Oncology Research, Vol. 26, pp. 753–764
Printed in the USA.

0965-0407/18 $90.00 + .00
DOI: https://doi.org/10.3727/096504017X15024946480113
E-ISSN 1555-3906
www.cognizantcommunication.com

lncRNA C2dat1 Promotes Cell Proliferation, Migration, and Invasion by Targeting miR-34a-5p in Osteosarcoma Cells

Daofu Jia,* Yanping Niu,† Dongling Li,‡ and Zhaorui Liu§

*Department of Emergency Medicine, Jinan Central Hospital, Jinan, Shandong, P.R. China
†Department of Outpatient Service Center, Qianfoshan Hospital of Shandong University, Jinan, Shandong, P.R. China
‡Department of Operation Room, Qilu Children's Hospital of Shandong University, Jinan, Shandong, P.R. China
§Department of Orthopedic Surgery, Jinan Central Hospital, Jinan, Shandong, P.R. China

Osteosarcoma is a highly aggressive malignant bone tumor with poor prognosis. Evidence has suggested that lncRNAs are deregulated in multiple cancers. In this study, we investigated the role of the lncRNA C2dat1 on the biological functions of osteosarcoma cells. The expressions of C2dat1, miR-34a-5p, and Sirt1 in human osteosarcoma cells were altered by transfection with their specific vectors/shRNA or mimic/inhibitor. Cell viability, migration, invasion, and apoptosis were assessed posttransfection. The mRNA and protein levels of C2dat1, miR-34a-5p, and Sirt1 were detected by qRT-PCR and Western blot. The results showed that C2dat1 suppression reduced cell viability, invasion, and migration, whereas it increased cell apoptosis in OS-732 cells. The expression of miR-34a-5p was downregulated when C2dat1 was overexpressed, whereas it negatively regulated Sirt1 expression. miR-34a-5p overexpression inhibited cell viability, migration, and invasion and promoted cell apoptosis in osteosarcoma cells by downregulation of Sirt1. Furthermore, miR-34a-5p overexpression deactivated the p38/ERK/AKT and Wnt/β-catenin signaling pathways by inhibition of Sirt1.

Key words: Osteosarcoma; C2dat1; miR-34a-5p; Sirt1

INTRODUCTION

Osteosarcoma is a highly aggressive malignancy that is common among children and adolescents under the age of 20[1] and accounts for 2% of all childhood cancers[2]. With advances in the treatment of osteosarcoma, the 5-year survival rate of patients with localized disease has increased to between 60% and 70%[3,4]. However, metastases occur within a few months when the prognosis of the disease is very poor, and the 5-year survival rate is between 15% and 30%[5]. Therefore, new and effective methods for early diagnosis and treatment are urgently required.

Although approximately 80% of the human genome is transcribed, only 2% encodes proteins[6]. These nonprotein coding RNA (ncRNA) molecules include long noncoding RNAs (lncRNAs) consisting of more than 200 nucleotides and microRNAs (miRNAs) comprising approximately 22 nucleotides. Both types of molecules play important roles in regulating gene expression and major cellular functions such as differentiation, proliferation, and death, all of which are critical in physiologic and pathologic processes[7]. Furthermore, there is now evidence of mutual cross-regulation between lncRNAs and miRNAs[8–10]. Evidence has also suggested that lncRNAs are dysregulated in multiple cancers and are involved in the regulation of tumor cell functions such as proliferation, apoptosis, cell cycle progression, migration, and invasion[11]. The mechanisms underlying the regulation of tumor cells by lncRNAs are complex, including binding to protein, RNA, or DNA to regulate the expression of oncogenes of tumor suppressors[12]. Although the roles of lncRNAs in osteosarcoma are unclear, a recent study demonstrated that the lncRNA FGFR3-AS1 promotes osteosarcoma[13]. The lncRNA C2dat1 had been shown to promote neuronal survival following cerebral ischemia[14]. However, the potential involvement of C2dat1 in the regulation of tumor cell growth is unclear. Therefore, in this study we investigated the role of lncRNA C2dat1 in osteosarcoma cell functions and the underlying mechanisms.

MATERIALS AND METHODS

Cell Culture and Treatment

Human osteosarcoma cell lines MG63 and OS-732 and the human osteoblast cell line hFOB1.19 were purchased from the American Type Culture Collection (ATCC)

Address correspondence to Zhaorui Liu, Department of Orthopedic Surgery, Jinan Central Hospital, No. 105 Jiefang Road, Lixia District, Jinan 250013, Shandong, P.R. China. E-mail: liuzhaorui517@126.com

(Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM; Solarbio, Beijing, P.R. China) containing 10% heat-inactivated fetal bovine serum (FBS). The cells were plated in tissue culture dishes at 37°C in a humidified 5% CO_2 incubator and cultured for 2–4 days until confluence was reached. Subcultures were prepared using 0.05% trypsin solution and seeded in 6- or 96-well tissue culture plates.

qRT-PCR Analysis

Total RNA was extracted from cells and tissues using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The One-Step SYBR® PrimeScript® PLUS RT-RNA PCR Kit (TaKaRa Biotechnology, Dalian, P.R. China) was used for real-time (RT)-PCR analysis of C2dat1 expression. The TaqMan MicroRNA Reverse Transcription Kit and TaqMan Universal Master Mix II with the TaqMan Micro RNA Assay of miR-34a-5p and U6 (Applied Biosystems, Foster City, CA, USA) were used for the analysis of miR-34a-5p expression levels in cells and tissues. RNA PCR Kit (AMV) Ver.3.0 (TaKaRa Biotechnology) was used to analyze Sirt1 expression. Data were normalized against GAPDH. Fold changes were calculated by relative quantification using the ($2^{-\Delta\Delta Ct}$) method.

Transfection and Generation of Stably Transfected Cell Lines

Short-hairpin RNA (shRNA) directed against human lncRNA C2dat1 was ligated into the U6/GFP/Neo plasmid (GenePharma, Shanghai, P.R. China) and was designated sh-C2dat1. For the analysis of Sirt1 functions, the full-length Sirt1 sequence and shRNA directed against Sirt1 were cloned into the pEX-2 and U6/GFP/Neo plasmids (GenePharma), respectively; these constructs were named pEX-Sirt1 and sh-Sirt1. Cell transfections were performed using Lipofectamine 3000 (Life Technologies) according to the manufacturer's instructions. Plasmid carrying a nontargeting sequence was used as a negative control (NC) of sh-C2dat1 and sh-Sirt1; this construct was referred to as sh-NC. The stably transfected cells were selected by culture in medium containing 0.5 mg/ml G418 (Sigma-Aldrich, St. Louis, MO, USA), and G418-resistant cell clones were established after approximately 4 weeks. miR-34a-5p mimics, inhibitors, and their respective NC were synthesized (Life Technologies) and used in cell transfections. The highest transfection efficiency was detected at 48 h. Thus, cells were harvested at 72 h post-transfection for use in experiments.

Cell Viability Assay

For cell viability assays, 1×10^5 cells were seeded in duplicate in 60-mm dishes. At the indicated time points, cells were washed, and the numbers of live cells were determined using the trypan blue exclusion method.

Apoptosis Assay

Cell apoptosis analysis was performed using propidium iodide (PI) and fluorescein isothiocynate (FITC)-conjugated annexin V staining. Briefly, cells were washed in phosphate-buffered saline (PBS) and fixed in 70% ethanol. Fixed cells were then washed twice in PBS and stained in PI/FITC-annexin V in the presence of 50 µg/ml RNase A (Sigma-Aldrich). After incubation for 1 h at room temperature in the dark, cells were analyzed using a FACScan flow cytometer (Beckman Coulter, Fullerton, CA, USA). The data were analyzed using FlowJo software.

Migration and Invasion Assays

Cell migration was determined using a modified two-chamber migration assay with a pore size of 8 µm. Cells suspended in 200 ml of serum-free DMEM were seeded in the upper compartment of 24-well Transwell culture chambers, and 600 ml of complete medium supplemented with 10% FBS (Sijiqing, Hangzhou, P.R. China) was added to the lower compartment. After incubation at 37°C, cells were fixed with methanol. Nonmigrated cells were removed carefully from the upper surface of the filter with a cotton swab. The migrated cells on the lower side of the filter were stained with crystal violet solution and counted under a light microscope.

The invasion behavior of cells was determined using 24-well Millicell Hanging Cell Culture inserts with 8-µm PET membranes (Millipore, Bedford, MA, USA). Briefly, after the cells were treated as indicated, 5.0×10^4 cells in 200 µl of serum-free DMEM medium were plated onto BD BioCoat™ Matrigel™ Invasion Chambers (8-µm pore size polycarbonate filters; BD Biosciences), and complete medium containing 10% FBS was added to the lower chamber. After incubation of the invasion chambers for 48 h at 37°C under 5% CO_2 in accordance with the manufacturer's protocol, the noninvading cells were carefully removed with a cotton swab. The invading cells were then fixed in 100% methanol, stained with crystal violet solution, and counted under a light microscope. The data were presented as the mean number of cells attached to the bottom surface in five randomly selected fields.

Reporter Vector Constructs and Luciferase Reporter Assay

The fragment of the C2dat1 sequence containing the predicted miR-34a-5p binding site was amplified by PCR and then cloned into a pmirGlO Dual-luciferase miRNA Target Expression Vector (Promega, Madison, WI, USA) to generate the C2dat1 wild-type reporter vector (C2dat1-wt). A corresponding construct was prepared

containing a mutated version of the putative miR-34a-5p binding site (C2dat1-Mt). These vectors and miR-34a-5p mimics were cotransfected into HEK 293T cells, and the Dual-Luciferase Reporter Assay System (Promega) was used to determine the luciferase activity.

Western Blotting

For Western blotting analyses, total cellular proteins were extracted using RIPA lysis buffer (Beyotime Biotechnology, Shanghai, P.R. China) supplemented with protease inhibitors (Roche, Guangzhou, P.R. China). The proteins were quantified using the BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). Proteins were then separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) using a Bio-Rad Bis-Tris Gel system according to the manufacturer's instructions and transferred to polyvinylidene difluoride (PVDF) membranes that were incubated at 4°C overnight in the presence of appropriate primary antibodies (diluted 1:1,000 in 5% blocking buffer). After washing, membranes were incubated with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies. After rinsing, the PVDF membranes were transferred into the Bio-Rad ChemiDoc™ XRS system for the addition of 200 µl of Immobilon Western Chemiluminescent HRP Substrate (Millipore). The signals were captured using Image Lab™ Software (Bio-Rad, Shanghai, P.R. China).

Statistical Analysis

All experiments were repeated three times. The results of multiple experiments are presented as the mean ± SD. Statistical analyses were performed using GraphPad Prism 6.0 statistical software (GraphPad Software Inc., La Jolla, CA, USA). Differences between groups were evaluated by one-way analysis of variance (ANOVA). A value of $p<0.05$ was considered to indicate statistical significance.

RESULTS

High Expression of C2dat1 in Osteosarcoma Cell Lines

Total RNA was isolated from the human osteosarcoma cell lines MG63 and OS-732 and the human osteoblast cell line hFOB1.19. Quantitative (q)RT-PCR analysis revealed high expression of C2dat1 in all three cell lines, and higher expression was detected in OS-732 cells compared with hFOB1.19 and MG63 cells (Fig. 1). Therefore, OS-732 cells were selected to further investigate the function of C2dat1 in osteosarcoma cells.

C2dat1 Suppression Reduced Cell Viability, Migration, and Invasion and Increased Apoptosis in OS-732 Cells

To investigate the function of C2dat1 in osteosarcoma cells, C2dat1 expression was suppressed in OS-732 cells by transfection with sh-C2dat1 constructs (#1 and #2) or sh-NC as a NC. C2dat1 expression was analyzed by qRT-PCR. Compared with the untransfected and sh-NC-transfected controls, C2dat1 expression was significantly inhibited by transfection with sh-C2dat1 #1 and #2 ($p<0.05$ or $p<0.001$) (Fig. 2A). C2dat1 #2 mediated the most effective suppression of C2dat1 expression and was selected to use in further investigations.

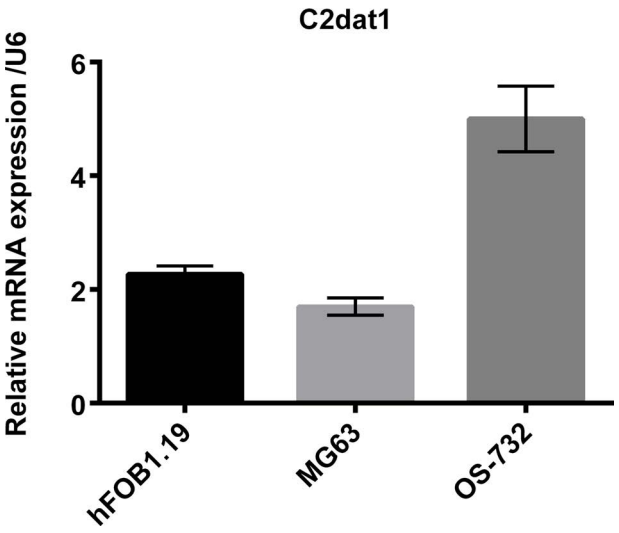


Figure 1. Expression of C2dat1 in osteosarcoma cell lines. Quantitative real-time (qRT)-PCR analysis of C2dat1 expression in the human osteosarcoma cell lines MG63 and OS-732 and the human osteoblast cell line hFOB1.19.

OS-732 cells were then transfected with sh-C2dat1 #2 or sh-NC to investigate the functions of C2dat1 on osteosarcoma cell viability and apoptosis. Trypan blue exclusion revealed that cell viability was significantly reduced in cells transfected with sh-C2dat1 compared to the controls ($p<0.01$) (Fig. 2B). The effects of C2dat1 suppression on the migration and invasion abilities of osteosarcoma cells were investigated using modified Transwell assays. Compared with the controls, sh-C2dat1 suppression suggested that the migration and invasion abilities were decreased in the OS-732 cells (both $p<0.05$) (Fig. 2C and D). Annexin V/PI staining showed that cell apoptosis was significantly increased ($p<0.005$) (Fig. 2E). Furthermore, Western blot analysis of apoptosis-related proteins demonstrated a downregulated expression of the antiapoptotic protein Bcl-2, whereas expression of the proapoptotic protein Bax was upregulated. Expression of the cleaved (active) forms of caspase 3 and caspase 9 was also upregulated by C2dat1 suppression (Fig. 2F).

Identification of miR-34a-5p as a Target of C2dat1

miR-34a-5p has been implicated in the initiation and progression of numerous cancers[15]. OS-732 cells were

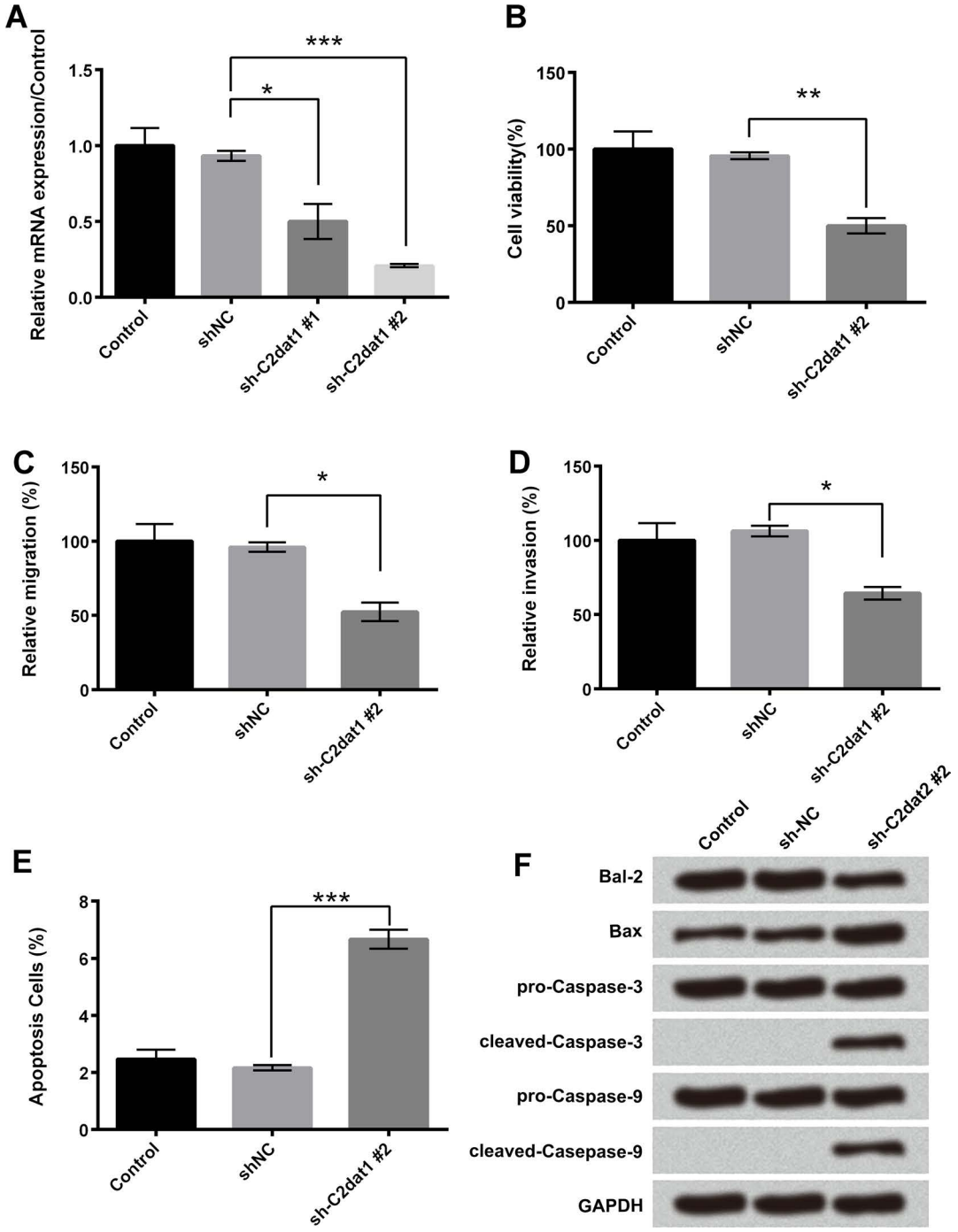


Figure 2. Effects of C2dat1 suppression on cell viability, migration, invasion, and apoptosis. To investigate the function of C2dat1 in osteosarcoma cells, C2dat1 expression was suppressed in OS-732 cells by transfection with sh-C2dat1 constructs (#1 and #2) or sh-NC as a negative control. (A) Quantitative PCR analysis of C2dat1 mRNA expression. (B) Evaluation of cell viability by trypan blue exclusion. (C) Transwell assay of migration. (D) Transwell assay of invasion. (E) Evaluation of apoptosis by flow cytometric analysis of annexin V/propidium iodide (PI) staining. (F) Western blot analysis of apoptosis-related proteins. *$p<0.05$, **$p<0.01$, ***$p<0.001$. Control, untransfected cells; NC, negative control.

then transfected with sh-C2dat1 #2 or sh-NC to investigate the association between C2dat1 and miR-34a-5p expression. The potential binding sequences between lncRNA C2dat1 and miR-34a-5p by bioinformatics analysis are shown in Figure 3A. qRT-PCR analysis showed that miR-34a-5p expression was significantly higher following silencing of C2dat1 expression in OS-732 cells compared with that detected in the control and sh-NC-transfected cells ($p<0.005$) (Fig. 3B). Reporter constructs containing the wild-type sequence or a mutated version of the putative miR-34a binding site in the C2dat1 sequence were generated to evaluate the role of miR-34a-5p as a target of C2dat1 in luciferase reporter assays. As shown in Figure 3C, the relative luciferase activity was significantly reduced in OS-732 cells transfected with C2dat1-wt compared with those transfected with sh-NC ($p<0.01$), whereas transfection with C2dat1-mt had no effect on luciferase activity.

Suppression of C2dat1 Inhibited Cell Viability, Migration, and Invasion and Promoted Apoptosis by Upregulation of miR-34a-5p

The mechanism of miR-34a-5p in the C2dat1 regulatory function of osteosarcoma cells was investigated. OS-732 cells were treated with a miR-34a-5p inhibitor after C2dat1 expression was silenced by transfection with sh-C2dat1 #2. As shown in Figure 4A–C, the cell viability, migration, and invasion were significantly reduced by cells transfected with sh-C2dat1 and was enhanced by cotransfection with the miR-34a-5p inhibitor ($p<0.05$ or $p<0.01$). Furthermore, C2dat1 silencing significantly increased OS-732 cell apoptosis compared with the controls, whereas cells cotransfected with the miR-34a-5p inhibitor significantly ameliorated the effect of increase ($p<0.01$ or $p<0.05$) (Fig. 4D). In accordance with this, Western blot analysis of apoptosis-related protein expression revealed that the downregulation of Bcl-2 and the upregulation of Bax were observed following shRNA-mediated C2dat1 silencing, which were reversed by cotransfection with the miR-34a-5p inhibitor (Fig. 4E).

miR-34a-5p Regulated Sirt1 Expression and Sirt1 Was a Target of miR-34a-5p

miR-34a-5p functions as a tumor suppressor via the Sirt1–p53 signaling pathway. To investigate Sirt1 as a potential target of miR-34a-5p, OS-732 cells were treated with a specific mimic or inhibitor of miR-34a-5p. miR-34a-5p was predicted to reversely bind to the 3′-untranslated region (3′-UTR) of Sirt1 (Fig. 5A). qRT-PCR and Western blot showed that Sirt1 expression was significantly inhibited by the miR-34a-5p mimic at both the mRNA and protein levels, whereas expression was

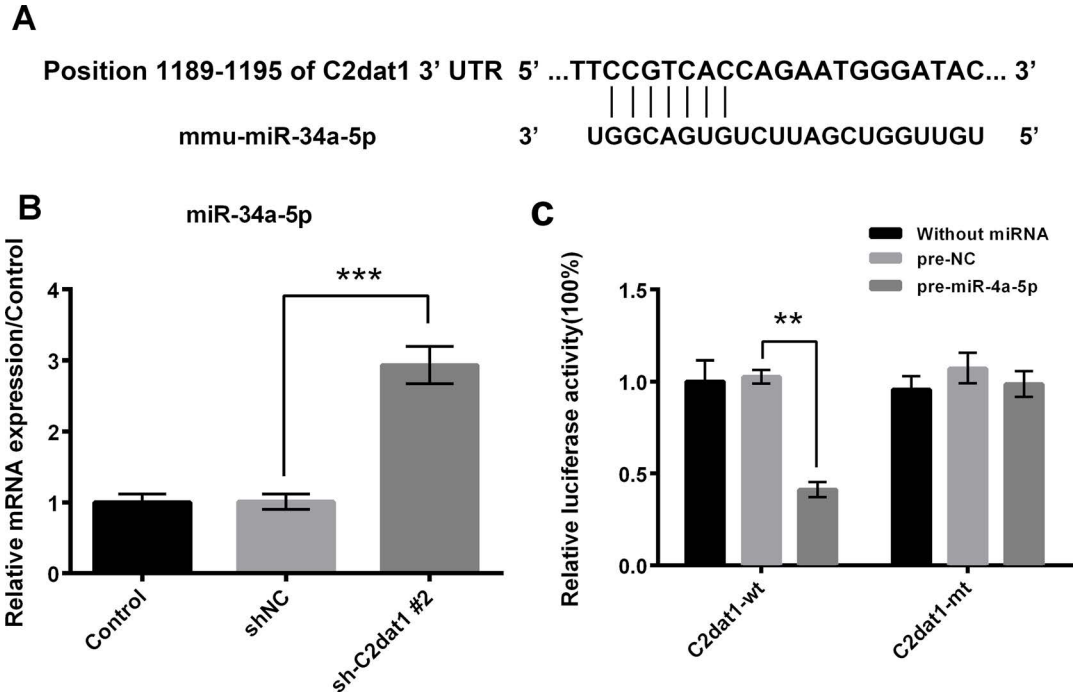


Figure 3. Identification of miR-34a-5p as a target of C2dat1. (A) Bioinformatics analysis was used to predict the potential binding sequences between long noncoding RNA (lncRNA) C2dat1 and miR-34a-5p. (B) qRT-PCR analysis of miR-34a-5p expression in OS-732 cells transfected with sh-C2dat1 #2 or sh-NC. (C) Dual-luciferase reporter assays of OS-732 cells transfected with luciferase reporter constructs containing the wild-type sequence of the putative miR-34a binding site in the C2dat1 sequence (C2dat1-wt) or a mutated version (C2dat1-mt). $^{**}p<0.01$, $^{***}p<0.001$.

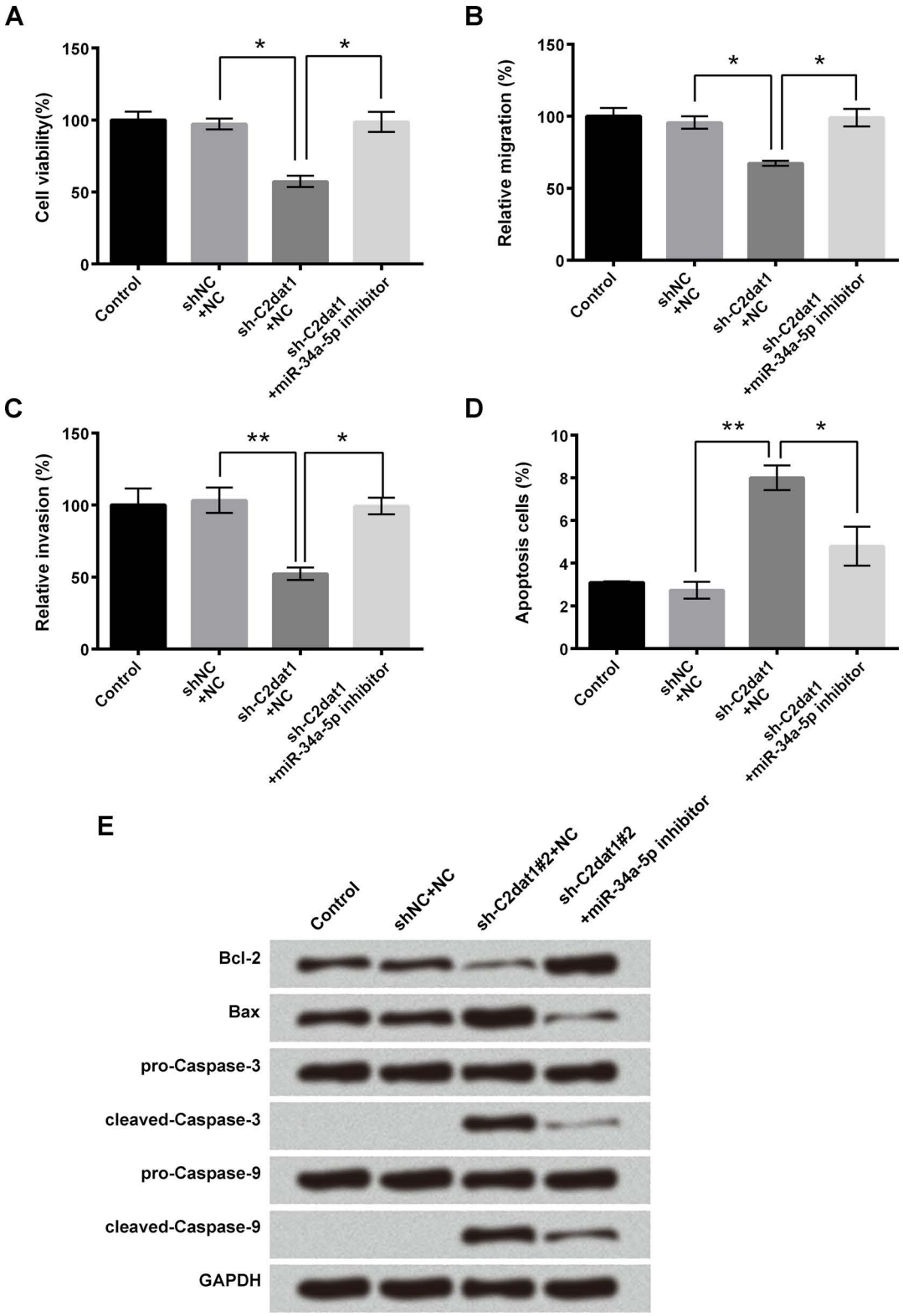


Figure 4. Suppression of C2dat1 inhibits cell viability, migration, and invasion and promotes apoptosis by upregulation of miR-34a-5p. OS-732 cells in which C2dat1 expression was silenced by transfection with sh-C2dat1 #2 were treated with a miR-34a-5p inhibitor. (A) Evaluation of cell viability by trypan blue exclusion. (B) Transwell assay of migration. (C) Transwell assay of invasion. (D) Evaluation of apoptosis by flow cytometric analysis of annexin V/PI staining. (E) Western blot analysis of apoptosis-related proteins. *$p<0.05$, **$p<0.01$.

significantly upregulated by the corresponding inhibitor (both $p<0.05$) (Fig. 5B and D). Furthermore, dual-luciferase activity was significantly inhibited by cells cotransfected with the Sirt1-wt and miR-34a-5p mimic ($p<0.01$), and this effect was absented in cells cotransfected with the Sirt1-mt and miR-34a-5p mimic (Fig. 5C). These observations indicated that miR-34a-5p mediates negative regulation of Sirt1 expression.

miR-34a-5p Overexpression Inhibited Cell Viability, Migration, and Invasion and Promoted Cell Apoptosis in Osteosarcoma Cells by Downregulation of Sirt1

The relationship between the expression of miR-34a-5p and Sirt1 was further investigated by evaluating the effects of miR-34a-5p mimics and Sirt1 overexpression in OS-732 cells. As shown in Figure 6A, the inhibitory effect of the miR-34a-5p mimic on Sirt1 gene expression was abolished by cotransfection with pEX-Sirt1 ($p<0.01$ or $p<0.05$). Western blot analysis revealed similar effects on Sirt1 protein expression (Fig. 6F). Functional studies showed that cell viability, migration, and invasion were increased in OS-732 cells treated with pEX-Sirt1 when miR-34a-5p was overexpressed (all $p<0.05$) (Fig. 6B–D), whereas cell apoptosis was significantly reduced ($p<0.005$) (Fig. 6E). As shown in Figure 6G, Western blot analysis of the expression of apoptosis-related proteins showed that OS-732 cells cotransfected with the miR-34a-5p inhibitor and pEX-Sirt1 upregulated the expression of Bcl-2, whereas Bax and the cleaved forms of caspase 3 and caspase 9 expressions were downregulated.

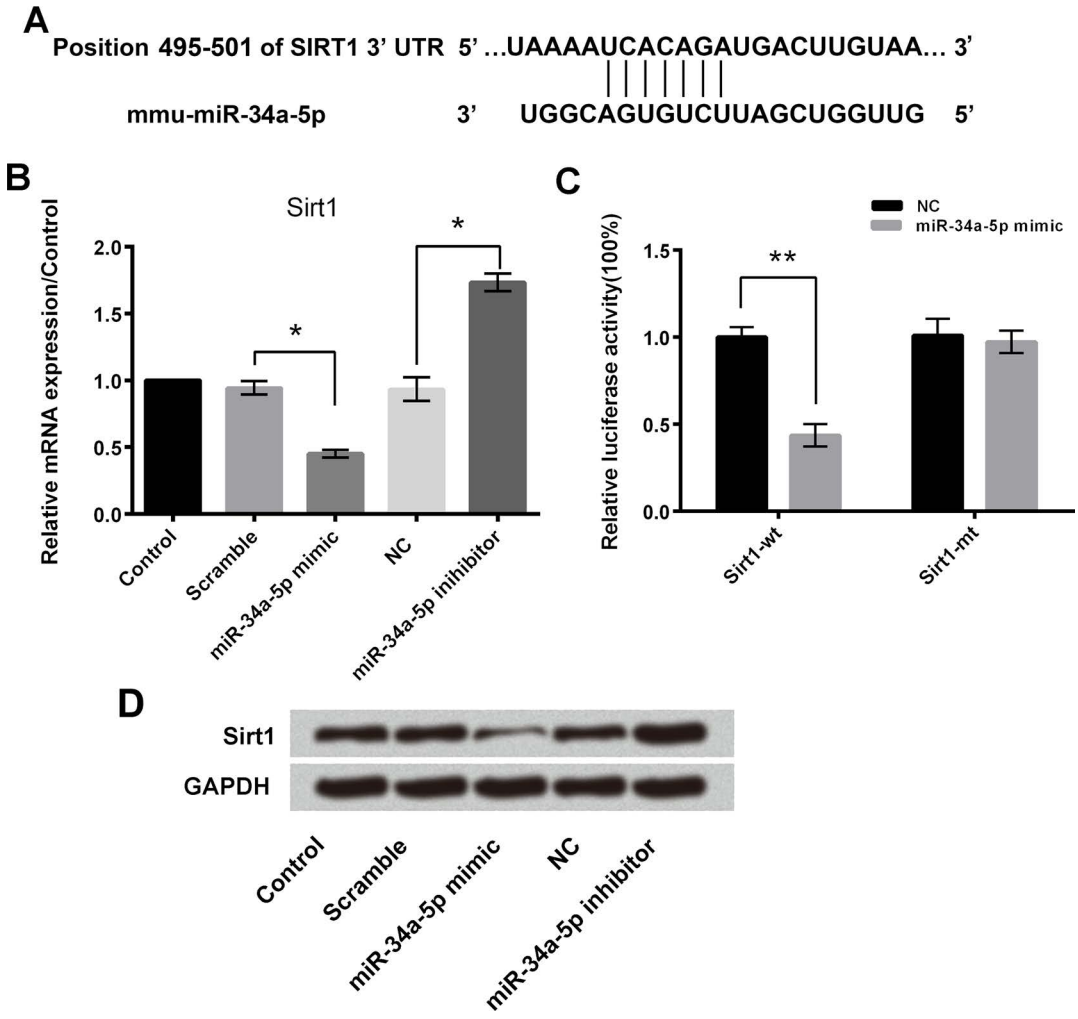
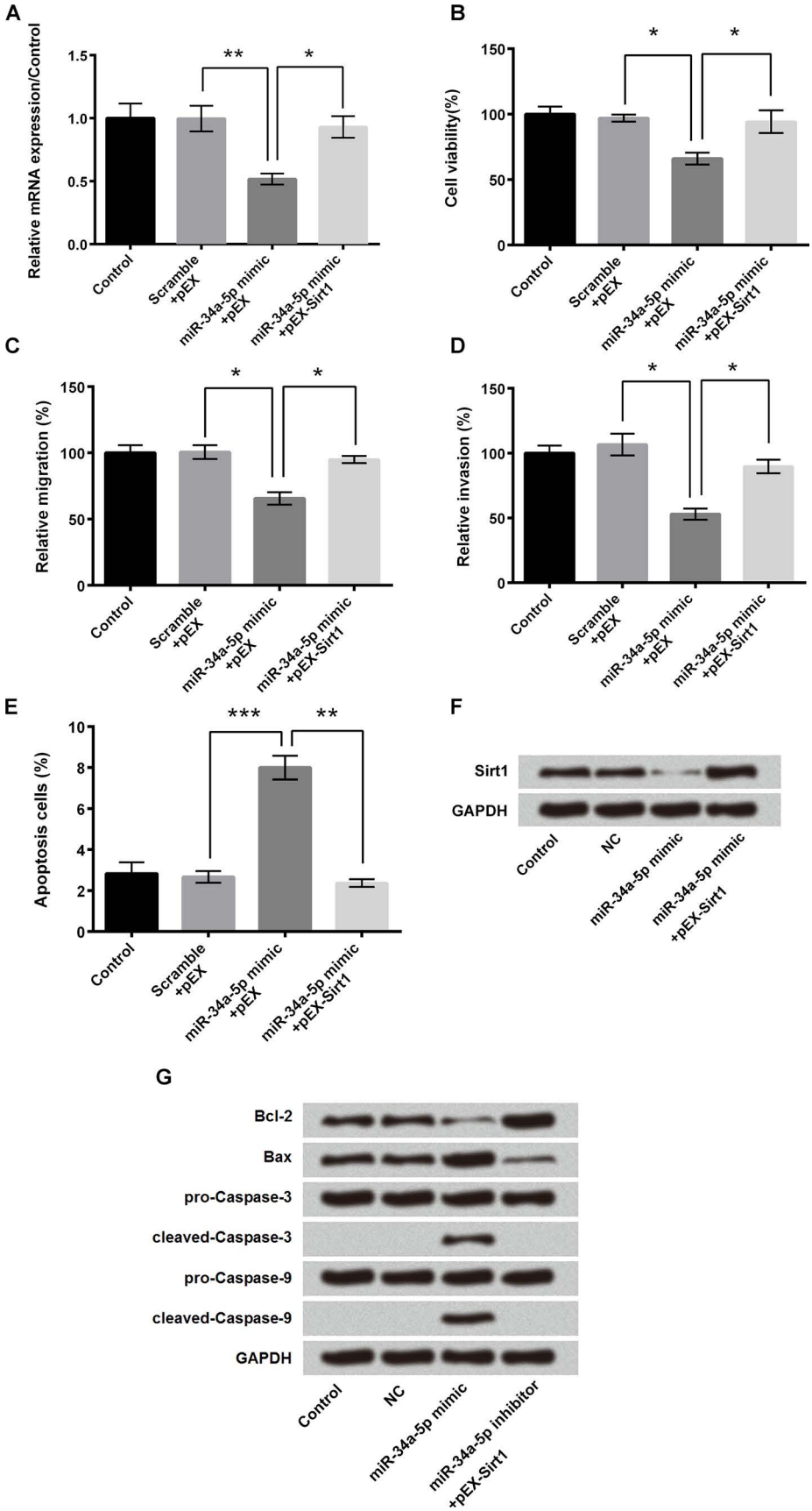


Figure 5. miR-34a-5p regulates Sirt1 expression, and Sirt1 is a target of miR-34a-5p. OS-732 cells were treated with a specific mimic or inhibitor of miR-34a-5p to investigate Sirt1 as a potential target of miR-34a-5p. (A) Bioinformatics analysis was used to predict the potential binding sequences between miR-34a-5p and Sirt1. (B) qRT-PCR analysis of Sirt1 mRNA expression. (C) Dual-luciferase reporter assays of the effects of the miR-34a-5p mimic or an NC on cells transfected with the Sirt1-wt constructs. (D) Western blot analysis of Sirt1 protein expression. *$p<0.05$, **$p<0.01$.


A
Relative mRNA expression/Control
1.5
1.0
0.5
0.0
**
*
Control
Scramble +pEX
miR-34a-5p mimic +pEX
miR-34a-5p mimic +pEX-Sirt1
B
Cell viability(%)
150
100
50
0
*
*
Control
Scramble +pEX
miR-34a-5p mimic +pEX
miR-34a-5p mimic +pEX-Sirt1
C
Relative migration (%)
150
100
50
0
*
*
Control
Scramble +pEX
miR-34a-5p mimic +pEX
miR-34a-5p mimic +pEX-Sirt1
D
Relative invasion (%)
150
100
50
0
*
*
Control
Scramble +pEX
miR-34a-5p mimic +pEX
miR-34a-5p mimic +pEX-Sirt1
E
Apoptosis cells (%)
10
8
6
4
2
0
***
**
Control
Scramble +pEX
miR-34a-5p mimic +pEX
miR-34a-5p mimic +pEX-Sirt1
F
Sirt1
GAPDH
Control
NC
miR-34a-5p mimic
miR-34a-5p mimic +pEX-Sirt1
G
Bcl-2
Bax
pro-Caspase-3
cleaved-Caspase-3
pro-Caspase-9
cleaved-Caspase-9
GAPDH
Control
NC
miR-34a-5p mimic
miR-34a-5p inhibitor +pEX-Sirt1

miR-34a-5p Overexpression Deactivated the p38/ERK/AKT and Wnt/β-Catenin Signaling Pathways by Downregulation of Sirt1

The involvement of the p38/ERK/AKT and Wnt/β-catenin signaling pathways in the mechanism by which miR-34a-5p regulates osteosarcoma cell function was investigated by Western blot analysis (Fig. 7). Overexpression of miR-34a-5p reduced the levels of the phosphorylated forms of p38MAPK, ERK, and AKT. In contrast, the levels of p-p38, p-ERK, and p-AKT were clearly upregulated by the suppression of miR-34a-5p; this effect was partially abrogated by concomitant siRNA-mediated silencing of Sirt1 (Fig. 7A). Similarly, miR-34a-5p overexpression inhibited expression of Wnt3a, Wn5a, and β-catenin. In contrast, expressions of these signaling molecules were markedly upregulated by cells transfected with the miR-34a-5p inhibitor, and these effects were abolished by concomitant siRNA-mediated silencing of Sirt1 (Fig. 7B). These findings indicated that overexpression of miR-34a-5p deactivated the p38/ERK/AKT and Wnt/β-catenin signaling pathways by downregulation of Sirt1, leading to decreased cell viability, migration, and invasion and increased cell apoptosis.

DISCUSSION

Osteosarcoma is a complex malignancy of the bone that is associated with a poor prognosis in patients, and metastases are already present at the time of diagnosis. Therefore, an improved understanding of the mechanism of this malignancy is urgently required to identify biomarkers for effective monitoring and diagnosis as well as to aid in the development of new and effective therapies. In this study, we investigated the effects of lncRNA C2dat1 on osteosarcoma cell function and the underlying mechanisms. Compared with the hFOB1.19 human osteoblast cell line, C2dat1 proved to be expressed at high levels in the MG63 and OS-732 osteosarcoma cell lines, while the latter was particularly highly expressed. Suppression of C2dat1 in OS-732 cells significantly inhibited cell viability, migration, and invasion and promoted apoptosis. Further studies confirmed that miR-34a-5p was a target of C2dat1, and the effects of C2dat1 on cell viability, migration, invasion, and apoptosis were mediated by upregulation of miR-34a-5p. In addition, miR-34a-5p negatively regulated the expression of Sirt1, and Sirt1 overexpression promoted cell proliferation, migration, and invasion by activation of the p38/ERK/AKT and Wnt/β-catenin signaling pathways. Thus, the present study provides new insight into the treatment of osteosarcoma.

lncRNAs play important roles in the control of cancer cell functions[16]. Furthermore, lncRNAs have been reported to be involved in the pathogenesis of osteosarcoma[17–20]. In the current study, we showed that C2dat1 was highly upregulated in OS-732 cells compared with the levels detected in human osteoblast cells. Similarly, the lncRNA TUG1 was upregulated and positively regulated cell proliferation in osteosarcoma cell lines[17]. A recent meta-analysis showed that altered lncRNA expression could predict poor outcome in osteosarcoma patients[21].

Further investigations of the role of C2dat1 in osteosarcoma indicated that sh-RNA-mediated suppression resulted in the reduction of OS-732 cell viability, migration, and invasion. Moreover, cell apoptosis was promoted with concomitantly downregulated expression of Bcl-2 and upregulated expression of Bax and the cleaved (active) forms of caspase 3 and caspase 9. These findings indicated that C2dat1 might function as an oncogene in osteosarcoma. This is consistent with other reports on the effects of lncRNAs, such as TUG1[22], SPRY4-IT1[23], and ZEB1-AS1[24].

Functional interactions between lncRNA and miRNA molecules have been well established[8]. Dysregulation of miR-34a, which was identified as part of the p53 tumor suppressor network, has been implicated in the development of some forms of cancer[25,26] and shown to promote chemoresistance in osteosarcoma cell lines[27]. Furthermore, miR-34a inhibits proliferation and metastasis of osteosarcoma cells[28]. Our investigations revealed that C2dat1 targeted miR-34a-5p directly, and the effects of C2dat1 on OS-732 cell function were mediated by upregulation of miR-34a-5p. miR-34a functions as a tumor suppressor via the Sirt1–p53 signaling pathway[29]. In accordance with this evidence, we demonstrated that miR-34a-5p regulated Sirt1 expression by directly targeting a region of the *Sirt1* gene. Investigations of the importance of the interaction between miR-34a-5p and Sirt1 on the biological functions of osteosarcoma cells using miR-34a-5p mimics and Sirt1 overexpression in OS-732 cells indicated that miR-34a-5p reduced cell viability, migration, and invasion and promoted cell apoptosis of osteosarcoma cells by downregulation of Sirt1 expression. miR-34a-5p was found to mediate negative regulation of Sirt1 by the activation of the p38/ERK/AKT

FACING PAGE

Figure 6. Sirt1 regulates cell viability, migration, invasion, and apoptosis of osteosarcoma cells. OS-732 cells were transfected with miR-34a-5p mimics and the pEX-Sirt1 overexpression vector. (A) qRT-PCR analysis of Sirt1 expression. (B) Evaluation of cell viability by trypan blue exclusion. (C) Transwell assay of migration. (D) Transwell assay of invasion. (E) Evaluation of apoptosis by flow cytometric analysis of annexin V/PI staining. (F) Western blot analysis of Sirt1 protein expression. (G) Western blot analysis of apoptosis-related proteins. $*p<0.05$, $**p<0.01$, $***p<0.001$.

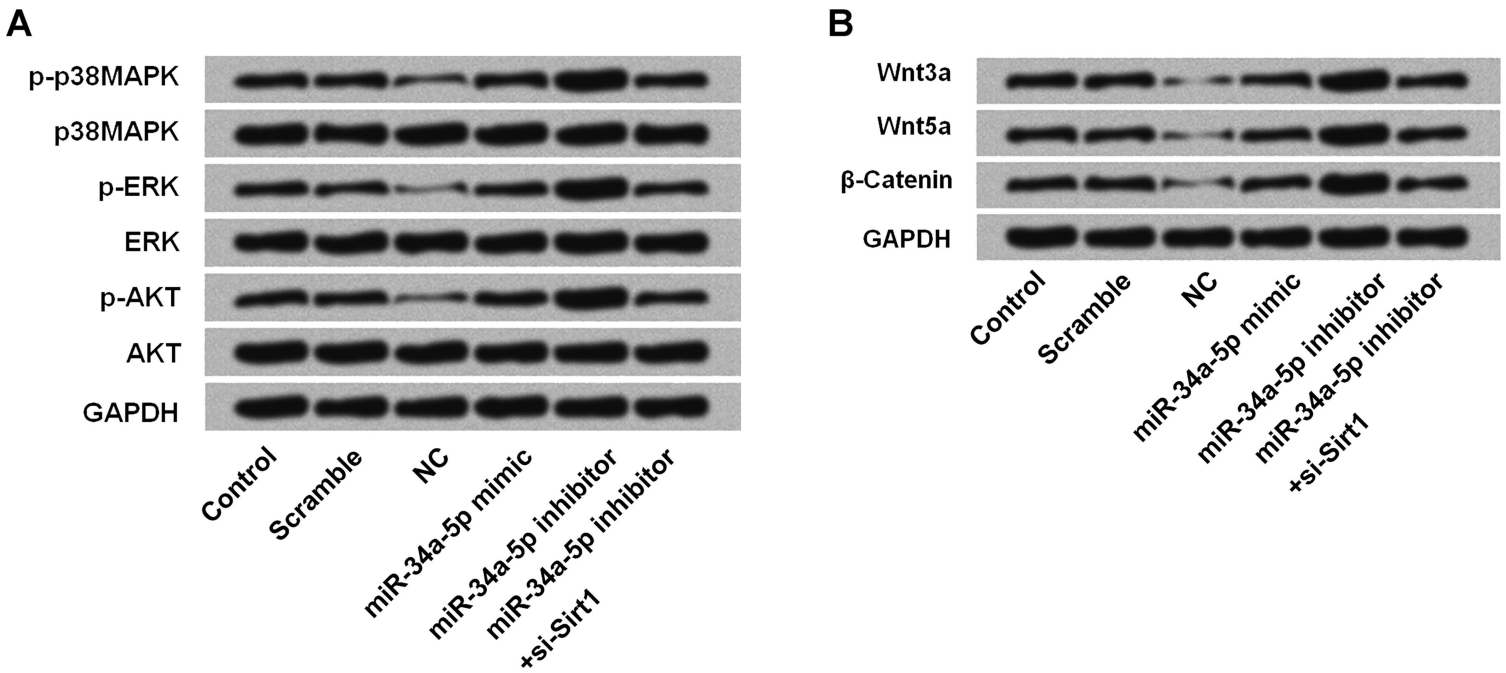


Figure 7. miR-34a-5p overexpression deactivates p38/ERK/AKT and Wnt/β-catenin signaling pathways by downregulation of Sirt1. Western blot analysis of the involvement of the (A) p38/ERK/AKT and (B) Wnt/β-catenin signaling pathways in the mechanism by which miR-34a-5p regulates osteosarcoma cell function.

and Wnt/β-catenin signaling pathways. Similarly, miR-133b targets Sirt1 directly to inhibit osteosarcoma cell proliferation and invasion via a mechanism that involves the Wnt/β-catenin pathway[30]. It has been reported that Sirt1 is significantly upregulated in primary osteosarcoma tumors and promotes metastasis in human osteosarcoma cells[31]. Thus, the interaction between miR-34a-5p and Sirt1 is implicated as an important therapeutic and diagnostic target in osteosarcoma. The study by Zhao et al. revealed that a bioengineered form of a miR-34 prodrug acts synergistically with doxorubicin to inhibit the growth of osteosarcoma tumors in a xenografted mouse model by downregulating expression of oncogenes including Sirt1[32].

In this study, we demonstrated that C2dat1 suppression reduced osteosarcoma cell viability, migration, and invasion and enhanced cell apoptosis. This process was mediated by the upregulation of miR-34a-5p, which mediated negative regulation of Sirt1 by activation of the p38/ERK/AKT and Wnt/β-catenin signaling pathways. Thus, C2dat1 and the miR34a-5p/Sirt1 axis are implicated as therapeutic targets in osteosarcoma.

ACKNOWLEDGMENTS: The authors declare no conflicts of interest.

REFERENCES

1. Geller DS, Gorlick R. Osteosarcoma: A review of diagnosis, management, and treatment strategies. Clin Adv Hematol Oncol. 2010;8(10):705–18.
2. Moore DD, Luu HH. Osteosarcoma. Cancer Treat Res. 2014;162:65–92.
3. Heare T, Hensley MA, Dell'Orfano S. Bone tumors: Osteosarcoma and Ewing's sarcoma. Curr Opin Pediatr. 2009; 21(3):365–72.
4. 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(4):523–32.
5. Longhi A, Errani C, De PM, Mercuri M, Bacci G. Primary bone osteosarcoma in the pediatric age: State of the art. Cancer Treat Rev. 2006;32(6):423–36.
6. Djebali S, Davis CA, Merkel A, Dobin A, Lassmann T, Mortazavi A, Tanzer A, Lagarde J, Lin W, Schlesinger F. Landscape of transcription in human cells. Nature 2012; 489(7414):101–8.
7. Geisler S, Coller J. RNA in unexpected places: Long non-coding RNA functions in diverse cellular contexts. Nat Rev Mol Cell Biol. 2013;14(11):699–712.
8. Yoon JH, Abdelmohsen K, Gorospe M. Functional interactions among microRNAs and long noncoding RNAs. Semin Cell Dev Biol. 2014;34(4):9–14.
9. Ponting CP, Oliver PL, Reik W. Evolution and functions of long noncoding RNAs. Cell 2009;136(4):629–41.
10. Fatica A, Bozzoni I. Long non-coding RNAs: New players in cell differentiation and development. Nat Rev Genet. 2014;15(1):7–21.
11. Huarte M. The emerging role of lncRNAs in cancer. Nat Med. 2015;21(11):1253–61.
12. Bhat SA, Ahmad SM, Mumtaz PT, Malik AA, Dar MA, Urwat U, Shah RA, Ganai NA. Long non-coding RNAs: Mechanism of action and functional utility. Noncoding RNA Res. 2016;1(1):43–50.
13. Sun J, Wang X, Fu C, Wang X, Zou J, Hua H, Bi Z. Long noncoding RNA FGFR3-AS1 promotes osteosarcoma growth through regulating its natural antisense transcript FGFR3. Mol Biol Rep. 2016;43(5):427–36.
14. Xu Q, Deng F, Xing Z, Wu Z, Cen B, Xu S, Zhao Z, Nepomuceno R, Bhuiyan MIH, Sun D. Long non-coding RNA C2dat1 regulates CaMKIIδ expression to promote neuronal survival through the NF-κB signaling pathway following cerebral ischemia. Cell Death Dis. 2016;7(3): e2173.
15. Ghandadi M, Sahebkar A. MicroRNA-34a and its target genes: Key factors in cancer multidrug resistance. Curr Pharm Des. 2016;22(7):933–9.
16. Prensner JR, Chinnaiyan AM. The emergence of lncRNAs in cancer biology. Cancer Discov. 2011;1(5):391–407.
17. Yunbo F, Xiaopo L, Xiaoli L, Guolong C, Pei Z, Faming T. LncRNA TUG1 is upregulated and promotes cell proliferation in osteosarcoma. Open Med. (Wars) 2016;11(1): 163–7.
18. Li Z, Yu X, Shen J. Long non-coding RNAs: Emerging players in osteosarcoma. Tumour Biol. 2016;37(3):2811–6.
19. Wei L, Peng X, Ruan W. Overexpression of lncRNA UCA1 promotes osteosarcoma progression and correlates with poor prognosis. J Bone Oncol. 2016;5(2):80–5.
20. Sun XH, Yang LB, Geng XL, Wang R, Zhang ZC. Increased expression of lncRNA HULC indicates a poor prognosis and promotes cell metastasis in osteosarcoma. Int J Clin Exp Pathol. 2015;8(3):2994–3000.
21. Yang Y, Wang S, Teng L. Altered long non-coding RNAs predict worse outcome in osteosarcoma patients: Evidence from a meta-analysis. Oncotarget 2017;8(21):35234–43.
22. Wang Y, Yang T, Zhang Z, Lu M, Zhao W, Zeng X, Zhang W. Long non-coding RNA TUG1 promotes migration and invasion by acting as a ceRNA of miR-335-5p in osteosarcoma cells. Cancer Sci. 2017;108(5):859–67.
23. Xu J, Ding R, Xu Y. Effects of long non-coding RNA SPRY4-IT1 on osteosarcoma cell biological behavior. Am J Transl Res. 2016;8(12):5330–7.
24. Liu C, Lin J. Long noncoding RNA ZEB1-AS1 acts as an oncogene in osteosarcoma by epigenetically activating ZEB1. Am J Transl Res. 2016;8(10):4095–105.
25. He L, He X, Lim LP, De SE, Xuan Z, Liang Y, Xue W, Zender L, Magnus J, Ridzon D. A microRNA component of the p53 tumour suppressor network. Nature 2007;447 (7148):1130–4.
26. Mraz M, Malinova K, Kotaskova J, Pavlova S, Tichy B, Malcikova J, Stano KK, Smardova J, Brychtova Y, Doubek M. miR-34a, miR-29c and miR-17-5p are downregulated in CLL patients with TP53 abnormalities. Leukemia 2009; 23(6):1159–63.
27. Pu Y, Zhao F, Wang H, Cai S. MiR-34a-5p promotes multichemoresistance of osteosarcoma through down-regulation of the DLL1 gene. Sci Rep. 2017;10(7):44218.
28. Yan K, Gao J, Yang T, Ma Q, Qiu X, Fan Q, Ma B. MicroRNA-34a inhibits the proliferation and metastasis of osteosarcoma cells both in vitro and in vivo. PLoS One 2012;7(3):e33778.
29. Yamakuchi M, Ferlito M, Lowenstein CJ. miR-34a repression of SIRT1 regulates apoptosis. Proc Natl Acad of Sci USA 2008;105(105):13421–6.
30. Ying S, Jianjun H, Xue Y, Shuwei Y, Liyuan Z, Jie W, Lixian C. MicroRNA-133b inhibits cell proliferation and

invasion in osteosarcoma by targeting to Sirt1. Oncol Res. 2017;25(9):1421–30.
31. Zhang N, Xie T, Xian M, Wang YJ, Li HY, Ying MD, Ye ZM. SIRT1 promotes metastasis of human osteosarcoma cells. Oncotarget 2016;7(48):79654–69.
32. Zhao Y, Tu MJ, Yu YF, Wang WP, Chen QX, Qiu JX, Yu AX, Yu AM. Combination therapy with bioengineered miR-34a prodrug and doxorubicin synergistically suppresses osteosarcoma growth. Biochem Pharmacol. 2015; 98(4):602–13.