphogenetic proteins signaling in osteoblast differentiation of mesenchymal stem cells. *J Biol Chem* 2004;279:55958-55968.
- 81 Zhang W, Deng ZL, Chen L, Zuo GW, Luo Q, Shi Q, Zhang BQ, Wagner ER, Rastegar F, Kim SH, Jiang W, Shen J, Huang E, Gao Y, Gao JL, Zhou JZ, Luo J, Huang J, Luo X, Bi Y, Su Y, Yang K, Liu H, Luu HH, Haydon RC, He TC, He BC: Retinoic acids potentiate bmp9-induced osteogenic differentiation of mesenchymal progenitor cells. *PLoS One* 2010;5:e11917.
- 82 Peng Y, Kang Q, Luo Q, Jiang W, Si W, Liu BA, Luu HH, Park JK, Li X, Luo J, Montag AG, Haydon RC, He TC: Inhibitor of DNA binding/differentiation helix-loop-helix proteins mediate bone morphogenetic protein-induced osteoblast differentiation of mesenchymal stem cells. *J Biol Chem* 2004;279:32941-32949.
- 83 Tang N, Song WX, Luo J, Luo X, Chen J, Sharff KA, Bi Y, He BC, Huang JY, Zhu GH, Su YX, Jiang W, Tang M, He Y, Wang Y, Chen L, Zuo GW, Shen J, Pan X, Reid RR, Luu HH, Haydon RC, He TC: Bmp9-induced osteogenic differentiation of mesenchymal progenitors requires functional canonical wnt/beta-catenin signaling. *J Cell Mol Med* 2009;13:2448-2464.
- 84 Huang E, Zhu G, Jiang W, Yang K, Gao Y, Luo Q, Gao JL, Kim SH, Liu X, Li M, Shi Q, Hu N, Wang L, Liu H, Cui J, Zhang W, Li R, Chen X, Kong YH, Zhang J, Wang J, Shen J, Bi Y, Statz J, He BC, Luo J, Wang H, Xiong F, Luu HH, Haydon RC, Yang L, He TC: Growth hormone synergizes with bmp9 in osteogenic differentiation by activating the jak/stat/igf1 pathway in murine multilineage cells. *J Bone Miner Res* 2012;27:1566-1575.
- 85 Luo J, Tang M, Huang J, He BC, Gao JL, Chen L, Zuo GW, Zhang W, Luo Q, Shi Q, Zhang BQ, Bi Y, Luo X, Jiang W, Su Y, Shen J, Kim SH, Huang E, Gao Y, Zhou JZ, Yang K, Luu HH, Pan X, Haydon RC, Deng ZL, He TC: Tgfbeta/bmp type i receptors alk1 and alk2 are essential for bmp9-induced osteogenic signaling in mesenchymal stem cells. *J Biol Chem* 2010;285:29588-29598.
- 86 Hu N, Jiang D, Huang E, Liu X, Li R, Liang X, Kim SH, Chen X, Gao JL, Zhang H, Zhang W, Kong YH, Zhang J, Wang J, Shui W, Luo X, Liu B, Cui J, Rogers MR, Shen J, Zhao C, Wang N, Wu N, Luu HH, Haydon RC, He TC, Huang W: Bmp9-regulated angiogenic signaling plays an important role in the osteogenic differentiation of mesenchymal progenitor cells. *J Cell Sci* 2013;126:532-541.
- 87 He BC, Gao JL, Luo X, Luo J, Shen J, Wang L, Zhou Q, Wang YT, Luu HH, Haydon RC, Wang CZ, Du W, Yuan CS, He TC, Zhang BQ: Ginsenoside rg3 inhibits colorectal tumor growth through the down-regulation of wnt/ss-catenin signaling. *Int J Oncol* 2011;38:437-445.
- 88 Schwartz M: A biomathematical approach to clinical tumor growth. *Cancer* 1961;14:1272-1294.
- 89 Luther GA, Lamplot J, Chen X, Rames R, Wagner ER, Liu X, Parekh A, Huang E, Kim SH, Shen J, Haydon RC, He TC, Luu HH: Igfbp5 domains exert distinct inhibitory effects on the tumorigenicity and metastasis of human osteosarcoma. *Cancer Lett* 2013;336:222-230.

- 90 Zhang Y, Chen X, Qiao M, Zhang BQ, Wang N, Zhang Z, Liao Z, Zeng L, Deng Y, Deng F, Zhang J, Yin L, Liu W, Zhang Q, Ya Z, Ye J, Wang Z, Zhou L, Luu HH, Haydon RC, He TC, Zhang H: Bone morphogenetic protein 2 inhibits the proliferation and growth of human colorectal cancer cells. *Oncol Rep* 2014;32:1013-1020.
- 91 Chen L, Jiang W, Huang J, He BC, Zuo GW, Zhang W, Luo Q, Shi Q, Zhang BQ, Wagner ER, Luo J, Tang M, Wietholt C, Luo X, Bi Y, Su Y, Liu B, Kim SH, He CJ, Hu Y, Shen J, Rastegar F, Huang E, Gao Y, Gao JL, Zhou JZ, Reid RR, Luu HH, Haydon RC, He TC, Deng ZL: Insulin-like growth factor 2 (igf-2) potentiates bmp-9-induced osteogenic differentiation and bone formation. *J Bone Miner Res* 2010;25:2447-2459.
- 92 Haydon RC, Luu HH, He TC: Osteosarcoma and osteoblastic differentiation: A new perspective on oncogenesis. *Clin Orthop Relat Res* 2007;454:237-246.
- 93 Luu HH, Song WX, Luo X, Manning D, Luo J, Deng ZL, Sharff KA, Montag AG, Haydon RC, He TC: Distinct roles of bone morphogenetic proteins in osteogenic differentiation of mesenchymal stem cells. *J Orthop Res* 2007;25:665-677.
- 94 Muramatsu Y, Kamegai A, Shiba T, Shrestha P, Takai Y, Mori M, Ilg E, B. S, Heizmann C: Histochemical characteristics of calcium binding s100 proteins and bone morphogenetic proteins in chondro-osseous tumors. *Oncol Rep* 1997;49-53.
- 95 Weterman MA, Stoop GM, van Muijen GN, Kuznicki J, Ruiter DJ, Bloemers HP: Expression of calcyclin in human melanoma cell lines correlates with metastatic behavior in nude mice. *Cancer Res* 1992;52:1291-1296.
- 96 Yang YQ, Zhang LJ, Dong H, Jiang CL, Zhu ZG, Wu JX, Wu YL, Han JS, Xiao HS, Gao HJ, Zhang QH: Upregulated expression of s100a6 in human gastric cancer. *J Dig Dis* 2007;8:186-193.
- 97 Hwang R, Lee EJ, Kim MH, Li SZ, Jin YJ, Rhee Y, Kim YM, Lim SK: Calcyclin, a ca2+ ion-binding protein, contributes to the anabolic effects of simvastatin on bone. *J Biol Chem* 2004;279:21239-21247.
- 98 Tsoporis JN, Izhar S, Parker TG: Expression of s100a6 in cardiac myocytes limits apoptosis induced by tumor necrosis factor-alpha. *J Biol Chem* 2008;283:30174-30183.
- 99 He TC: Distinct osteogenic activity of bmps and their orthopaedic applications. *J Musculoskelet Neuronal Interact* 2005;5:363-366.
- 100 Deng ZL, Sharff KA, Tang N, Song WX, Luo J, Luo X, Chen J, Bennett E, Reid R, Manning D, Xue A, Montag AG, Luu HH, Haydon RC, He TC: Regulation of osteogenic differentiation during skeletal development. *Front Biosci* 2008;13:2001-2021.
- 101 Wang RN, Green J, Wang Z, Deng Y, Qiao M, Peabody M, Zhang Q, Ye J, Yan Z, Denduluri S, Idowu O, Li M, Shen C, Hu A, Haydon RC, Kang R, Mok J, Lee MJ, Luu HL, Shi LL: Bone morphogenetic protein (bmp) signaling in development and human diseases. *Genes Dis* 2014;1:87-105.