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Sine oculis homeobox homolog 1 promotes α5β1-mediated invasive migration and metastasis of cervical cancer cells

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ABSTRACT

Sine oculis homeobox homolog 1 (SIX1) has been supposed to be correlated with the metastasis and poor prognosis of several malignancies. However, the effect of SIX1 on the metastatic phenotype of tumor cells and the underlying mechanisms were still unclear to date. Here we report that SIX1 can promote α5β1-mediated metastatic capability of cervical cancer cells. SIX1 promoted the expression of α5β1 integrin to enhance the adhesion capacity of tumor cells *in vitro* and tumor cell arrest in circulation *in vivo*. Moreover, higher expression of SIX1 in tumor cells resulted in the increased production of active MMP-2 and MMP-9, up-regulation of anti-apoptotic genes (BCL-XL and BCL2) and down-regulation of pro-apoptotic genes (BIM and BAX), thus promoting the invasive migration and anoikis-resistance of tumor cells. Importantly, blocking α5β1 abrogated the regulatory effect of SIX1 on the expression of these genes, and also abolished the promotional effect of SIX1 on invasive capability of tumor cells. Furthermore, knockdown of α5 could abolish the promoting effect of SIX1 on the development of metastatic lesions in both experimental and spontaneous metastasis model. Therefore, by up-regulating α5β1 expression, SIX1 not only promoted the adhesion capacity, but also augmented ECM-α5β1-mediated regulation of gene expression to enhance the metastatic potential of cervical cancer cells. These results suggest that SIX1/α5β1 might be considered as valuable marker for metastatic potential of cervical cancer cells, or a therapeutic target in cervical cancer treatment.

1. Introduction

Sine oculis homeobox homolog 1 (SIX1) is a development-related transcription factor that promotes proliferation and inhibits apoptosis in the embryonic development of organs [1,2]. SIX1 is rarely expressed in most adult tissues. However, the overexpression of SIX1 has been observed in various malignancies [3–7]. By transcriptionally regulating the expression of numerous genes, SIX1 could modulate cell cycle, proliferation, and stem cell-like features of tumor cells. Moreover, recent studies showed that the enforced expression of SIX1 in non-invasive breast cancer cells could promote the metastatic capability of the cells [2,7]. Nevertheless, the mechanisms underlying the effect of SIX1 on the metastatic phenotype of tumor cells have not been fully understood to date.

Integrin expression is crucial for metastatic capability of tumor cells [8]. In addition to disrupting homophilic adhesion and mediating the interaction of tumor cells with extracellular matrix (ECM) [9], integrins could promote the production and activation of matrix metalloproteinases (MMPs), which accelerate the invasive migration of tumor cells by degrading the ECM and exposing new cell-binding sites [9,10]. Moreover, the crosstalk of integrins with growth factor receptors or oncogenes could inhibit apoptosis to promote tumor cell survival [11]. Therefore, the expression levels of integrins are critical for the metastatic capability of tumor cells. Over 24 distinct integrins are formed due to different combinations of α-subunit and β-subunit. Highly invasive tumor cells frequently express high levels of one or several types of integrins. It has been found that extra-cellular factors could augment the invasive capability of tumor cells by up-regulating the expression of the integrins such as αvβ3, α3β1, α5β1, etc. [9,11–13]. However, it was unknown whether the increased expression of the transcription factor such as SIX1 could regulate the expression of integrins, and whether the promotional effect of SIX1 on tumor metastasis might be dependent on the alteration of integrin expression.

In cervical cancer, SIX1 expression was reported to be potentially induced by human papillomavirus oncoproteins [14,15]. But the effect of SIX1 on the metastatic phenotype of cervical

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cancer cells and the underlying mechanisms were still unknown. In this study we investigated the effect of SIX1 on the metastatic phenotype of cervical cancer cells by analyzing the regulation of integrin expression and integrin-mediated function. The resultant data showed that SIX1 overexpression could promote invasive and metastatic capability of cervical cancer cells by upregulating the expression of α5β1. Through up-regulating α5β1 expression, SIX1 enhanced the adhesion of tumor cells to ECM molecules, the invasion of tumor cells into target organ, and the anoikis-resistance of tumor cells, thus promoting tumor metastasis.

2. Materials and methods

2.1. Cells and transfection

Human cervical cancer cell lines Siha and Hela were purchased from the American Type Culture Collection (Manassas, VA). The cells were transduced with CMV-Fluc-IRES-RFP lentiviral particles (GeneChem, shanghai, China). RFP/luciferase-expressing cells were isolated by FACS and used in living imaging. In flow cytometry analysis, RFP-free cells were used.

Siha cells were transfected with pcDNA3.1-SIX1 expression plasmid (a kind gift from Kong-Ming Wu, Thomas Jefferson University, Philadelphia) or pcDNA3.1 plasmid using Lipofectamine 2000 (Invitrogen, Life Technologies), and screened by G418. NshSIX1(1) and NshSIX1(2) shRNA lentiviral particles (GeneChem) were used to knock-down SIX1 expression, targeting 5′-CCAGCTCAGAAGAG GAATT-3′ and 5′-CACGCCAGGAGCTCAAACT-3′, respectively. "N" indicated neomycin. Pshα5(1) and Pshα5(2) lentiviral particles (GeneChem) were used to knock-down α5 expression, targeting 5′-CTCCCTGAAGCCTTTGCAT-3′ and 5′-GAGCAGATTGCAGAATCTT-3′, respectively. "P" indicated puromycin. Nshcon and Pshcon, not targeting any known gene, were used as controls. The cells with stable transfection of shRNA were selected with G418 and/or puromycin.

2.2. Western blot assay

Western blot assay was done as described previously [16]. Abs were purchased from Sigma–Aldrich, Millipore and Proteintech.

2.3. Assay of gene expression by real-time RT-PCR

Total RNA was extracted from cells or tumor tissues with TRIzol reagent (Invitrogen). The relative quantity of mRNA was determined by real-time RT-PCR. *Gapdh* and *EEF1A1* were chosen as reference genes, which were reported as the most reliable combination in cervical cancer [17]. The primer sequences were shown in the Supplementary Table. The expression of gene was quantified using the comparative C_T method. The expression level of each mRNA was normalized to that of *GAPDH* and *EEF1A1* mRNAs, and expressed as *n*-fold difference relative to the control.

2.4. Migration and invasion assay

Migration and invasion assay was performed using Transwell chamber (Corning, NY). In invasion assay, the transwell filters was coated with matrigel (BD Biosciences) containing 10 μg/ml fibronectin (Invitrogen). The cells (4×10^4) were seeded into the upper chambers. After 8-h (migration assay) or 24-h (invasion assay) incubation at 37 °C in a humidified incubator with 5% CO_2, the cells that passed through the membrane were fixed and stained with 0.1% crystal violet, and counted under a microscope from three randomly chosen fields in each membrane. The average number of the cells per field was calculated.

2.5. Adhesion assay

Tumor cells were added (1×10^4/well) to 96-well plates pre-coated with fibronectin. After 2-h incubation at 37 °C and the removal of non-adherent cells, the adherent cells were quantified by using Cell Counting Kit (CCK8). The wells used for counting total number of cells proceeded to the counting step without the removal of non-adherent cells. The results were expressed as the percentage of the adherent cells in total cells.

2.6. MMP assay by gelatin zymography

Tumor cells were cultured for 48 h in DMEM medium containing 1% FBS in matrigel/fibronectin-coated plates. The MMP-2 and MMP-9 in supernatants were measured by gelatin zymography as described previously [13].

2.7. Flow cytometric analysis

Tumor cells were incubated with mouse anti-human α5β1 antibody (Millipore) or isotype control at 4 °C for 0.5 h. The secondary antibody was FITC-conjugated anti-mouse IgG. Parameters were acquired on a FACS Calibur flow cytometer (BD Biosciences) and analyzed with CellQuest software. Percent staining was defined as the percentage of cells in the gate which was set to exclude ~99% of isotype control cells. The expression index was calculated by using the formula: mean fluorescence × percentage of positively stained cells [8,13].

2.8. Soft agar assay

Cells were suspended in 0.35% agar in DMEM (20% FBS) and plated (1.5×10^3 per well) on a layer of 0.5% agar in culture medium in 6-well plates. After two-week culture, colonies were photographed and counted by phase-contrast microscopy (Nikon, Japan).

2.9. Assay of anoikis

Tumor cells were cultured (1×10^6/well) for 24 h in 6-well plates pre-coated with poly-HEMA (10 mg/ml, Sigma). The cells were then stained with Annexin V-FITC/Propidium Iodide (PI) apoptosis detection kit (BD Biosciences), and analyzed by flow cytometry as described previously [18].

2.10. Experimental metastasis model

Female NOD/SCID mice (4 weeks old) were purchased from Beijing HFK Bio-Technology Co., Ltd. (Beijing, China) for studies approved by the Committee on the Ethics of Animal Experiments of Tongji Medical College. The mice were maintained in the accredited animal facility of Tongji Medical College. Tumor cells were injected into mice via tail vein (2×10^6 cells/mouse). At each time option, mice were anaesthetization with 3% pentobarbital sodium, and imaged 10 min after intraperitoneal injection of 100 mg/kg D-luciferin using the IVIS SPECTRUM system (Caliper, Xenogen USA). Total flux (photons/s) was quantified using Living Image version 4.3.1 software.

2.11. Statistical analysis

Each experiment was repeated for at least three times independently. SPSS (version 13.0) software package was used for statistical analysis. Results were expressed as mean value ± standard error

of the mean (SEM), and interpreted by one-way ANOVA. Differences were considered to be statistically significant when $P < 0.05$.

3. Results

3.1. SIX1 promotes invasive and metastatic capability of cervical cancer cells

SIX1 expression was limited in normal cervical epithelium, but the increased expression of SIX1 was observed in many cervical cancer specimens (Supplementary Fig. 1). The risk (OR) of lymph-node metastasis in the patients with strong SIX1 expression was 8.36-fold (95% CI:1.77∼39.44; $P = 0.007$) of that in other patients, suggesting that overexpression of SIX1 might correlate with lymph-node metastasis in cervical cancers. In line with this, over-expression of SIX1 in Siha cells (Fig. 1A), a cervical cancer cell line with low level of SIX1 expression, significantly promoted the migration and invasion of the cells, and increased lymph-node metastasis after implantation of tumor cells into the claw pads of mice (Supplementary Fig. 2). Correspondingly, knock-down of SIX1 in Hela cells, a cervical cancer cell line with high level of SIX1 expression, resulted in the decrease of migration and invasion of tumor cells *in vitro* as well as lymph-node metastasis *in vivo* (Supplementary Fig. 3). These results demonstrate that SIX1 could promote invasion and metastasis of cervical cancer.

3.2. SIX1 up-regulates α5β1 expression in cervical cancer cells

The integrins that are associated with poor prognosis of cervical cancer and other malignancies include αvβ3, αvβ6, α5β1, α3β1, α4β1, α6β4 and αvβ5 [11,19–21]. We therefore investigated whether SIX1 could regulate the expression of these integrins. The results showed that SIX1 could up-regulate the expression of α5 and β1, but not other integrin subunits (Fig. 1B). The increased protein levels of α5 and β1 (Fig. 1C) and cell surface α5β1 (Fig. 1D) were confirmed by Western bolt and flow cytometry respectively. Correspondingly, knock-down of SIX1 in Hela cells decreased the protein levels of α5 and β1 (Fig. 1E) and cell surface α5β1 (Fig. 1F). These results demonstrate that SIX1 could promote the expression of α5β1 integrin in cervical cancer cells.

3.3. SIX1 augments α5β1-mediated adhesion and invasion of cervical cancer cells

Based on the above results, we next investigated whether the promotional effect of SIX1 on the metastatic capability was dependent on α5β1 integrin. Compared with control cells, Siha-SIX1 cells displayed the increased attachment to fibronectin (Fig. 2A), the ligand of α5β1. Besides α5β1, a variety of integrins can bind to fibronectin. However, when function-blocking antibody was used to block α5β1, the ability of Siha-SIX1 cells to bind fibronectin was reduced to the levels of that in Siha-3.1 group (Fig. 2A). Similarly, blocking α5β1 resulted in the decrease of the adhesion of Hela cells to fibronectin, similar to knocking down the expression of SIX1 (Fig. 2A). Moreover, knock-down of α5 in Siha-SIX1 cells (Supplementary Fig. 4A) resulted in the same effect (Supplementary Fig. 4B). These results indicated that SIX1 promoted the adhesion of cervical cancer cells to fibronectin by increasing the expression of α5β1.

α5β1 not only mediates the binding of tumor cells to ECM molecules, but also mediates signal transduction to promote the production and activation of MMP-2 and MMP-9 to accelerate the degradation of extra-cellular matrix, thus accelerating the invasive migration of tumor cells [9,22,23]. Consistently, the up-regulation of α5β1 by SIX1 could promote the production of active MMP-2 (Fig. 2B) and MMP-9 (Fig. 2C), which was abolished by blocking α5β1. The same effect was also observed in Siha-SIX1 cells if α5 expression was knocked down (Supplementary Fig. 4C). In line with this, the invasion of SIX1-expressing tumor cells could be suppressed by either blocking α5β1 (Fig. 2D) or knocking down the expression of α5 (Supplementary Fig. 4D). Taken together, these results suggested that the up-regulated α5β1 expression was responsible for the promoting effect of SIX1 on the adhesive ability and invasive capacity of cervical cancer cells.

3.4. α5β1 is required for SIX1 to enhance anoikis-resistance of cervical cancer cells

Enhancing anoikis-resistance is another key mechanism underlying integrin-mediated metastatic dissemination, which is necessary for the survival and colonization of tumor cells in distant

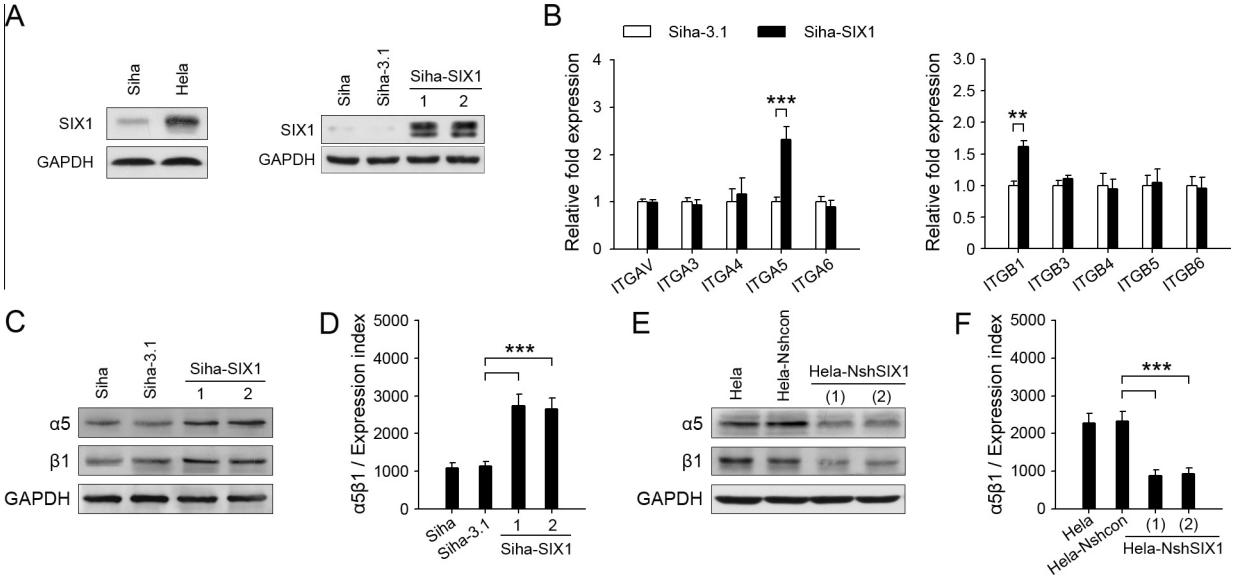

Fig. 1. SIX1 promotes the expression of α5β1 integrin in cervical cancer cells. (A) The expression of SIX1 was detected by Western blot. (B) The expression levels of indicated genes coding for integrin subunits were detected by real-time RT-PCR. (C, D) The expression of α5β1 in Siha control cells and the Siha cells stably transfected with the indicated vector. The protein levels of α5 and β1 were detected by Western Blot (C). Cell surface α5β1 was measured by flow cytometric analysis (D). (E, F) The protein levels of α5 and β1 (E) and cell surface α5β1 (F) in the indicated Hela cells were detected by Western blot and flow cytometric analysis, respectively.

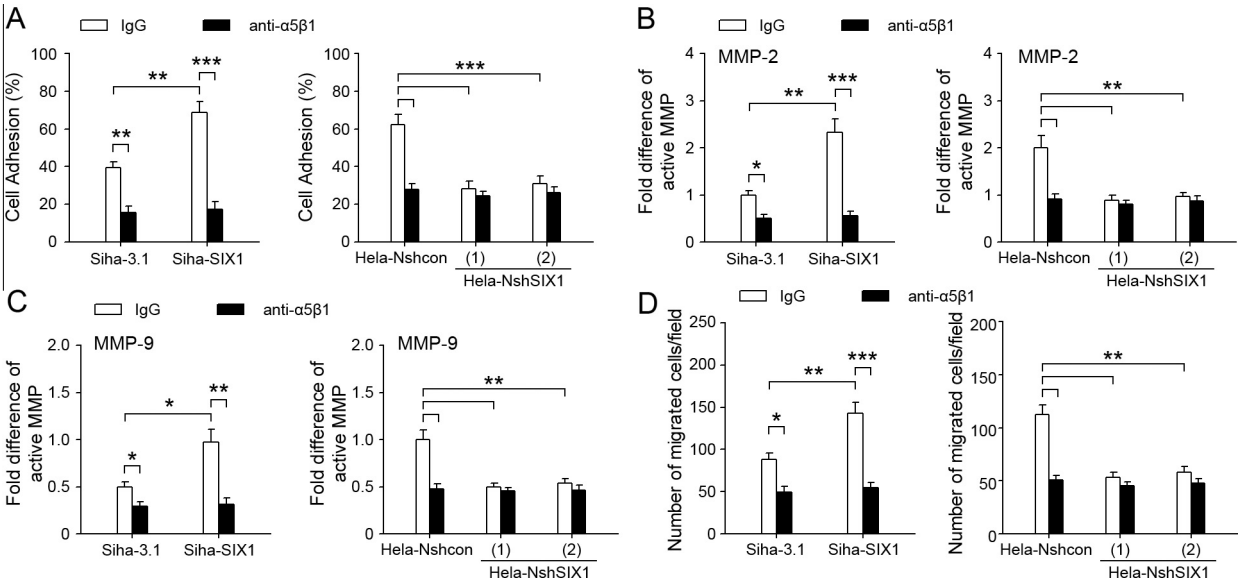

Fig. 2. α5β1 mediates the effect of SIX1 on adhesion and invasion of cervical cancer cells. Corresponding Siha and Hela cells were pre-incubated with IgG-control or function-blocking antibody of α5β1 (anti-α5β1, Millipore) for 1 h, and then used in following experiments. (A) The adhesion of the cells to fibronectin-coated plates was measured as described in Section 2. (B, C) The cells were then cultured in matrigel/fibronectin-coated plates for 48 h in presence of the antibody (20 μg/ml). The MMP-2 (B) and MMP-9 (C) in supernatants were detected by zymography assay. The fold differences of active MMPs were calculated after densitometric analysis of the gel. (D) Invasion assay of tumor cells was performed as described in Section 2 in presence of the antibody.

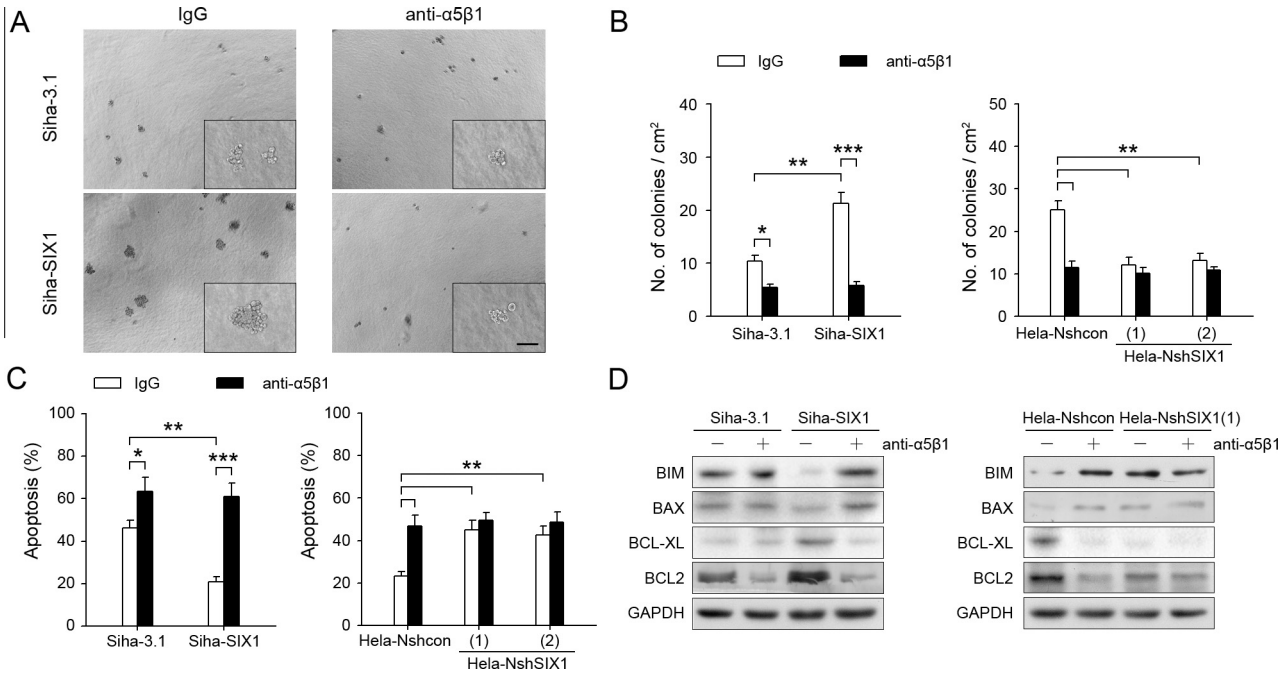

Fig. 3. α5β1 is required for SIX1 to enhance anoikis-resistance of cervical cancer cells. Corresponding Siha and Hela cells were pre-incubated with IgG-control or anti-α5β1 antibody for 1 h, and then used in following experiments. (A, B) The cells were allowed to grow in soft agar for 2 weeks. The representative photographs of colony formation were shown at ×40 and ×200 (inserts) magnification. Bar, 100 μm (A). The average number of colonies per cm^2 was calculated (B). (C, D) The cells were cultured in presence of antibody (20 μg/ml) for 48 h. Then, the cells were incubated in polyHEMA-coated plates for 24 h. Apoptosis was measured by flow cytometric analysis (C). Or the expression of apoptosis-related proteins was detected by Western blot (D).

organs [24,25]. We therefore further investigated the effect of SIX1 on the anoikis-resistance of cervical cancer cells. SIX1 significantly promoted the colony formation (Fig. 3A and B) and reduced the apoptosis (Fig. 3C) of cervical cancer cells under anchorage-independent condition, indicating that SIX1 could enhance the anoikis-resistance of tumor cells. The promotional effect of SIX1 on anoikis-resistance of tumor cells was abolished by blocking α5β1. Moreover, higher expression of SIX1 resulted in the decreased expression of BIM and BAX, the main pro-apoptotic proteins in anoikis, and the increased expression of BCL-XL and BCL-2, the main anti-apoptotic proteins in anoikis (Fig. 3D and Supplementary Fig. 5). The effect of SIX1 on the expression of these

genes was abrogated by blocking α5β1. These results demonstrate that SIX1 promoted the expression of α5β1 to augment anoikis-resistance of cervical cancer cells.

3.5. SIX1 promotes the metastatic potential of cervical cancer cells by up-regulating α5β1 expression

We next investigated the effect of SIX1 on the invasive capacity of cervical cancer cells in target organ. Tumor cell arrest and extravasation in the lung of mice were assessed 5 h and 24 h, respectively, after i.v. injection of tumor cells. Overexpression of SIX1 increased the arrest and extravasation of Siha cells in the lung of mice (Fig. 4A and B). Consistently, knock-down of SIX1 decreased the arrest and extravasation of Hela cells in the lung of mice (Fig. 4B). When α5β1 was blocked, the arrest and extravasation of Siha-SIX1 cells and Hela cells was reduced to the levels of those in corresponding control group (Fig. 4A and B).

The augmented anoikis-resistance of tumor cells is in favor of not only the invasion of tumor cells into the target organ but also the development of metastatic lesions. Compared with Siha-3.1 cells, Siha-SIX1 cells developed faster-growing metastatic lesions in the lung of mice (Fig. 4C and Supplementary Fig. 6). The effect of SIX1 was abolished by knocking down the expression of α5 integrin. Moreover, knock-down of α5 in Siha-SIX1 cells also abolished the promotional effect of SIX1 on spontaneous lymph-node metastasis (Supplementary Fig. 7). Consistently, similar effects were observed when either SIX1 or α5 integrin was knocked down in Hela cells (Fig. 4D and Supplementary Fig. 8). Taken together, these results demonstrate that the up-regulation of α5β1 expression is necessary for SIX1 to promote the metastatic capability of cervical cancer cells.

4. Discussion

Although SIX1 has been supposed to be associated with the metastatic potential of several malignancies [2–4], the mechanisms underlying the effect of SIX1 on the metastatic phenotype of tumor cells were still unclear to date. The data in this study show that SIX1 overexpression can promote α5β1-mediated metastatic capability of cervical cancer cells. In cervical cancer, αvβ3, αvβ6 and α5β1 are main integrins that contribute to poor prognosis. Intriguingly, SIX1 could up-regulate the expression of α5 and β1, but not other integrin subunits that were identified to affect prognosis of various cancers. The effect of SIX1 on the metastatic potential of cervical cancer cells was mainly mediated by α5β1. By up-regulating α5β1 expression, SIX1 enhanced the adhesion, invasion and anoikis-resistance of tumor cells to facilitate metastasis of cervical cancer.

After reaching the bloodstream, either directly or through the lymphatic system, the interaction of tumor cells with vascular endothelium and subendothelial ECM is essential for tumor cell arrest and extravasation into distant organs [9]. The binding of integrins to ECM molecules such as fibronectin plays an important role in cell adhesion, migration and invasion of tumor cells. Fibronectin is the ligand for α5β1. Although several other integrins also mediate the binding of tumor cells to fibronectin, blocking α5β1 could completely abrogate the promoting effect of SIX1 on the cell attachment to fibronectin *in vitro* and tumor cell arrest in circulation *in vivo*. Therefore, SIX1 promoted the interaction of cervical cancer cells with fibronectin by up-regulating the expression of α5β1.

The interaction of integrins with ECM molecules not only mediates the adhesion and migration of tumor cells, but also modulates the metastatic capability of tumor cells through integrin-mediated signaling [9,11]. Our data showed that SIX1 overexpression could promote the production of active MMP-2 and MMP-9, up-regulate the expression of anti-apoptotic genes, and down-regulate the expression of pro-apoptotic genes. However, SIX1 could not directly modulate the expression of the above-mentioned genes, although it is a transcription factor that modulates the expression of numerous genes. Blocking α5β1 could abrogate the regulatory effect of SIX1 on the expression of these genes, suggesting that

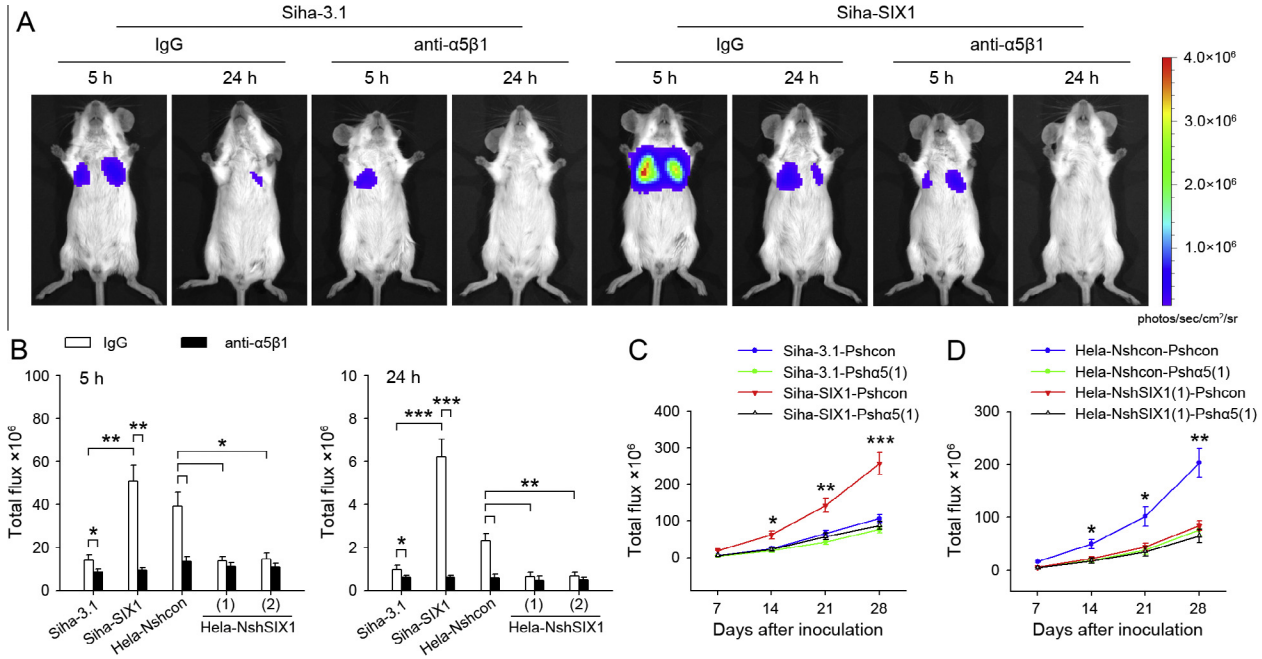

Fig. 4. SIX1 promotes the metastatic potential of cervical cancer cells by up-regulating α5β1 expression. (A, B) Corresponding Siha and Hela cells were pre-incubated with IgG-control or function-blocking antibody of α5β1 for 1 h, and were subsequently injected into NOD/SCID mice via tail vein. The cell arrest and extravasation in the lung of mice were assayed by *in vivo* bioluminescence images. Representative images of mice were shown (A). Signal intensity was measured for regions of interest and normalized as total flux (B, $n = 10$ per group). (C, D) After i.v. inoculation of indicated Siha (C) and Hela (D) cells, the development of metastatic lesions in the lung of mice was dynamically monitored by *in vivo* bioluminescence images ($n = 10$ per group).

SIX1 enhanced α5β1-mediated signaling to modulate the expression of these genes. Therefore, the up-regulation of α5β1 expression not only promoted the adhesion, arrest and migration of tumor cells, but also enhanced ECM-α5β1-mediated signal transduction to regulate gene expression in tumor cells. The increased production and activation of MMPs play the leading role in integrin-induced degradation of ECM, which is necessary for both intravasation and extravasation of tumor cells [26]. Increasing the production of active MMPs might be one of the mechanisms through which SIX1 promoted α5β1-mediated invasion and extravasation of tumor cells. On the other hand, anoikis-resistance is also required for tumor cells to intravasate into circulation and extravasate into distant organs [27]. Anoikis-resistance of tumor cells is crucial for metastasis to occur, since many disseminated tumor cells fail to develop metastasis due to anoikis [27,28]. The up-regulation of anti-apoptotic genes and the down-regulation of pro-apoptotic genes might be the important mechanisms by which SIX1 facilitated tumor cell dissemination, promoted the survival and growth of disseminated tumor cells in new microenvironments, and accelerated the development of metastatic lesions in distant organs.

Blocking α5β1 could completely abolish the promotional effect of SIX1 on the adhesion, invasion and metastatic capability of tumor cells, which was further confirmed by knocking down the expression of α5 expression. β1 could combine with various α-subunits to form different integrins, but α5 could only combine with β1 to form heterodimer integrin. Thus, knock-down of α5 could reduce the level of α5β1 without affecting the expression of other integrins containing β1 subunit. When α5 was knocked down in Siha-SIX1 cells, the cell adhesion, production of active MMPs, invasive capability, as well as the ability to form spontaneous and experimental metastasis were reduced to the levels of those in Siha cells. These results further demonstrated that the promotional effect of SIX1 on metastatic capability of tumor cells was dependent on α5β1 integrin.

In summary, in this study we demonstrated that SIX1 promoted metastatic capability of cervical cancer cells. SIX1 promoted the expression of α5β1 in tumor cells to augment multiple functions that are necessary for metastatic phenotype. The up-regulation of α5β1 is critical for SIX1 to enhance the adhesion of tumor cells to ECM, promote the invasion of tumor cells into target organs, and accelerate the development of metastatic lesions. These results suggest that SIX1/α5β1 might be considered as valuable marker for metastatic potential of cervical cancer cells, or a therapeutic target in cervical cancer treatment.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.03.002.

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