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Leptin promotes proliferation and invasion of osteosarcoma cells by upregulating the expression of SIRT1

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Abstract: Osteosarcoma (OS) is a primary high-grade malignant bone neoplasm, and the prognosis of OS remains poor due to early metastasis. Leptin plays an essential role in tumorigenesis, but the role of leptin in the development of OS is still not fully understood. In this study, we used a human osteosarcoma MG-63 cell line as an experimental model. MG-63 cells were treated with leptin, and cell proliferation, apoptosis, adhesion, invasion, and gene expression, were evaluated. The results showed that leptin promoted proliferation, decreased adhesion, suppressed apoptosis, and promoted invasion, of MG-63 cells. Moreover, the expression of SIRT1 was upregulated in MG-63 cells exposed to leptin. Furthermore, MMP-2, 8, and 9 were significantly upregulated by SIRT1, while SIRT1 knockdown inhibited the proliferation and invasion of MG-63 cells. In conclusion, our results suggest that leptin promotes OS cell proliferation and invasion by inducing the expression of SIRT1.

Introduction

Osteosarcoma (OS) is a malignant tumor that is highly prevalent in children and adolescents, and the prognosis of OS is very poor (Durfee *et al.*, 2016). The metastasis tendency of OS is the main cause of poor prognosis, despite the development in therapeutic strategies combining surgery, irradiation, and chemotherapy (Carrle and Bielack, 2006; Ottaviani and Jaffe, 2009). The underlying mechanism for OS development is not fully clarified.

Obesity has become a public health problem, which is related to many chronic diseases such as atherosclerosis, diabetes, and tumorigenesis (Arnold *et al.*, 2016). Leptin is a hormone secreted by fat cells and a biomarker of obesity (de Luis *et al.*, 2009). Leptin helps to regulate energy balance by inhibiting hunger, reducing weight, increasing calories, and storing glucose to regulate energy balance. The physiological expression of leptin is limited in several kinds of tissues such as bone marrow, ovaries, lymphoid tissues and epithelium (Friedman and Halaas, 1998; Nalabolu *et al.*, 2014; Seoung *et al.*, 2018). It has also been reported that leptin contributes to the occurrence and development of tumors. In a previous study, we found that a high level of leptin indicated higher metastatic potential and poor prognosis in OS patients (Feng *et al.*, 2016).

Sirtuin1 (SIRT1) is the NAD^+-dependent deacetylase that plays an important role in cell differentiation and senescence, metabolism, and carcinogenesis (Knight and Milner, 2012). Changes in SIRT1 expression could affect multiple cellular functions, including proliferation, apoptosis, and metastasis (Cheng *et al.*, 2016; Ford *et al.*, 2005; Lee *et al.*, 2013). It is indicated that the expression of SIRT1 is sensitive to leptin (Sasaki, 2015). Our previous study also demonstrated the correlation between the expression of leptin and SIRT1, both of which were significantly associated with shorter overall survival in OS (Feng *et al.*, 2016). In this study, we aimed to investigate the mechanism by which leptin regulates the proliferation and invasion of OS cells.

Materials and Methods

Cell culture

The human osteosarcoma MG-63 cell line was purchased from the Chinese Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured with Dulbecco's modified Eagle's

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medium (DMEM; Invitrogen, Carlsbad, CA, USA) with a supplement of 10% fetal bovine serum (FBS, Life Technologies, Beijing, China), streptomycin (100 mg/mL) and penicillin (100 mg/mL), in a humid incubator with 5% CO_2 at 37°C. Cells at the exponential growth phase were treated with 100 ng/mL leptin (Sigma, St. Louis, MO, USA) or transfected with SIRT1 shRNA or control shRNA (provided by Sangon Biotech, Shanghai, China) following the manufacturer's protocols.

MTT assay

MG-63 cells (5×10^3/well) were seeded in a 96-well plate and cultured for 24 h. The cells were washed phosphate-buffered saline (PBS) three times, and then 100 ng/mL leptin was added. After culture for a defined time, the medium in each well was removed, PBS was used to wash the cells, and then 5 mg/mL MTT solution was added. After incubation for 2 h, the MTT solution was discarded, and dimethyl sulfoxide (DMSO, 150 μL) was added in each well. Then the absorbance of each well was measured by spectrophotometer at 490 nm.

Analysis of apoptosis by flow cytometry

Cells were harvested by digestion with trypsin (Invitrogen, Carlsbad, CA, USA), washed with PBS, and resuspended to be stained in the dark using Annexin-FITC-PI apoptosis detection kit (KeyGen, Nanjing, China) according to the manufacturer's protocol. The stained cells were immediately analyzed on a flow cytometer.

Cell adhesion

MG-63 cells (1×10^5/well) were seeded in a 6-well plate and then cultured at 37°C for 30 min. After incubation, the cells were washed by PBS three times, and the number of attached cells was counted by hemocytometer under a microscope. The test was conducted in triplicate wells and repeated three times.

Transwell assay

Matrigel was added to the upper chamber and incubated at 37°C for 1 h. Next, cells were seeded in the upper chamber. The medium with serum was added to the lower chamber. Then the plate was placed in a cell culture incubator for 24 h at 37°C with 5% CO_2. After exposure, the cells on the bottom side of the membrane were fixed with methanol and stained with gentian violet (0.1%). The cells were counted under a microscope.

Real time PCR

Total mRNA was extracted by Trizol (Invitrogen, CA, USA). Then, real-time PCR was performed to examine gene expression by using SYBR Green PCR Kit (Applied BI). The primers were as follows: VEGF 5'-TAGGCGCTTGATGGTAAT-3' and 5'-ATGGGTTCTTCTAAACTTGGACT-3'; SIRT1 5'-TAGGCGCTTGATGGTAAT-3' and 5'-ATGGGTTCTTCTAAACTTGGACT-3'; GAPDH 5'-CCGGCAAATTACCCATCC-3' and 5'-TGGGATTTCCATTGATGACAAG-3'. GAPDH was used as an internal control.

Western blot analysis

The proteins in each group were separated by SDS-PAGE (10%) gel and transferred to nitrocellulose membranes (Millipore). The membranes were blocked by 5% non-fat-milk and then incubated with primary antibodies against SIRT1 and GAPDH (Boshide Biotech, Wuhan, China) at

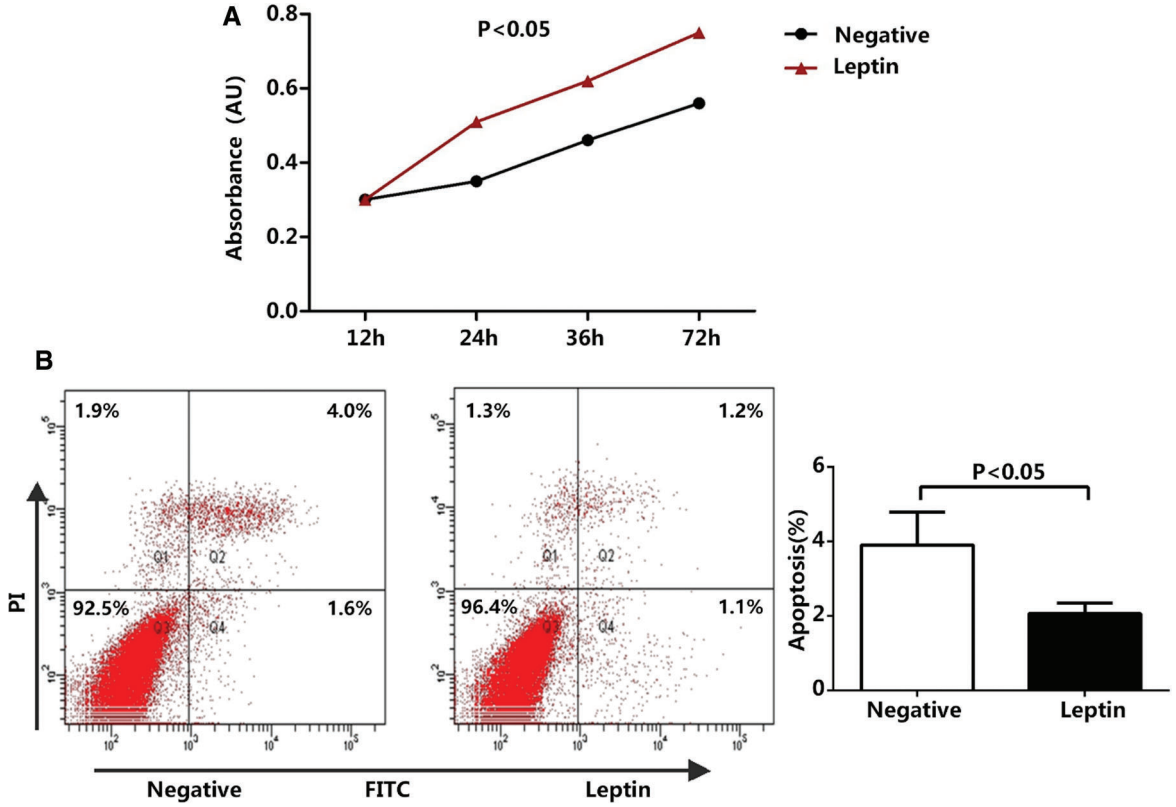

FIGURE 1. Leptin promoted the proliferation and suppressed apoptosis of MG-63 cells.
(A) MTT assay of MG-63 cell proliferation. (B) Flow cytometry analysis of the apoptosis of MG-63 cells. The data are presented as mean ± SD (n = 3). *$P < 0.05$.

4°C overnight. Then the membranes were washed in TBS-Tween 20 for 3 times and incubated with secondary antibodies labeled with horseradish peroxidase (Boshide Biotech, Wuhan, China) at room temperature. The membranes were developed by using an enhanced chemiluminescence detection system. GAPDH was used as a loading control.

Enzyme-linked immunosorbent assay (ELISA)

The conditioned media (CM) was collected from MG-63 cells. The levels of matrix metalloproteinase-2, 8, and 9 (MMP-2, 8, and 9) in CM were determined by using ELISA kit (R & D SYSTEMS, USA).

Statistical analysis

All data were presented as the mean ± standard deviation (SD) and analyzed by one-way ANOVA or Student's *t*-test using the SPSS 19.0 statistical software. $P < 0.05$ was considered statistically significant.

Results

Leptin promoted the proliferation and suppressed apoptosis of MG-63 cells

First, we examined the effect of leptin on the proliferation of MG-63 cells by MTT assay. As shown in Fig. 1(A), leptin (100 ng/mL) could significantly enhance the proliferation of MG-63 cells in a time-dependent manner. Next, we performed flow cytometric to examine the apoptotic status of MG-63 cells exposed to leptin. We found that the apoptotic rate at 24 h was 3.91 ± 0.88 % in the control group and 2.10 ± 0.23 % in the leptin-treated group (Fig. 1(B)). These results indicated that leptin promoted the proliferation and suppressed apoptosis of MG-63 cells.

Leptin decreased the adhesion and promoted the invasion of MG-63 cells

The effect of leptin on the adhesion of MG063 cells was also investigated. The result showed that leptin could effectively suppress the adhesion of MG-63 cells (Fig. 2(A)). We employed transwell assays to evaluate the invasion of MG-63 cells exposed to leptin. To prevent cell proliferation from influencing the invasion capacity of MG-63 cells, the medium was FBS free. As shown in Fig. 2(B), MG-63 cells in leptin treated group demonstrated a higher potential in cell invasion. These results demonstrated that leptin significantly reduced cell adhesion and induced cell invasion of MG-63 cells *in vitro*.

Leptin upregulated SIRT1 expression and SIRT1 overexpression promoted the proliferation and invasion of MG-63 cells

SIRT1 has been reported to play a key role in carcinogenesis. As shown in Figs. 3(A) and 3(B), leptin effectively upregulated the

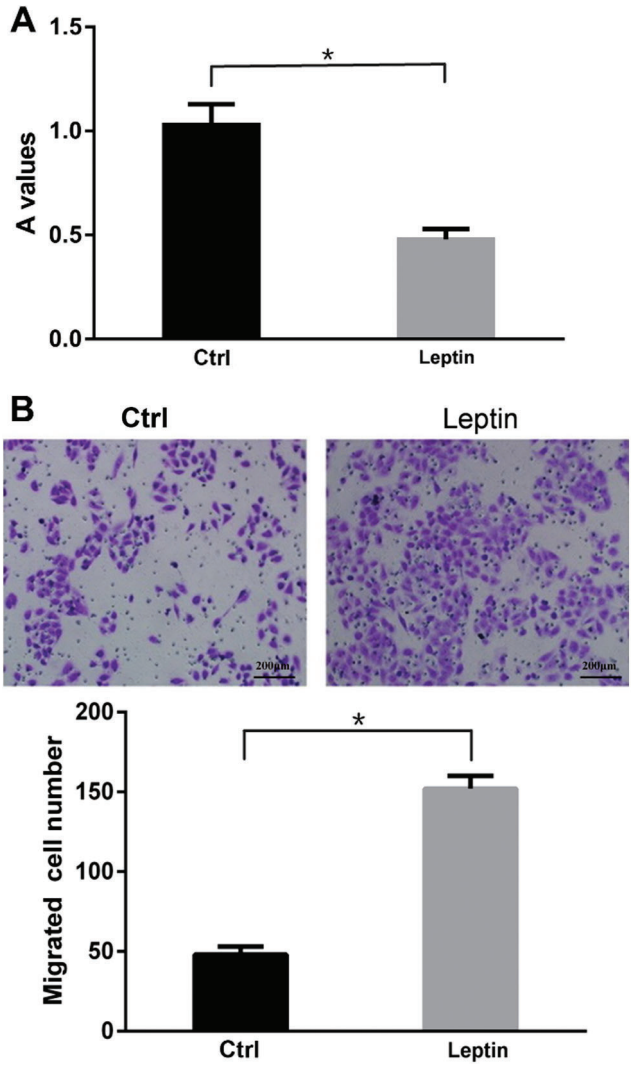

FIGURE 2. Leptin decreased the adhesion and promoted the invasion of MG-63 cells.
(A) Cell adhesion assay. (B) Transwell assay. The number of cells that had invaded into matrigel was counted in 10 random fields (200×). The data are presented as mean ± SD (n = 3). *$P < 0.05$.

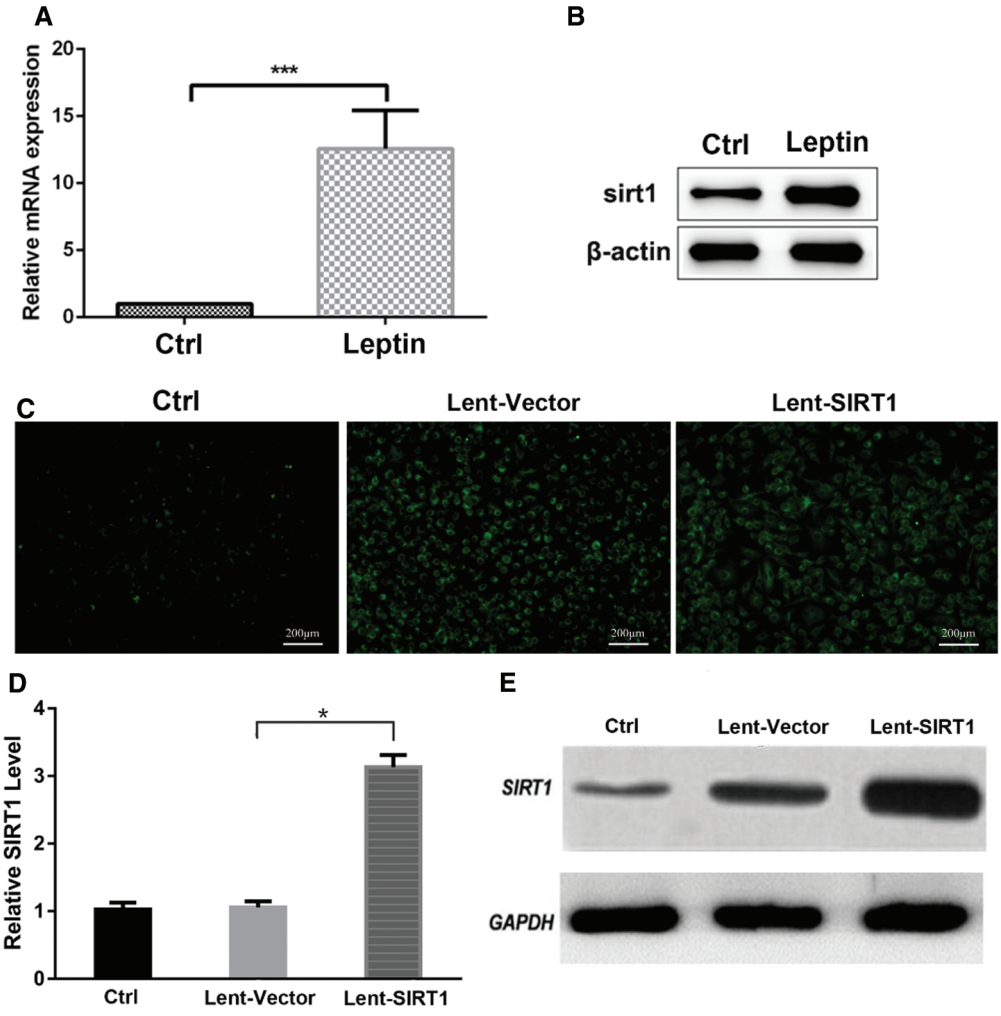

FIGURE 3. Leptin upregulated SIRT1 expression in MG-63 cells. (A) Real-time PCR analysis of SIRT1 expression. (B) Western blot analysis of SIRT1 protein levels in MG-63 cells. (C) MG-63 cells were transfected by lentivirus vector, and transfection efficiency was determined by observing GFP in a fluorescence microscope. (D) Real-time PCR and (E) Western blot analysis of SIRT1 expression in MG-63 cells infected by lentivirus. The data are presented as mean ± SD (n = 3). ***P < 0.001, *P < 0.05.

expression of SIRT1. To investigate the role of SIRT1 in OS, we overexpressed SIRT1 in MG-63 cells (Figs. 3(C)–3(E)).

We found that upregulation of SIRT1 significantly promoted the proliferation and suppressed the apoptosis of MG-63 cells (Figs. 4(A)–4(B)). In addition, cell adhesion was inhibited, and cell invasion was enhanced by SIRT1 overexpression (Figs. 4(C)–4(D)). These data suggested that leptin upregulated SIRT1 and high expression of SIRT1 significantly changed the proliferation, apoptosis, adhesion, and invasion of MG-63 cells.

SIRT1 knockdown inhibited the proliferation and invasion of MG-63 cells

In order to confirm the role of SIRT1 in the enhancement of proliferation and invasion of MG-63 cells induced by leptin, shRNA was designed to silence SIRT1 expression. The results demonstrated that SIRT1 shRNA could effectively knock down SIRT1 expression in MG-63 cells (Fig. 5(A)). Consequently, leptin-induced cell proliferation and invasion were attenuated by SIRT1 knockdown in MG-63 cells (Figs. 5(B)–5(C)). These results indicated that the role of leptin in promoting the proliferation and invasion of MG-63 cells would be mediated by SIRT1.

SIRT1 upregulated metastatic-associated gene expression

It is well-known that MMP-2 and 9 play a key role in cell invasion (Yang and Wu, 2018). Therefore, we examined MMP-2, 8, and 9 expressions by real-time PCR and ELISA in MG-63 cells. The results showed that SIRT1 could increase the expression of MMP-2, 8, and 9 (Fig. 6). Therefore, the upregulation of MMP-2, 8, and 9 by SIRT1 contributed to enhanced cell invasion induced by leptin treatment.

Discussion

It has been demonstrated that leptin promoted the invasion and progression of several cancer types such as breast cancer, pancreatic cancer and lung cancer (Fan *et al.*, 2015; Feng *et al.*, 2013; Strong *et al.*, 2015). This study aimed to investigate the effects of leptin on the proliferation and invasion of OS and the underlying mechanisms. Our data indicate that leptin could enhance OS cell proliferation and invasion by upregulating SIRT1.

Several reports have indicated that leptin contributes to aggressive tumor phenotypes by inducing cell growth, migration, and invasion (Schmidt *et al.*, 2015; Somasundar *et al.*, 2004). Leptin is produced by osteoblasts, and the level of leptin is associated with metastasis and poor prognosis in OS (Kushlinskii *et al.*, 2000). Our previous study indicated that patients with a high level of leptin had poor prognosis (Feng *et al.*, 2016; Guo *et al.*, 2016). Consistent with previous studies, we found that leptin could effectively enhance the proliferation and invasion of OS cells while inhibiting the apoptosis and adhesion of OS cells.

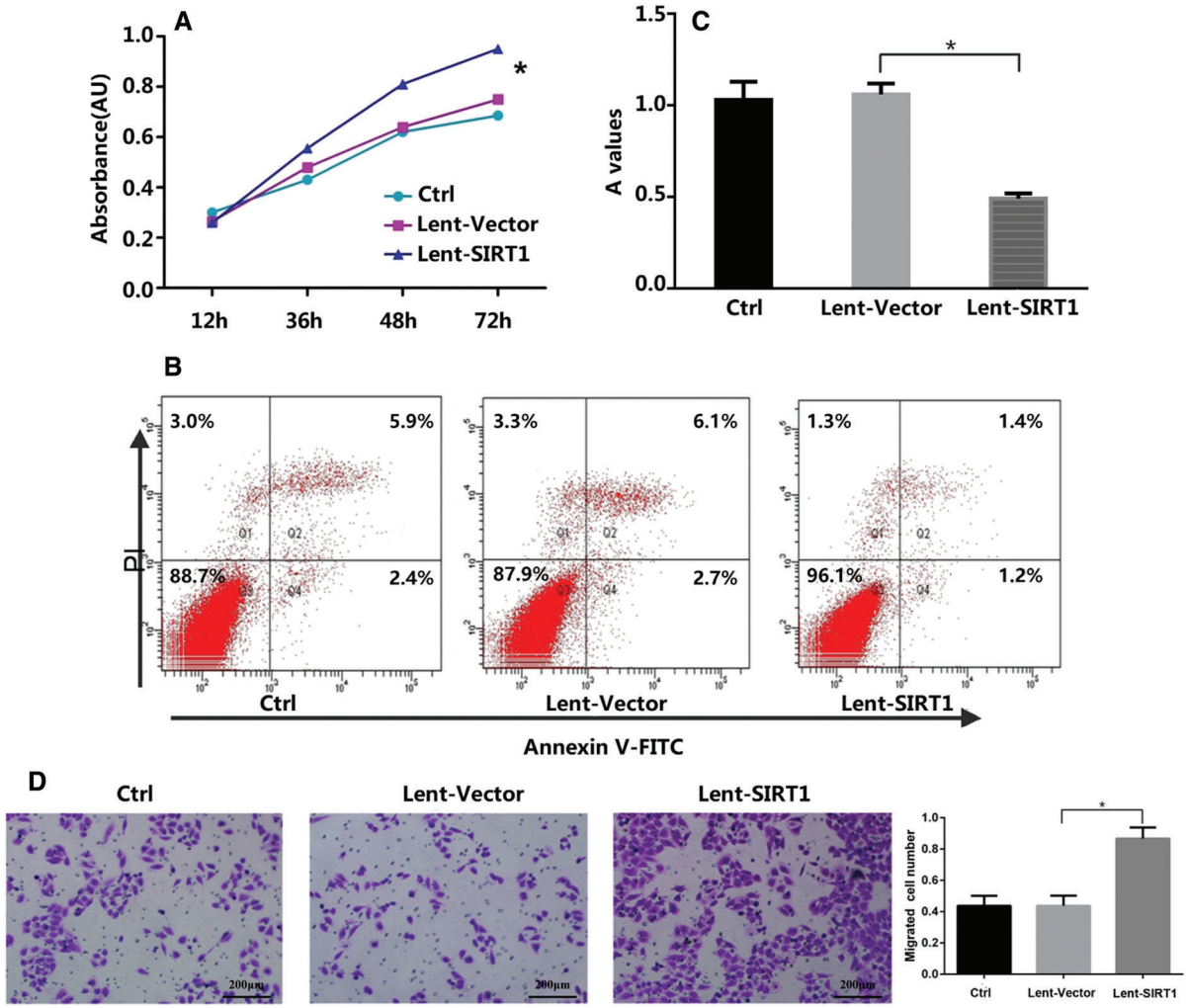

FIGURE 4. SIRT1 overexpression promoted the proliferation and invasion of MG-63 cells.
(A) MTT assay of cell proliferation. (B-C) Flow cytometry analysis of the apoptosis of MG-63 cells. (D) Transwell assay. The number of MG-63 cells in different groups that had invaded into matrigel was counted in 10 random fields (200×). The data are presented as mean ± SD (n = 3). *$P < 0.05$.

SIRT1 has been reported to be involved in many biologic functions by regulating transcription factors such as p53, NF-κB, and nuclear receptor (Vaziri *et al.*, 2001; Yeung *et al.*, 2004). SIRT1 is activated and expressed at a high level in a variety of cancers (Brooks and Gu, 2009). SIRT1 overexpression inhibited apoptosis and promoted cell proliferation (Huffman *et al.*, 2007; Kim *et al.*, 2008). Additionally, SIRT1 overexpression contributed to cancer metastasis (Shi *et al.*, 2017; Zhang *et al.*, 2016). In agreement with previous work, our results have demonstrated that overexpression of SIRT1 enhanced OS cells' proliferation and invasion while blocking apoptosis of OS cells.

It has been reported that leptin could induce the expression of SIRT1 in hypothalamic pro-opiomelanocortin (POMC) neurons (Ramadori *et al.*, 2010). Leptin also upregulates the SIRT1 level in primary cells isolated from the murine hypothalamus (Sasaki *et al.*, 2014). These data suggest that SIRT1 may be involved in the effects of leptin on cancer progression. Our data showed that leptin could induce SIRT1 overexpression in OS cells, with increased cell proliferation and invasion, as well as decreased cell apoptosis and adhesion. Furthermore, these effects of leptin on OS cells were diminished when SIRT1 was silenced by SIRT1 shRNA. A recent study reported that leptin promoted colon cancer by inducing Nrf2 dependent SIRT1 overexpression (Song *et al.*, 2018). These findings indicate that blocking SIRT1 expression might be a therapeutic target for OS.

Similar to other cancers, OS metastasis requires the degradation of the extracellular matrix via increased expression of MMPs (Guo *et al.*, 2016). MMPs including MMP-2, 8, and 9 have been shown to be overexpressed in several cancers. MMPs are reported to contribute to cancer development by promoting cancer metastasis (Brown and Murray, 2015; Leblais *et al.*, 2018). For example, MMP-2, 8, and 9 overexpression correlate with metastasis and poor prognosis of many cancers (Aroui *et al.*, 2016; Decock *et al.*, 2015). In this study, we found that MMP-2, 8, and 9 were upregulated by SIRT1 in OS cells. These results suggested that SIRT1 overexpression promoted OS cells invasion by increasing the expression of MMP-2, 8, and 9. Further studies are needed to investigate whether highly metastatic OS cells could generate endogenous leptin to induce the

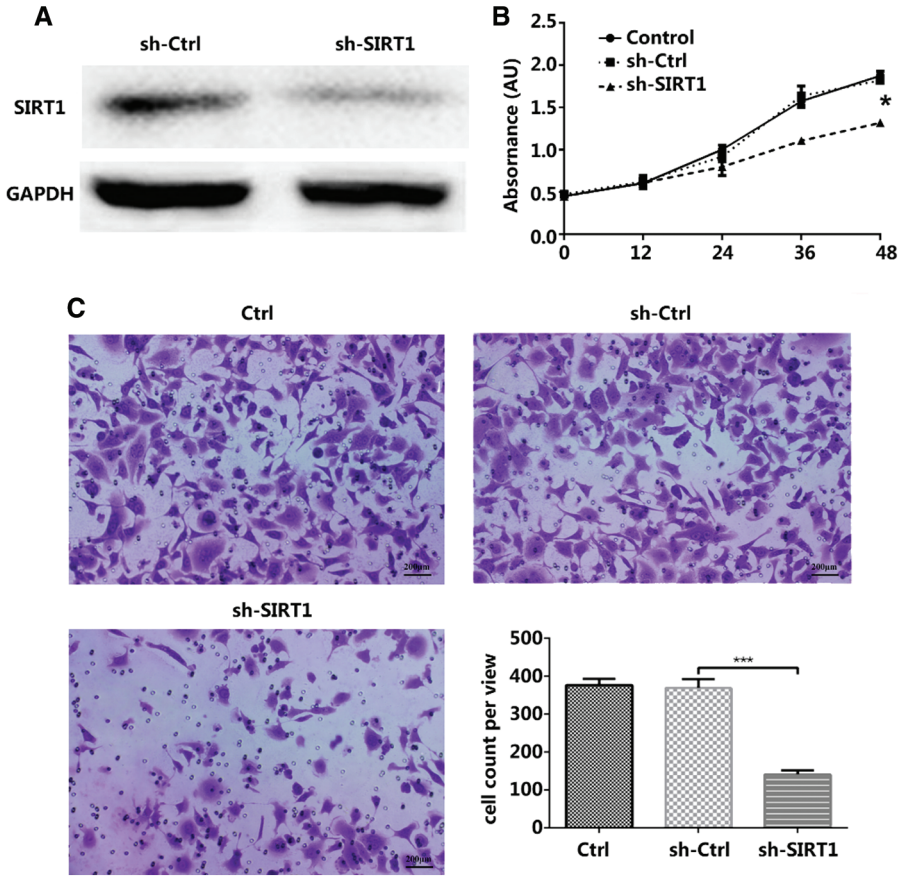

FIGURE 5. The knockdown of SIRT1 blocked the effects of leptin on the proliferation and invasion of MG-63 cells. (A) SIRT1 expression was examined by Western blot in MG-63 cells. (B) MTT assay was used to detect cell proliferation. (C) Transwell assay. The number of MG-63 cells in different groups that had invaded into matrigel was counted in 10 random fields (200×). The data are presented as mean ± SD (n = 3). $^{*}P < 0.05$, $^{***}P < 0.001$.

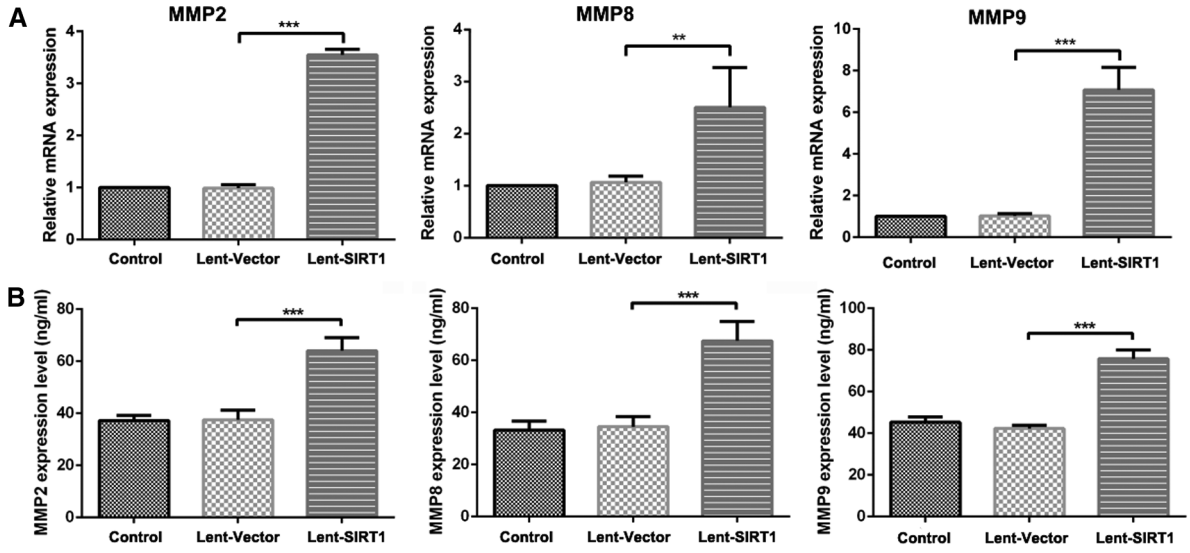

FIGURE 6. SIRT1 upregulated metastatic-associated gene expression.
(A) MMP-2, 8, and 9 expressions were determined by real-time PCR at the mRNA level. (B) MMP-2, 8, and 9 expressions were determined by ELISA assay. The data are presented as mean ± SD (n = 3). $^{**}P < 0.01$, $^{***}P < 0.001$.

expression of MMP-2, 8, and 9, and promote cell proliferation and invasion. In addition, *in vivo* animal studies are necessary to confirm the oncogenic role of leptin in OS. On the other hand, epithelial to mesenchymal transition (EMT) plays a crucial role in tumor metastasis (Ma *et al*., 2019; Yang and Tian, 2019). Therefore, it would be interesting to explore whether leptin could regulate EMT to promote OS metastasis.

Taken together, the results of our study demonstrated that leptin enhanced the proliferation and invasion of OS cells by upregulating the expression of SIRT1 and MMP-2, 8, and 9. Leptin is a potent therapeutic target for metastatic OS.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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