MicroRNA-133b Inhibits Cell Proliferation and Invasion in Osteosarcoma by Targeting Sirt1

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MicroRNAs are a class of small noncoding RNAs that function as critical gene regulators through targeting mRNAs for translational repression or degradation. In this study, we showed that the miR-133b expression level was decreased while the Sirt1 mRNA expression level was increased in osteosarcoma tissue and cell lines. A low expression of miR-133b was significantly associated with tumor size, distant metastasis, and advanced clinical stage. In addition, osteosarcoma patients with a low miR-133b expression showed a worse prognosis when compared to those with a high level of miR-133b expression. Thus, we identified Sirt1 as a novel direct target of miR-133b. Overexpression of miR-133b suppressed Sirt1 expression and attenuated cell proliferation and invasion. Forced expression of Sirt1 could partly rescue the inhibitory effect of miR-133b in osteosarcoma cells. Our finding also suggested that the inhibitory effects of the miR-133b/Sirt1 axis on osteosarcoma progression were involved in the Wnt/β-catenin pathway. Taken together, these findings will shed light on the role and mechanism of miR-133b in regulating osteosarcoma cell growth via the miR-133b/Sirt1 axis, and miR-133b may serve as a potential therapeutic target in osteosarcoma in the future.

Key words: miR-133b; Sirt1; Osteosarcoma; Proliferation; Invasion

INTRODUCTION

Treatment with a combination of chemotherapy and aggressive surgical resection has markedly improved the prognosis of osteosarcoma patients over the last few decades. However, patients with local relapse or distant metastasis still have a very poor prognosis[1]. It is therefore essential to develop novel approaches to treating recurrent or/and metastatic osteosarcoma. Recently, growing evidence has supported the cancer-related effects of miRNAs, a newly discovered class of noncoding small RNAs that function through negatively regulating a variety of gene expressions. Mature miRNAs exert their effects by integrating into an RNA-inducing silencing complex (RISC) and binding to specific complementary sites within 3′-untranslated regions (3′-UTR) of their target gene mRNA in order to inhibit translation or directly induce degradation[2].

In a variety of cancers, miRNA expression is significantly altered, and it has the potential to be a prominent diagnostic and prognostic tool[3]. It is important to elucidate the function of miRNAs in tumor pathogenesis and progression because miRNAs have the potential to regulate various critical biological processes, including the differentiation, progression, apoptosis, proliferation, and drug resistance of tumor cells[4]. However, not much is known about the expression and deregulation of miRNAs in osteosarcoma with very poor prognosis and a high possibility for tumor proliferation and migration. Although proliferation and migration have been acknowledged as the most lethal attributes of solid tumors, the knowledge of the molecular mechanism underlying them is still limited.

Bioinformatic algorithms have shown that all human miRNAs may regulate up to 30% of human genes, which represent the majority of genetic pathways[5]. Many studies have identified specific miRNA expression profiles of multiple cancer types compared to those of normal adjacent tissues. Depending on cellular contexts and target genes that they regulate, miRNAs may function as tumor suppressors or oncogenes[6]. Among these functional miRNAs, miR-133b has been demonstrated to be significantly decreased in multiple cancer types and play the role of tumor suppressor. For instance, miR-133b leads to the attenuation of melanoma proliferation and migration[7]. Another study found that miR-133b exhibited a lower expression in osteosarcoma cell lines when compared with osteoblast cell lines[8], which implied the

involvement of miR-133b in osteosarcoma proliferation and metastasis.

Sirtuins are nicotinamide adenine dinucleotide (NAD^{+})-dependent class III histone deacetylases that are conserved from bacteria to eukaryotes[9]. The mammalian sirtuin family consists of seven members, designated SIRT1 through SIRT7, which are characterized by a conserved 275-amino acid catalytic core and unique additional N-terminal and/or C-terminal sequences of variable length[10]. Among the seven human sirtuins, SIRT1 is the most studied and is known to have more than a dozen substrates, including Ku70, p53, NF-κB, and fork head (FOXO) transcription factors, to affect stress and DNA damage[11].

In the present study, we confirmed the regulatory relationship between miR-133b, a known tumor-suppressive miRNA, and an oncogene, Sirt1. We provided evidence that miR-133b could impede osteosarcoma cell proliferation and migration, at least in part, by targeting Sirt1.

MATERIALS AND METHODS

Tissue Samples, Cell Lines, and Cell Transfection

A total of 15 pairs of fresh primary osteosarcoma tissue and their matched adjacent normal bone and myeloid tissues were collected. All samples were obtained from patients who underwent surgical resection at Xiamen Haicang Hospital (Xiamen, P.R. China) and were snap frozen in liquid nitrogen, and then stored at −80°C until further use. An additional 80 cases of osteosarcoma tissue were purchased from Auragene Bioscience Co. (Changsha, P.R. China). The 80 cases of osteosarcoma tissue samples contained the clinical characteristic information, including age, gender, tumor size, histological grade, distant metastasis, clinical stage, and survival time, which was obtained by follow-up. This project was approved by the ethics committee of Xiamen Medical College.

The human osteoblast hFOB, HEK293 cells, and three human osteosarcoma cell lines, including MG-63, Saos-2, and U2OS, were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were grown routinely in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA) and cultured in a 37°C humidified atmosphere of 5% CO_2. Ectopic expression of miR-133b in cells was achieved by transfection with miR-133b mimics (GenePharma, Shanghai, P.R. China) using Lipofectamine 2000 (Invitrogen). Overexpression of Sirt1 was performed using the Sirt1 ORF expression clone (GeneCopoecia, Guangzhou, P.R. China). Cells were plated into 6-well clusters or 96-well plates and transfected for 24 or 48 h. Transfected cells were used in further assays or RNA/protein extraction.

RNA Extraction and SYBR Green Quantitative PCR Analysis

Total RNA was extracted from cells using TRIzol reagent (Invitrogen). Mature miR-133b expression in cells was detected using a Hairpin-it™ miRNAs qPCR Kit (GenePharma). Expression of RNU6B was used as an endogenous control. Sirt1 mRNA expression was measured by SYBR Green qPCR assay (Takara, Dalian, P.R. China). Data were processed using the $2^{-\Delta\Delta CT}$ method.

Immunohistochemical Staining

The expression of Sirt1 was evaluated using immunohistochemical (IHC) staining. The IHC staining was performed as previously described[12]. Briefly, the tissue sections cut at 4 μm were deparaffinized and hydrated and were then retrieved with citrate buffer in boiling water for 15 min. The sections were then incubated with primary antibodies (mouse monoclonal anti-Sirt1; 1:200; Sigma-Aldrich, St. Louis, MO, USA) overnight at 4°C. The sections were incubated with secondary antibody for 60 min at 37°C. The signaling was visualized using substrate diaminobenzidine (DAB) and counterstained with hematoxylin.

CCK-8 Cell Proliferation Assay

Cell proliferation rates were measured using the cell counting kit-8 (CCK-8; Beyotime, Hangzhou, P.R. China). Cells (0.5×10^4) were seeded into each 96-well plate for 24 h, transfected with the indicated miRNA or plasmids, and further incubated for 24, 48, and 72 h, respectively. CCK-8 reagents (10 μl) were added to each well 1 h before the endpoint of incubation. The optical density (OD) at 490 nm value in each well was determined with a microplate reader.

Colony Formation Assay

For each group, 4 ml of complete medium containing 200 cells was added to a 60-mm dish. After cells were cultured at 37°C with 5% CO_2 for 14 days, the supernatant was discarded, and cells were washed with phosphate-buffered saline (PBS) three times. Cells were then fixed with 4% paraformaldehyde for 15 min and stained with GIMSA (Solarbio, Beijing, P.R. China) for 20 min. Colonies were counted under an inverted microscope (Nikon, Japan), and the data were expressed as the percentage of colony number. This assay was repeated three times.

Flow Cytometric Analysis of Apoptosis With Annexin V/PI Double Staining

An Annexin-V Apoptosis Detection Kit (Life Technologies, Grand Island, NY, USA) was used for analysis of apoptosis. After the indicated treatment, MG-63 cells were trypsinized, collected, and resuspended. About

2×10^5 cells were harvested and washed twice with cold PBS, and then resuspended in 500 μl of binding buffer. Annexin-V-FITC (10 μl) and propidium iodide (PI; 10 μl) were added to the solution and mixed well. After 15 min of incubation, the cells were analyzed using flow cytometric analysis (BD Biosciences, San Jose, CA, USA).

Cell Invasion Assay

The invasive and migratory potential of cells was evaluated using Transwell inserts with 8-μm pores (Corning, Corning, NY, USA). For the invasion assay, 3.0×10^5 cells in serum-free medium precoated with Matrigel matrix (Becton, Dickinson and Company, East Rutherford, NJ, USA) were added to each upper insert 24 h after transfection, and 500 μl of 10% FBS medium was added to the matched lower chamber. After 48 h of incubation, cells that did not invade were removed from the upper surface of the Transwell membrane with a cotton swab, and cells on the lower membrane surface were fixed in methanol, stained with 0.1% crystal violet, and photographed. The staining was then dissolved in 5% acetic acid, and the OD at 490 nm value in each well was determined by a microplate reader.

Scratch Assay

Cells in each group were collected and resuspended in complete DMEM. Cells (1×10^5) were then seeded into each well of a six-well plate. When confluence reached 100%, the cells were scratched with the head of a 200-μl tip and washed with serum-free medium. After being cultured in serum-free medium for 24 h, the cells were continued to be cultured in complete DMEM at 37°C 5% CO_2. We photographed these cells in each group at 0 and 48 h.

Western Blot Analysis

Immunoblotting was performed to detect the protein expression in osteosarcoma cell lines. Cultured or transfected cells were lysed in RIPA buffer with 1% PMSF. Protein was loaded onto a SDS-PAGE minigel and transferred onto a PVDF membrane. After being probed with primary antibodies [anti-Sirt1, anti-c-PARP, anti-Bak, and anti-Bcl-xl (Sigma-Aldrich); anti-MMP7, anti-Wnt5a, anti-β-catenin, and anti-pGSK3β (Cell Signaling Technology, Danvers, MA, USA); and anti-β-actin (Abcam, Cambridge, UK)] at 4°C overnight, the blots were subsequently incubated with HRP-conjugated secondary antibody (1:5,000). Signals were visualized using ECL Substrates (Millipore, Boston, MA, USA). β-Actin was used as an endogenous protein for normalization.

Luciferase Reporter Assay

A fragment of 3′-UTR of Sirt1 [1,089 base pairs (bp)] containing the putative miR-133b binding site (403–409) was amplified by PCR using the following primers: wt-Sirt1, 5′-CCGCTCGAGCACCAGTAAAACAAGG AACTTG-3′ (forward) and 5′-GAATGCGGCCGCTTT ACAGAAACAAATGCAATGTTAC-3′ (reverse).

The PCR product was subcloned into a psiCHECK-2 vector (Promega, Madison, WI, USA) immediately downstream to the luciferase gene sequence. A psiCHECK-2 construct containing the 3′-UTR of Sirt1 with a mutant seed sequence of miR-133b was also synthesized using the following primers: mut-Sirt1, 5′-TTAAAATTTCCTA CTTGTGTATAGAAATGGAAAG-3′ (forward) and 5′-ACAAGTAGGAAATTTTAATACAGTGGTTCTC-3′ (reverse).

All constructs were verified by DNA sequencing. HEK293 cells were plated into 96-well clusters and then cotransfected with 100 ng of constructs with or without miR-133b precursors. At 48 h after transfection, luciferase activity was detected using a Dual-Luciferase Reporter Assay System (Promega) and normalized to *Renilla* activity.

Statistical Analysis

All data from three independent experiments were expressed as mean±SD and processed using SPSS17.0 statistical software (Chicago, IL, USA). The expressions of miR-133b in osteosarcoma tissues and their matched adjacent normal bone and myeloid tissues were compared by the Wilcoxon's paired test. The correlation between miR-133b and Sirt1 expression was analyzed by the Spearman's correlation analysis. The clinical association between miR-133b expression and clinicopathological variables in osteosarcoma patients was evaluated by the chi-square test. The difference among the groups was estimated by the Student's *t*-test or one-way ANOVA, depending on conditions. A value of $p<0.05$ was considered to be statistically significant.

RESULTS

miR-133b Is Frequently Downregulated in Osteosarcoma Tissues and Predictive of a Poor Prognosis in Osteosarcoma Patients

We performed SYBR Green Quantitative PCR (qPCR) analysis to detect the expression levels of miR-133b, miR-133a, and Sirt1 in osteosarcoma tissue and cell lines. In the large panel of 15 cases of primary osteosarcoma tissues and their adjacent normal bone and myeloid tissues, our results showed that miR-133b, but not miR-133a, was significantly decreased in 13 (86.6%) osteosarcoma tissues and Sirt1 mRNA was increased in 12 (80.0%) tissues when compared with that of paired adjacent normal tissues (Fig. 1A–C). By IHC staining, we also confirmed that the Sirt1 protein expression was significantly decreased in osteosarcoma tissues
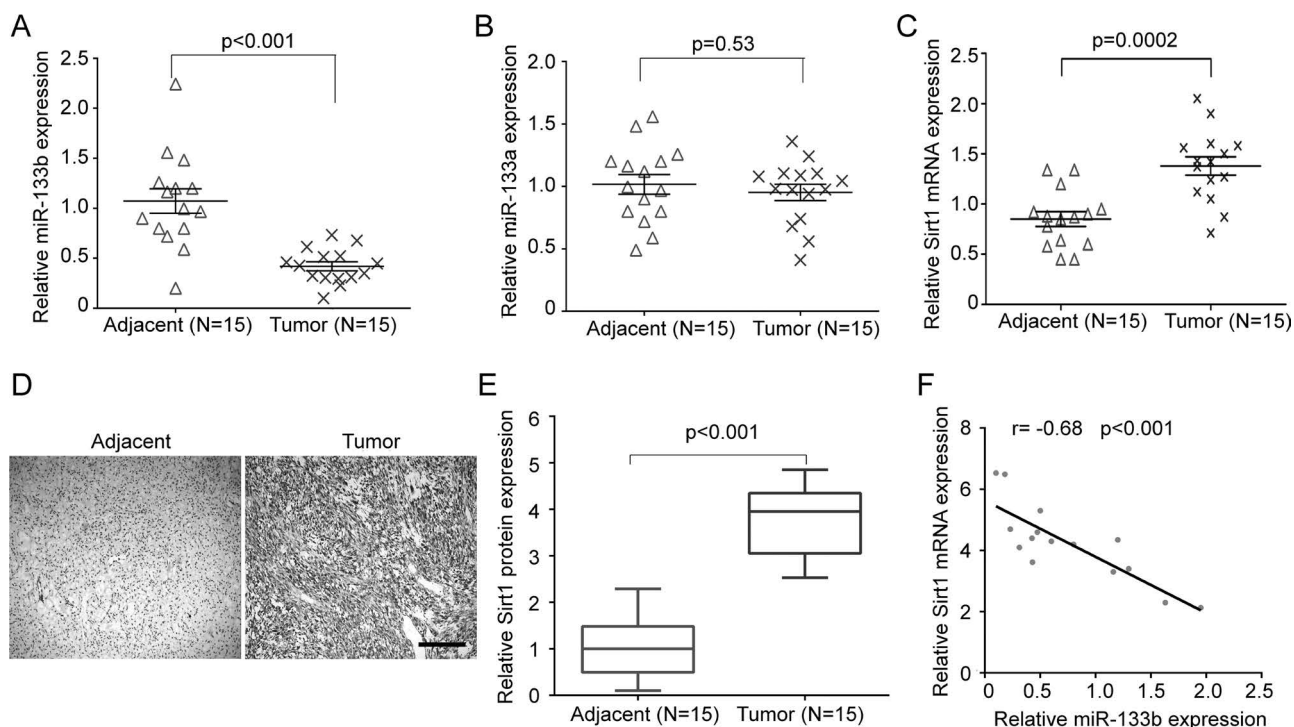
Figure 1. The expression of miR-133b and Sirt1 in osteosarcoma tissues. Quantitative PCR (qPCR) was used to detect the expression of (A) miR-133b, (B) miR-133a, and (C) Sirt1 mRNA in 15 osteosarcoma tissues and 15 adjacent tissues. (D) Representative images of immunostaining with Sirt1 in adjacent and osteosarcoma tissues. (E) Quantification of the integral optical density (IOD) for Sirt1 immunostaining in (D). (F) Spearman's correlation analysis for miR-133b and Sirt1 expression in 15 osteosarcoma tissues. Data are expressed as mean±standard deviation.

compared with that of adjacent normal tissues (Fig. 1D and E). Spearman's correlation test showed a significant inverse correlation of the miR-133b and Sirt1 mRNA expression levels in osteosarcoma tissues with Spearman's correlation ($r = -0.68$, $p < 0.001$), confirming that lower expression levels of miR-133b are significantly associated with higher levels of Sirt1 mRNA expression in the same set of osteosarcoma tissues (Fig. 1F). In addition, we extended our test to 80 cases of osteosarcoma tissue samples. qPCR was performed to determine the expression of miR-133b, and the association between miR-133b and the clinicopathological variables of osteosarcoma patients was analyzed. The miR-133b expression in osteosarcoma tissues was categorized as low expression ($n = 46$) or high expression ($n = 34$) in relation to the mean value. Low expression of miR-133b was significantly associated with tumor size ($p = 0.021$), distant metastasis ($p = 0.029$), and advanced clinical stage ($p = 0.013$). However, we found no association between miR-133b expression and age, gender, or histological grade of osteosarcoma patients ($p > 0.05$). We further analyzed the relationship between miR-133b expression and the survival time of osteosarcoma patients. Our data showed that osteosarcoma patients

with low miR-133b expression showed a worse prognosis when compared with those having a high level of miR-133b ($p = 0.02$) (Fig. 2). These findings suggest that the decreased expression of miR-133b is involved in the malignant progression of NSCLC.
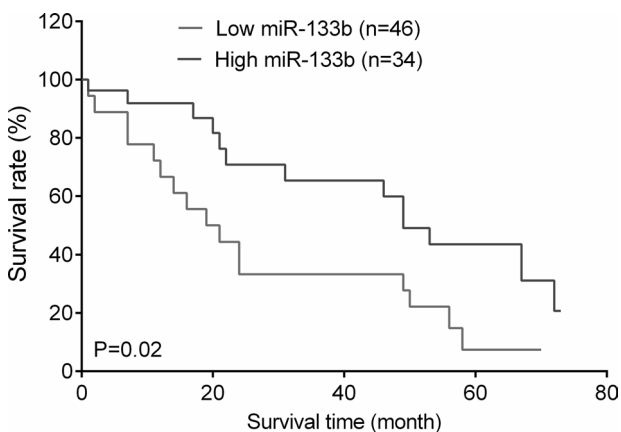

Figure 2. The relationship between miR-133b expression and overall survival rate of osteosarcoma patients. The patients with a low miR-133b expression have a shorter survival time than those with a high miR-133b expression ($p = 0.02$).

## miR-133b Efficiently Suppressed Sirt1 by Directly Targeting the Sirt1 3′-UTR

The combining sites of the Sirt1 3′-UTR with miR-133b were predicted by TargetScan (www. targetsan.org) and microRNA.org (www.microRNA.org); its position was 403–409 of Sirt1 3′-UTR. A sequential replacement of a 7-bp region was performed to produce a mutant vector (Fig. 3A). To further investigate whether the predicted binding site of miR-133b to 3′-UTR of Sirt1 was responsible for this regulation, we cloned the 3′-UTR of Sirt1 downstream to a luciferase reporter gene (wt-Sirt1). Its mutant version (mut-Sirt1) by binding site

mutagenesis was also constructed (Fig. 3B). We cotransfected wt-Sirt1 vector and miR-133b mimics or scramble control into MG-63 cells. The luciferase activity of miR-133b-transfected cells was significantly reduced in a dose-dependent manner when compared with scramble control cells. Moreover, miR-133b-mediated repression of luciferase activity was abolished by the mutant putative binding site (Fig. 3C and D).

To further confirm the inhibitory role of miR-133b in Sirt1 expression, we infected MG-63 cells with miR-133b mimics, miR-133b inhibitor, and their negative controls. Quantitative real-time (RT)-PCR and Western blot
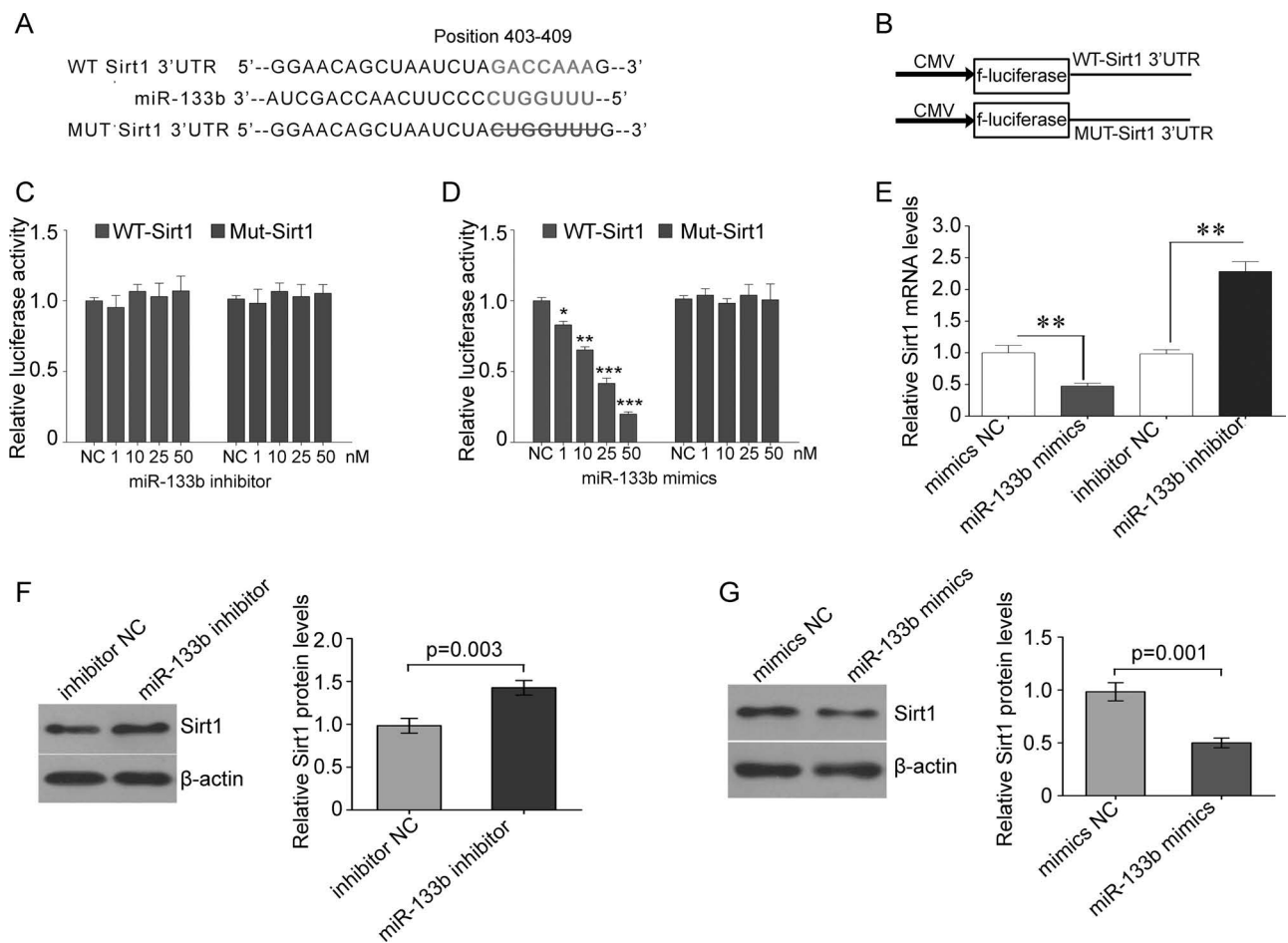
Figure 3. miR-133b directly targets Sirt1. (A) The predicted miR-133b binding site (gray) within the Sirt1 3′-UTR and its mutated version (through line). (B) Representation of the wt- and mut-Sirt1 3′-UTR vectors used in the luciferase assay. (C) There were no significant alterations in luciferase activity after being cotreated with the miR-133b inhibitor and either wt-Sirt1 3′-UTR or mut-Sirt1 3′-UTR. (D) miR-133b represses the luciferase activity of wt-Sirt1 3′-UTR in a dose-dependent manner. Mutated Sirt1 3′-UTR abrogated miR-133b-mediated repression luciferase activity. (E) qPCR detected the expression of Sirt1 mRNA after transfecting with the miR-1133b mimic, miR-133b inhibitor, or negative control in MG-63 cells. (F) Western blotting detected Sirt1 protein expression after miR-133b inhibitor or negative control treatment in MG-63 cells. β-Actin was used as the loading control. (G) Western blotting detected Sirt1 protein expression after miR-133b mimic or negative control treatment in MG-63 cells. β-Actin was used as the loading control. Data are expressed as mean±standard deviation. $*p<0.05$, $**p<0.01$, $***p<0.001$ versus negative control (NC). wt, wild type; mut, mutant.
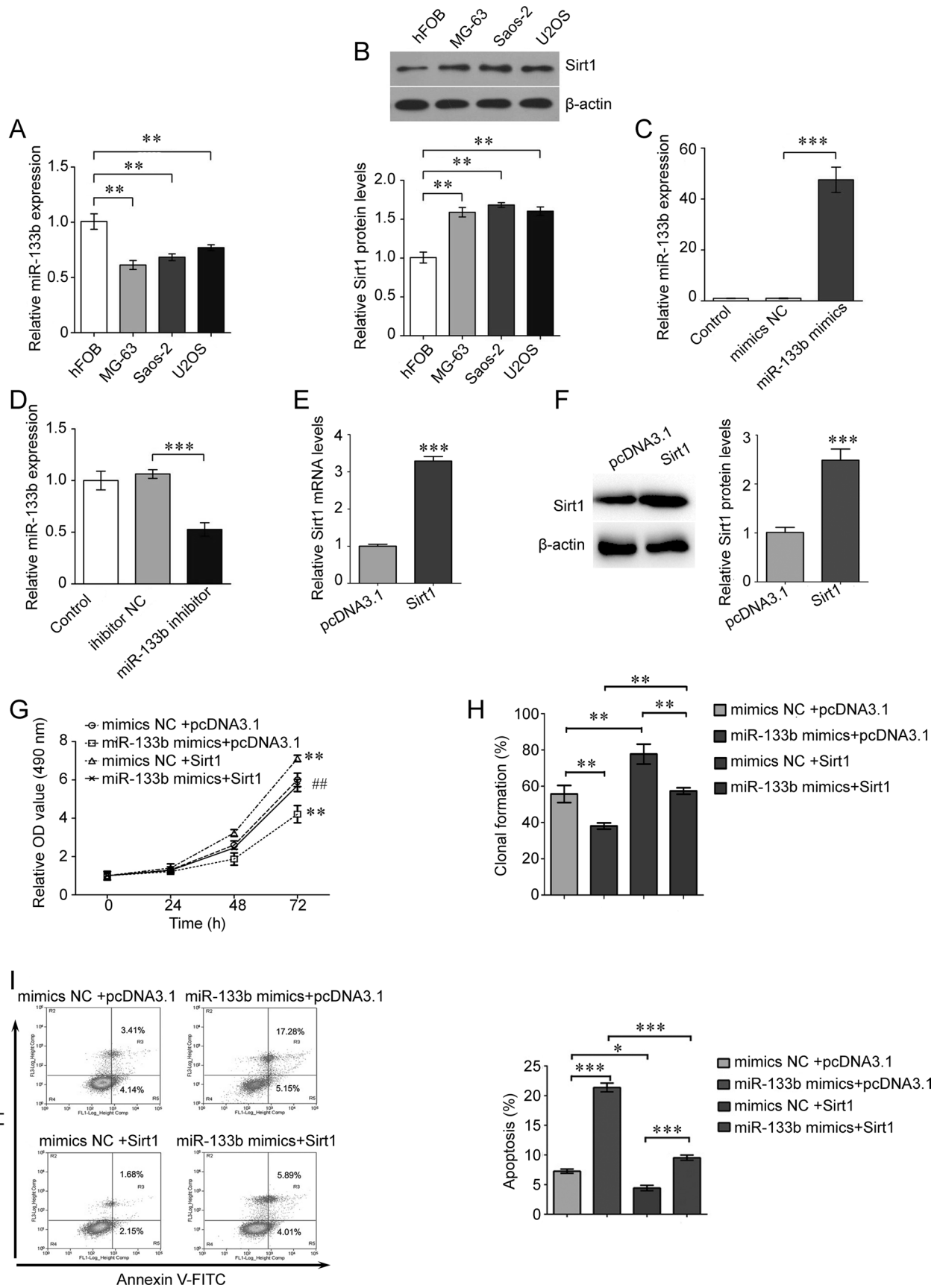
analysis showed that enhanced miR-133b in MG-63 cells significantly repressed Sirt1 mRNA and protein expression when compared with cells transfected with scramble control, whereas inhibited miR-133b expression had the opposite effect (Fig. 3E–G).

Effects of miR-133b on Osteosarcoma Cell Proliferation, Apoptosis, Migration, and Invasion

To further investigate the biological role of miR-133b in osteosarcoma progression, we detected the expression of miR-133b and Sirt1 in one human osteoblast and three human osteosarcoma cell lines. All three cell lines showed a notable loss of miR-133b and an obvious upregulation of Sirt1 protein expression, in comparison with the control human osteoblast cell (Fig. 4A and B). The MG-63 cells transfected with miR-133b mimics, miR-133b inhibitor, or Sirt1-overexpressed plasmid were used for further analysis (Fig. 4C–F). This showed that the increased expression of miR-133b induced significant inhibition on cell growth and colony number and dramatically increased the apoptotic rate, while the inhibitory role of miR-133b was reversed by cotreatment with the Sirt1-overexpressed plasmid (Fig. 4G–I).

In addition, we tested the role of miR-133b on cell migration and invasion. Compared with the scramble control, miR-133b mimic-transfected MG-63 cells exhibited significant impairment of migratory ability (Fig. 5). The corresponding effect on the invasive ability was observed in a parallel invasion assay. To further confirm the potential relationship between miR-133b and the downstream gene Sirt1, we tested cell motility under the condition of overexpression of Sirt1. Once Sirt1 expression was effectively upregulated, transfected MG-63 cells exhibited upregulated cell migration and invasion abilities (Fig. 5A and B), which sufficiently reversed the inhibitory effects induced by the upregulation of miR-133b. Taken together, these results indicate that miR-133b functions as a potent tumor suppressor through regulating Sirt1 expression.

We further determined the expression of some key genes involved in cell growth and cell apoptosis. The expression of proapoptosis genes, including cleaved PARP (c-PARP) and Bak, was notably upregulated, but

the levels of proproliferative gene Bcl-xl and proinvasive gene MMP7 were significantly reduced after overexpression of miR-133b in MG-63 cells (Fig. 6A). However, the effects of miR-133b overexpression were rescued by Sirt1-expressed vector transfection. In addition, we tested whether the Wnt/β-catenin pathway that is activated in many types of cancers was affected by the miR-133b/Sirt1 axis. Our results showed that the expression of Wnt5a and β-catenin was significantly repressed, and the expression of pGSK3β was enhanced by miR-133b overexpression. Once Sirt1 expression was upregulated in MG-63 cells transfected with miR-133b mimics, the effect of miR-133b on the Wnt/β-catenin pathway was attenuated (Fig. 6B). These findings suggest that the inhibitory effect of the miR-133b/Sirt1 axis on osteosarcoma progression is involved in the Wnt/β-catenin pathway.
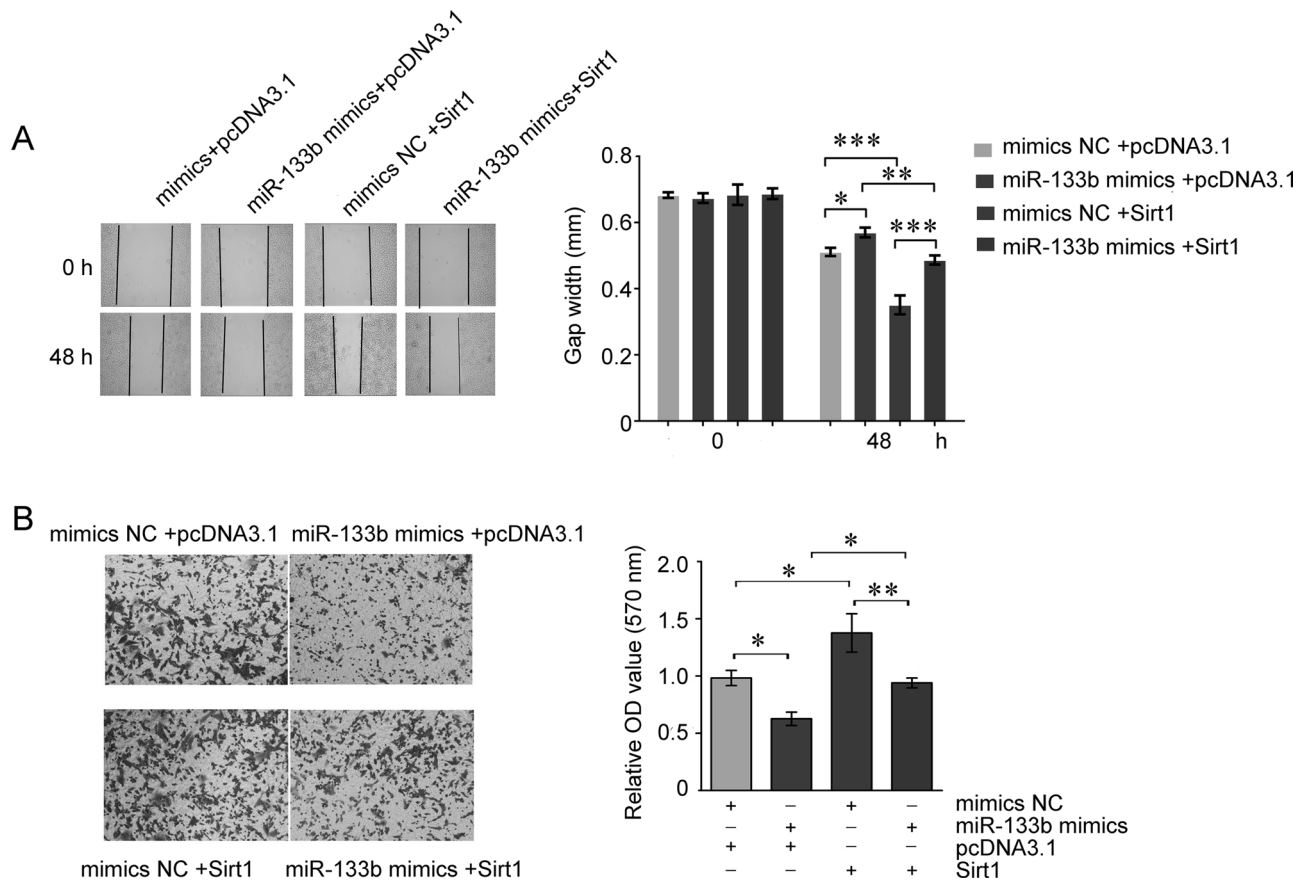
DISCUSSION

Most deaths from cancer are caused by complications arising from metastasis. Therefore, targeting metastatic disease is a pivotal anticancer strategy. Studies on tumor invasion and metastasis have revealed the critical role of miRNA in these processes by the mechanism that miRNA could regulate a variety of genes pivotal for invasion or metastasis[13]. Quite recently, some miRNAs have been identified to promote[14] or suppress[15] tumor invasion or metastasis, providing potential therapeutic targets for an antimetastasis strategy.

In osteosarcoma, miR-199a-3p expression had a significant effect on osteosarcoma cell growth in vitro. Overexpression of miR-199a-3p by transfection significantly decreased osteosarcoma cell growth and migration[8]. In addition, miR-199a-3p was reported to regulate mTOR and Met to influence the doxorubicin sensitivity in liver cancer cells[16]. In this study, we raised the hypothesis that miR-133b might be involved in the osteosarcoma metastatic process. Finally, we confirmed the link between miR-133b and Sirt1, a positive tumor metastasis-related gene, and found that miR-133b inhibited osteosarcoma cell growth, invasion, and migration via targeting Sirt1. The results obtained from quantitative RT-PCR validated

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Figure 4. Forced miR-133b expression represses MG-63 cell growth by targeting Sirt1. (A) qPCR detected the expression of miR-133b in osteosarcoma cell lines, including MG-63, Saos-2, and U2OS, and the human osteoblasts (hFOB). (B) Western blot detected the expression of Sirt1 in osteosarcoma cell lines, including MG-63, Saos-2, and U2OS, and the human osteoblasts (hFOB), and quantified. (C) qPCR detected the expression of miR-133b in MG-63 cells after miR-133b mimic or negative control transfection. (D) qPCR detected the expression of miR-133b in MG-63 cells after miR-133b inhibitor or negative control transfection. (E) qPCR detected the expression of Sirt1 in MG-63 cells after Sirt1-overexpressed vector or negative control transfection. (F) Western blot detected the expression of Sirt1 in MG-63 cells after Sirt1-overexpressed vector or negative control transfection. (G) Cell counting kit-8 (CCK-8) was used to measure the cell proliferation after indicated treatment. **$p<0.01$ versus negative control (NC); ##$p<0.01$, miR-133b mimics+Sirt1 versus miR-133b mimics+pcDNA3.1. (H) Colony formation assay was used to measure the ability to form colonies after the indicated treatment. (I) Flow cytometry analysis was used to quantify the percentage of apoptosis after the indicated treatment. Data are expressed as mean±standard deviation. *$p<0.05$, **$p<0.01$, ***$p<0.001$ versus NC.

Figure 5. Forced miR-133b expression inhibits the ability of migration and invasion of MG-63 cells by targeting Sirt1. (A) Scratch assay was used to measure the ability of MG-63 cells to migrate after the indicated treatment. (B) Transwell assay was used to measure the ability of MG-63 cells to invade after the indicated treatment. Data are expressed as mean±standard deviation. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

that miR-133b was commonly downregulated in osteosarcoma cell lines and in 13 out of 15 (86.6%) enrolled osteosarcoma patients, which were consistent with previous studies. We restored the miR-133b expression in MG-63 cells and found that miR-133b inhibited cell proliferation and invasion. Moreover, a low expression of miR-133b was significantly associated with tumor size, distant metastasis, and advanced clinical stage. These findings suggest that miR-133b was involved in the process of metastasis. Interestingly, the levels of miR-133b were negatively correlated with Sirt1 mRNA levels in osteosarcoma tissue. It was reported that Sirt1 was identified to be an independent prognostic indicator of metastasis formation and metastasis-free survival. Our results confirmed a vital molecular relationship between miR-133b and Sirt1. We showed that, at both the mRNA and protein levels, upregulation of miR-133b expression in MG-63 cells effectively suppressed Sirt1 expression, and forced expression of Sirt1 could reverse the expression of Sirt1. The results suggest a potential inverse relevance of

miR-133b and Sirt1 in osteosarcoma and the main effect of Sirt1 on the cells is an autocrine affect because miR-133b downregulated the level of cellular Sirt1 mRNA and protein.

Furthermore, with the luciferase reporter assay study, we verified that miR-133b directly targets the Sirt1 gene through binding to a specific complementary site within its 3′-UTR. These results verified that miR-133b plays a suppressive role in cellular proliferation, migration, and invasion, at least in part, due to directly inhibiting Sirt1 expression. Our study revealed the inhibitory effect of miR-133b on Sirt1 and partly elucidated a potential molecular mechanism by which miR-133b participates in osteosarcoma aggressiveness. More recently, Chan et al. proposed a novel field of cancer-related miRNAs, termed metastamir, that are associated with metastatic processes[17]. For example, miR-21 is a mastermind of metastasis that promotes cell survival, migration, invasion, in vivo intravasation, and metastasis[18], whereas the miR-200 family is delinquent and whose absence contributes to the EMT
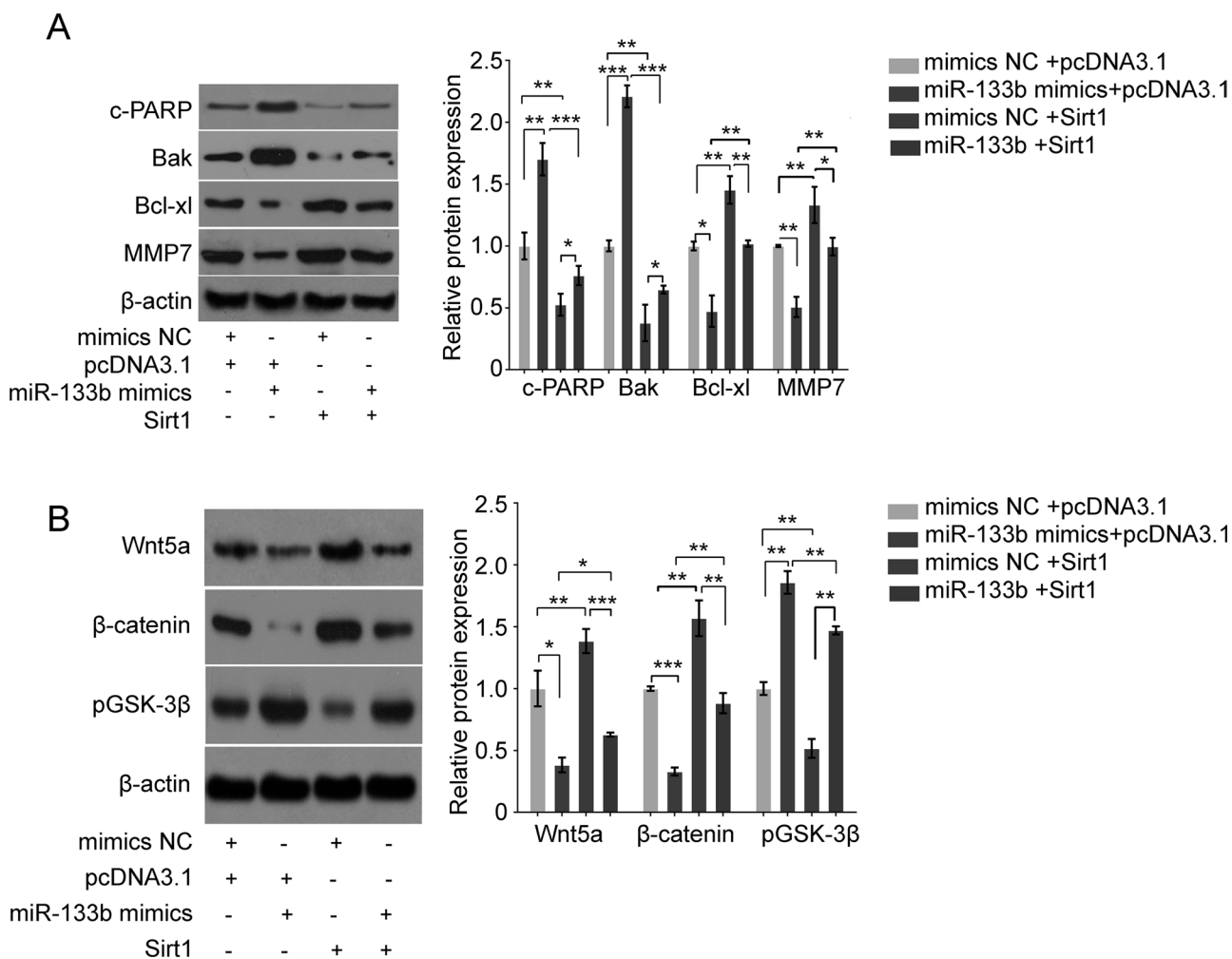
Figure 6. miR-133b regulates the apoptotic-related gene expression and Wnt/β-catenin signaling through Sirt1. (A) MG-63 cells were transfected with miR-133b mimics or Sirt1-overexpressed plasmid, or cotreated with them for 48 h. Then the expression of cleaved PARP (c-PARP), Bak, Bcl-xl, and MMP7 protein was measured by Western blot and quantified. (B) The expression of Wnt5a, β-catenin, and pGSK3β protein was measured by Western blot after the indicated treatment and quantified. Data are expressed as mean±standard deviation. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

phenotype[19]. miR-204 has recently been recognized as a direct posttranscriptional repressor of Snai1 mRNA and, consistent with its postulated tumor-suppressive role, was found to be underexpressed in different cancer cell lines. Attenuated expression of miR-204 resulted in a loss of cell–cell adhesion, supporting the EMT-related properties of Snail[18]. These metastamir are potential candidate cancer prognostic markers and therapeutic targets for metastatic cancers. Our findings suggest that miR-133b could function as a metastamir via targeting Sirt1. Secreted signaling molecules of the Wnt family have been widely investigated, and studies suggest that the Wnt signaling pathway is closely associated with bone malignancies, including breast cancer-induced bone metastasis, as well as osteosarcoma[20]. Small RNA technology to knock down

aberrant Wnt/β-catenin or inhibit β-catenin expression by small molecular inhibitors has shown promising effects against bone cancer[21]. In the current study, we demonstrated that the expression of Wnt5a and β-catenin was significantly repressed, the expression of pGSK3β was enhanced by miR-133b overexpression, and the effects of miR-133b on the Wnt/β-catenin pathway were attenuated by the restoration of Sirt1. These findings suggest that the inhibitory effect of the miR-133b/Sirt1 axis on osteosarcoma progression is involved in the Wnt/β-catenin pathway.

In conclusion, we newly described the miR-133b/Sirt1 link and provided a potential mechanism for Sirt1 deregulation and its contribution to osteosarcoma cell proliferation and invasion. As a result, restoration of miR-133b

expression could have an important implication for the clinical management of osteosarcoma.

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