



# Knockdown of Long Non-Coding RNA NEAT1 Inhibits Proliferation and Invasion and Induces Apoptosis of Osteosarcoma by Inhibiting miR-194 Expression

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**Purpose:** Long non-coding RNA (lncRNA) nuclear paraspeckle assembly transcript 1 (NEAT1) has been implicated as an oncogene in the development and progression of osteosarcoma. This study aims to explore the mechanism of NEAT1 in osteosarcoma.

**Materials and Methods:** Expressions of NEAT1 and miR-194 in osteosarcoma tissues and cells were detected by quantitative real-time PCR. The effects of NEAT1 knockdown or miR-194 overexpression on cell proliferation, invasion, and apoptosis were determined by 3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide (MTT) assay, transwell invasive assay, and flow cytometry analysis, respectively. Luciferase reporter assay was performed to observe the possible interaction between NEAT1 and miR-194.

**Results:** NEAT1 was upregulated and miR-194 was downregulated in osteosarcoma tissues and cells. Knockdown of NEAT1 or overexpression of miR-194 suppressed proliferation and invasion and induced apoptosis of osteosarcoma cells *in vitro*. Luciferase reporter assay validated that NEAT1 could interact with miR-194 and negatively modulated its expression. Furthermore, inhibition of miR-194 reversed the suppression of proliferation and invasion and the promotion of apoptosis induced by NEAT1 depletion in osteosarcoma cells.

**Conclusion:** Knockdown of NEAT1 suppressed proliferation and invasion and induced apoptosis in osteosarcoma cells by inhibiting miR-194 expression.

**Key Words:** lncRNA, tumorigenesis, osteosarcoma, miR-194

## INTRODUCTION

Osteosarcoma is the most widespread malignant bone tumor and a major cause of cancer death among adolescents and young adults, with a incidence of approximately 10 to 26 new cases per million worldwide annually.<sup>1,2</sup> Due to high aggressiveness and early systemic metastasis, the 5-year overall survival rate of those diagnosed with metastatic osteosarcoma is

only about 20–30%.<sup>1,3</sup> Although the combination of surgery resection and neoadjuvant chemotherapy has become a standard treatment strategy for osteosarcoma patients for more than 30 years, patients with recurrent or metastatic osteosarcoma still have an extremely poor outcome.<sup>4</sup> Till now, the molecular mechanisms involved in osteosarcoma origination, metastasis, and chemoresistance has remained largely unknown. Therefore, new prognostic biomarkers and treatment targets are expected to be identified to improve therapeutic efficacy for osteosarcoma patients.

MicroRNAs (miRNAs) are small non-coding RNAs with 18 to 25 nucleotides in length, which lead to translation inhibition or target mRNA degradation by interacting with the 3'-UTR or coding region of mRNA targets.<sup>5</sup> Multiple miRNAs, such as miR-199a-3p, miR-125b, and miR-29, have been shown to be implicated in cell proliferation and migration and patient prognosis in osteosarcoma.<sup>6-8</sup> miR-194 has been found to prevent metastasis and progression of various tumors, such as liver can-

**Received:** April 27, 2017 **Revised:** July 26, 2017

**Accepted:** July 26, 2017

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•The authors have no financial conflicts of interest.

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cer,<sup>9</sup> endometrial cancer,<sup>10</sup> and gastric cancer.<sup>11</sup> Moreover, a previous study showed that miR-194 partially inhibited the proliferation, migration, and invasion of osteosarcoma cells *in vitro*, as well as tumor growth and pulmonary metastasis of osteosarcoma cells *in vivo*.<sup>12</sup> Moreover, there were statistically significant relationships between miR-194 expression and clinical stage, distant metastasis, and patient mortality. Downregulation of miR-194 has been shown to be associated with poor prognosis in osteosarcoma patients.<sup>12</sup> However, how miR-194 is regulated in osteosarcoma is still unclear.

Long non-coding RNAs (lncRNAs) are a class of RNAs with length surpassing 200 nucleotides with little or no protein-coding ability.<sup>13</sup> lncRNAs are often dysregulated and are involved in the occurrence and development of human cancers,<sup>14</sup> including osteosarcoma: for instance, lncRNA urothelial carcinoma associated 1 contributed to the initiation and progression of osteosarcoma.<sup>15</sup> lncRNA tumor suppressor candidate 7 hindered cell proliferation and served as a tumor suppressor in osteosarcoma.<sup>16</sup> lncRNA taurine up-regulated gene 1 (TUG1) was overexpressed and improved cell proliferation in osteosarcoma.<sup>17</sup> lncRNA modified frailty index 2 knockdown suppressed cell proliferation, migration, and invasion, and induced cell apoptosis in osteosarcoma.<sup>18</sup> Nuclear paraspeckle assembly transcript 1 (NEAT1) has been identified as a nuclear-restricted lncRNA and could act as an oncogene in osteosarcoma progression.<sup>19,20</sup> However, the molecule mechanism through which NEAT1 exerts its role in osteosarcoma progression is still undefined. In the present study, we aimed to explore the function and molecule mechanism of lncRNA NEAT1 in osteosarcoma.

## MATERIALS AND METHODS

### Tissue specimens and cell culture

Fifteen osteosarcoma patient tissue samples and fifteen normal tissue samples were obtained from Zhoukou Central Hospital. This study was approved by the local Ethic Review Committees.

Human osteosarcoma cell lines (MG63 and U2OS) and the human normal osteoblastic cell line hFOB 1.19 were obtained from the American Type Culture Collection and cultured at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. All cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (Invitrogen).

### Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from cultured cells using TRIzol (Invitrogen). The expression levels of NEAT1 and miR-194 were evaluated using the SYBR-Green PCR Master Mix Kit (Takala, Dalian, China) and an ABI7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The primer sequences for  $\beta$ -actin were 5'-TGA GAG GGA AAT CGT GCG TGA C-3'

(forward primer) and 5'-AAG AAG GAA GGC TGG AAA AGA G-3' (reverse primer). The primer sequences for NEAT1 were 5'-CTT CCT CCC TTT AAC TTA TCC ATT CAC-3' (forward primer) and 5'-CTC TTC CTC CAC CAT TAC CAA CAA TAC-3' (reverse primer). The primer sequences for U6 were 5'-CTC GCT TCG GCA CA-3' (forward primer) and 5'-AAC GCT TCA CGA ATT TGC GT-3' (reverse primer). The primer sequences for miR-194 were 5'-ACA GCA ACT CCA TGT GG-3' (forward primer) and 5'-GAA CAT GTC TGC GTA TCT C-3' (reverse primer).  $\beta$ -actin and U6 were used as internal reference for NEAT1 and miR-194, respectively. The relative expression levels of NEAT1 and miR-194 were calculated and normalized using the 2<sup>- $\Delta\Delta$ Ct</sup> method.<sup>21</sup>

### Transfection

The scrambled control miR (miR-con), miR-194 mimics (UGUA ACAGCAACUCCAUGUGGA), scrambled control anti-miR (anti-miR-con), and anti-miR-194 (UCCACAUGGAGUUGCU GUUACA) were synthesized by Genepharma (Shanghai, China). NEAT1 (GenBank #EF177379.1) was amplified from the cDNA of MG63 and U2OS cells and cloned into the pcDNA3.1 plasmid, which was named pcDNA-NEAT1. The siRNA sequence targeting NEAT1 was 5'-GUGAGAAGUUGCUUAGAAACUU UCC-3' (si-NEAT1). si-NEAT1 and negative control siRNA (si-con) were obtained from GenePharma. Cells were transfected with plasmid or nucleotide sequences using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

### MTT assay

Transfected MG63 and U2OS cells (4×10<sup>3</sup> cells per well) were seeded in 96-well plates. After cell incubation for 24, 48, 72, and 96 h, 10  $\mu$ L of MTT (Sigma, St. Louis, MO, USA) was added to each well for another 4 h. Then, the cultural supernatant was discarded, and cells were treated with 150  $\mu$ L of dimethyl sulphoxide to resolve formazan crystals. Absorbance values at 490 nm were determined by a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

### Cell invasion assay

The invasion ability of MG63 and U2OS cells was determined by Transwell invasion assay (Becton Dickinson, Bedford, MA, USA). Cells were planted on the top side of the membrane pre-coated with 10  $\mu$ g/mL of Matrigel and incubated for 24 h. Then, the invaded cells in the lower membrane were fixed and stained with 0.5% crystal violet solution, and counted under a microscope (Olympus, Tokyo, Japan).

### Flow cytometry

Cell apoptosis was evaluated using Annexin V/fluorescein isothiocyanate (FITC) and propidium iodide (PI) apoptosis detection kits (Becton Dickinson, Franklin Lakes, NJ, USA). Briefly, MG63 and U2OS cells were resuspended and stained with 5  $\mu$ L of Annexin V/FITC and 5  $\mu$ L of PI (Sigma) for 15 min at room

temperature in the dark. Flow cytometry (Becton Dickinson) was used to detect apoptosis of MG63 and U2OS cells.

**Luciferase reporter assays**

MG63 and U2OS cells ( $5 \times 10^4$  cells per well) were cultured in a 24-well plate and co-transfected with the luciferase reporter plasmids pGL3 (Promega, Madison, WI, USA) containing wild-type or mutant NEAT1 putative sites at miR-194 binding domain (Wt-NEAT1 or Mut-NEAT1) and miR-con or miR-194. The luciferase activities were measured using a dual-luciferase reporter assay system (Promega) at 48 h post-transfection.

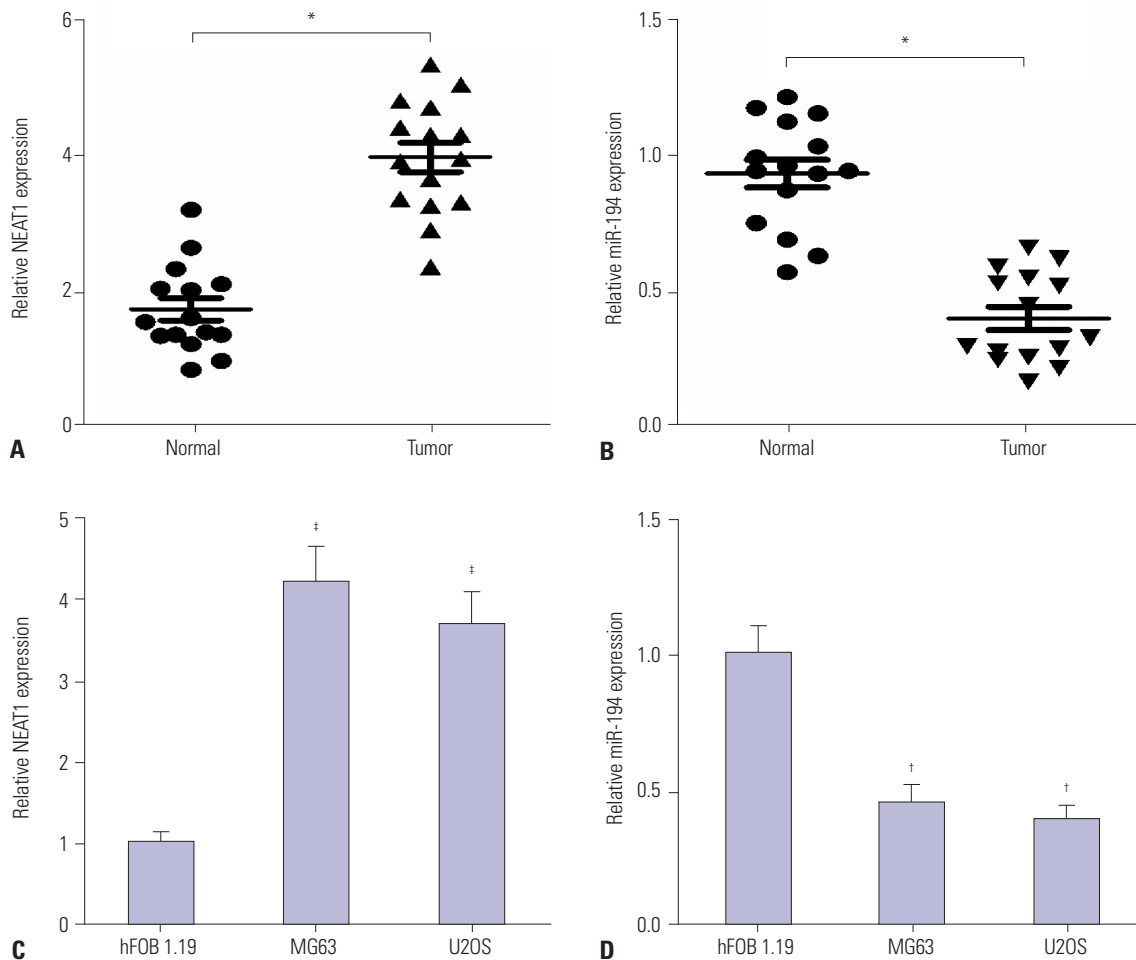
**Statistical analysis**

All data are presented as means $\pm$ SD and analyzed using SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). The significant differences among groups were calculated with Student's t-test or one-way ANOVA.  $p < 0.05$  was considered statistically significant.

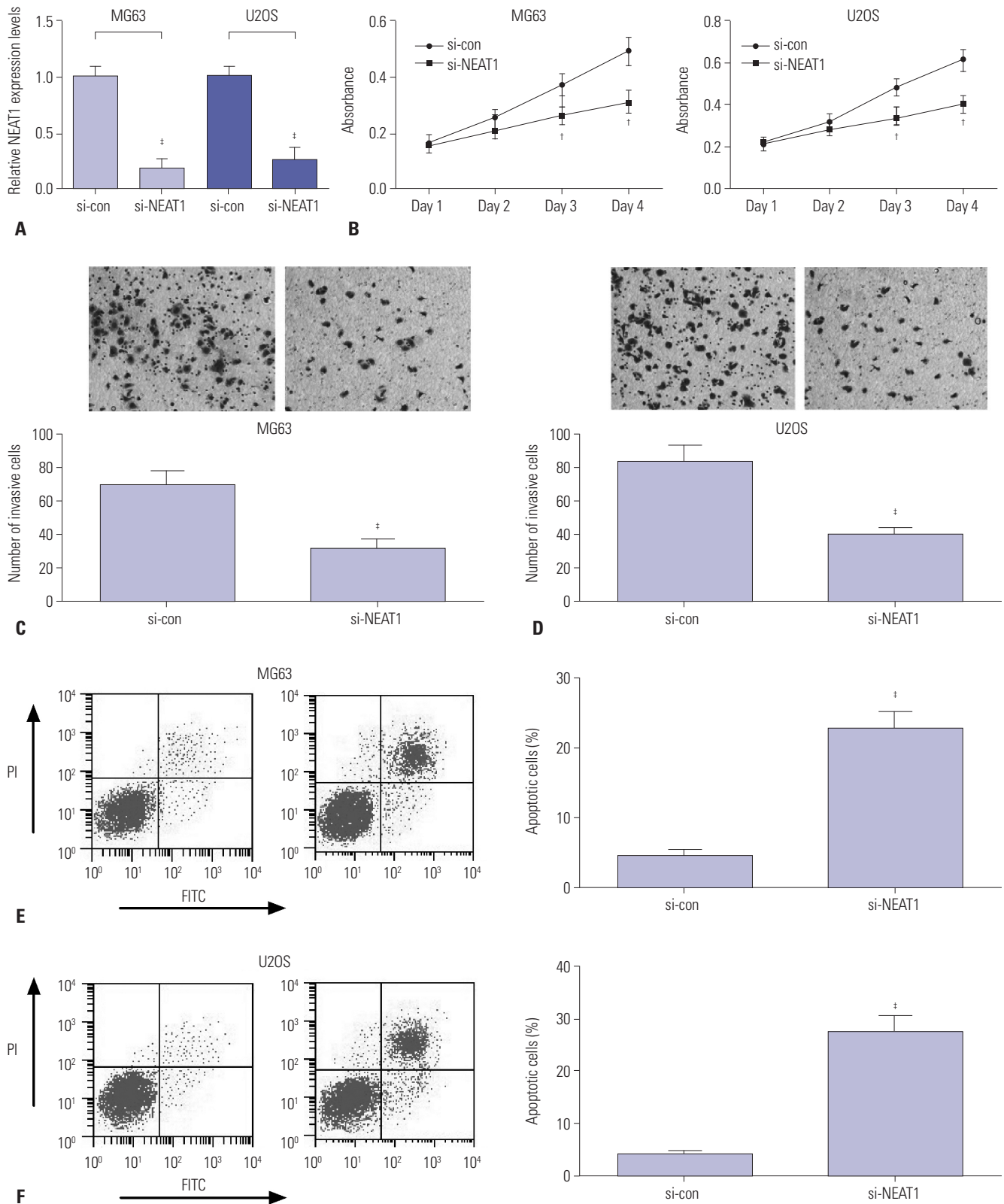
**RESULTS**

**NEAT1 is upregulated and miR-194 is downregulated in osteosarcoma tissues and cells**

Real-time PCR assay was performed to detect lncRNA NEAT1 and miR-194 expression in osteosarcoma tissue samples ( $n=15$ ) and normal tissue samples ( $n=15$ ). The level of NEAT1 was markedly increased and the expression of miR-125a was significantly reduced in osteosarcoma tumor tissues, compared with normal tissues (Fig. 1A and B). Moreover, the levels of NEAT1 and miR-194 were further detected in osteosarcoma cell lines MG63 and U2OS or the human normal osteoblastic cell line hFOB 1.19. The results indicated that NEAT1 expression was significantly elevated and miR-194 level was markedly decreased in MG63 and U2OS cells, compared with that in hFOB 1.19 cells (Fig. 1C and D). These results suggested that dysregulated expression of NEAT1 and miR-194 may be involved in the development of osteosarcoma.



**Fig. 1.** Expression of NEAT1 and miR-194 in osteosarcoma tissues and cells. (A and B) The expression levels of NEAT1 and miR-194 in osteosarcoma tissue samples ( $n=15$ ) and normal tissue samples ( $n=15$ ) by qRT-PCR analysis. (C and D) qRT-PCR analysis of NEAT1 and miR-194 in MG63 and U2OS cells. \* $p < 0.05$ , † $p < 0.01$ , ‡ $p < 0.001$ . NEAT1, nuclear paraspeckle assembly transcript 1; qRT-PCR, quantitative real-time PCR.



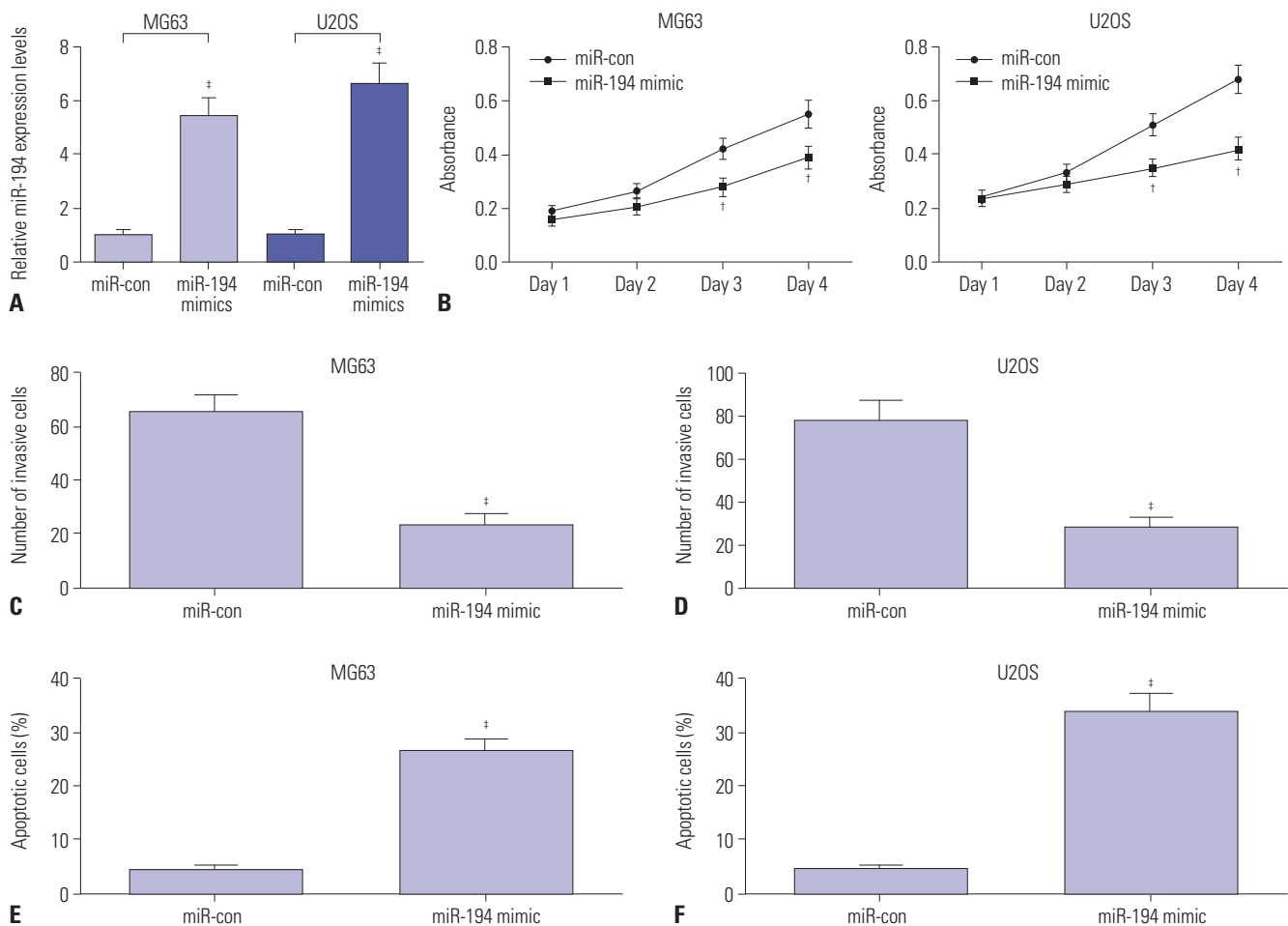
**Fig. 2.** Knockdown of NEAT1 inhibits proliferation and invasion and promotes apoptosis of MG63 and U2OS cells. MG63 and U2OS cells were transfected with si-NEAT1 or si-con. (A) qRT-PCR analysis was performed to detect NEAT1 levels in MG63 and U2OS cells. (B) MTT assay was performed to detect viability of MG63 and U2OS cells at days 1, 2, 3, and 4 after transfection. (C and D) Transwell invasive assay was carried out to assess invasive ability of MG63 and U2OS cells 24 h post transfection. (E and F) Flow cytometry analysis was conducted to determine the apoptotic rate of MG63 and U2OS cells 24 h after transfection. † $p < 0.01$ , ‡ $p < 0.001$  vs. si-con. NEAT1, nuclear paraspeckle assembly transcript 1; qRT-PCR, quantitative real-time PCR; MTT, 3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide; PI, propidium iodide; FITC, fluorescein isothiocyanate.

**Knockdown of NEAT1 suppresses proliferation and invasion and promotes apoptosis of osteosarcoma cells**

To elucidate the regulatory roles of NEAT1 on osteosarcoma, MG63 and U2OS cells were transfected with si-NEAT1 or si-con. The results of qRT-PCR analysis revealed that si-NEAT1 significantly reduced NEAT1 expression in MG63 and U2OS cells, compared with si-con (Fig. 2A). MTT assay showed that downregulation of NEAT1 led to a significant reduction of viabilities, compared with control group, in MG63 and U2OS cells (Fig. 2B). Transwell invasion assay indicated that the invasive ability of MG63 and U2OS cells was significantly suppressed by NEAT1 knockdown, compared with the si-con group (Fig. 2C and D). Flow cytometry analysis revealed that the apoptotic rate of MG63 and U2OS cells in si-NEAT1 transfection group was significantly increased, compared to the si-con group (Fig. 2E and F). These findings suggested that silencing of NEAT1 inhibited proliferation and invasion and induced apoptosis of osteosarcoma cells.

**miR-194 inhibits proliferation and invasion and induces apoptosis of osteosarcoma cells**

To further assess the effects of miR-194 on proliferation, invasion, and apoptosis of osteosarcoma cells, MG63 and U2OS cells were transfected with miR-194 or miR-con. The results of qRT-PCR analysis indicated that miR-194 was successfully overexpressed in MG63 and U2OS cells (Fig. 3A). MTT assay revealed that miR-194 transfection dramatically repressed the cell viability of MG63 and U2OS cells (Fig. 3B). Consistently, the invasive capacity of MG63 and U2OS cells was markedly hindered by miR-194 upregulation (Fig. 3C and D). Moreover, enforced expression of miR-194 resulted in a remarkable enhancement of apoptosis in MG63 and U2OS cells (Fig. 3E and F). Taken together, all these results demonstrated that overexpression of miR-194 impeded proliferation and invasion and induced apoptosis of osteosarcoma cells.



**Fig. 3.** miR-194 overexpression suppressed proliferation and invasion and induced apoptosis of MG63 and U2OS cells. MG63 and U2OS cells were transfected with miR-194 mimic or miR-control. (A) qRT-PCR analysis was performed to detect the miR-194 expression in MG63 and U2OS cells. (B) MTT assay of viability at days 1, 2, 3 and 4 in MG63 and U2OS cells. (C and D) Transwell invasive assay of invasive capacity 24 h post transfection in MG63 and U2OS cells. (E and F) Flow cytometry analysis of apoptosis of 24 h post transfection in MG63 and U2OS cells. †*p*<0.01, ‡*p*<0.001 vs. miR-con. qRT-PCR, quantitative real-time PCR; MTT, 3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide.



**NEAT1 inhibits miR-194 expression in osteosarcoma cells**

Bioinformatics analysis by using starBase v2.0 showed that NEAT1 contains a binding site of miR-194 (Fig. 4A). To confirm the relationship between NEAT1 and miR-194, the luciferase reporter assay was performed. The results displayed that transfection of miR-194 significantly reduced the luciferase activity of Wt-NEAT1, but not with Mut-NEAT1, in MG63 and U2OS cells (Fig. 4B). To further confirm whether NEAT1 could indeed regulate miR-194 expression, MG63 and U2OS cells were transfected with si-NEAT1 or pcDNA-NEAT1. The results demonstrated that knockdown of NEAT1 improved the expression of miR-194 and that overexpression of NEAT1 suppressed the level of miR-194 in MG63 and U2OS cells (Fig. 4C). Together, these results clarified that NEAT1 functioned as a sponge to modulate miR-194 expression in osteosarcoma cells.

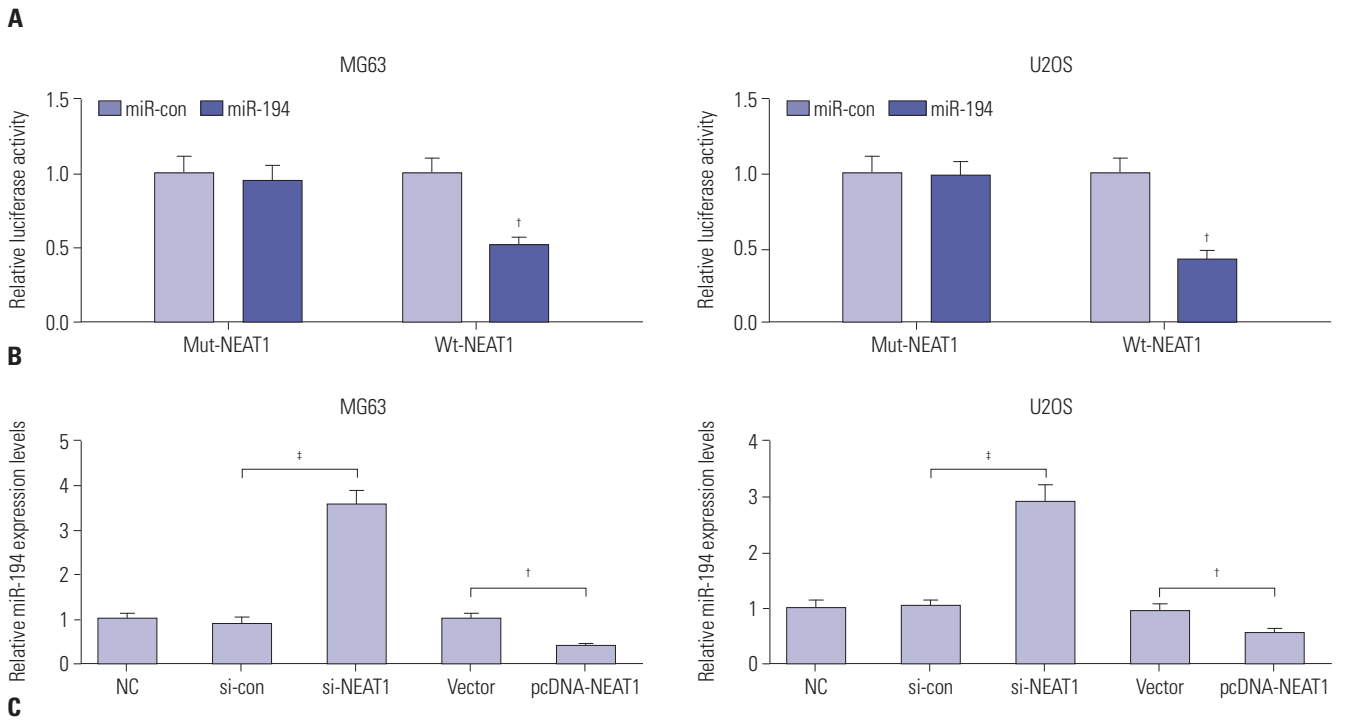
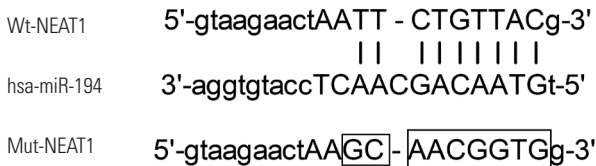
**miR-194 inhibition reverses the suppression of proliferation and invasion and the induction of apoptosis triggered by NEAT1 knockdown in osteosarcoma cells**

To further explore whether NEAT1 exerted its function in osteosarcoma cells through regulating miR-194 expression,

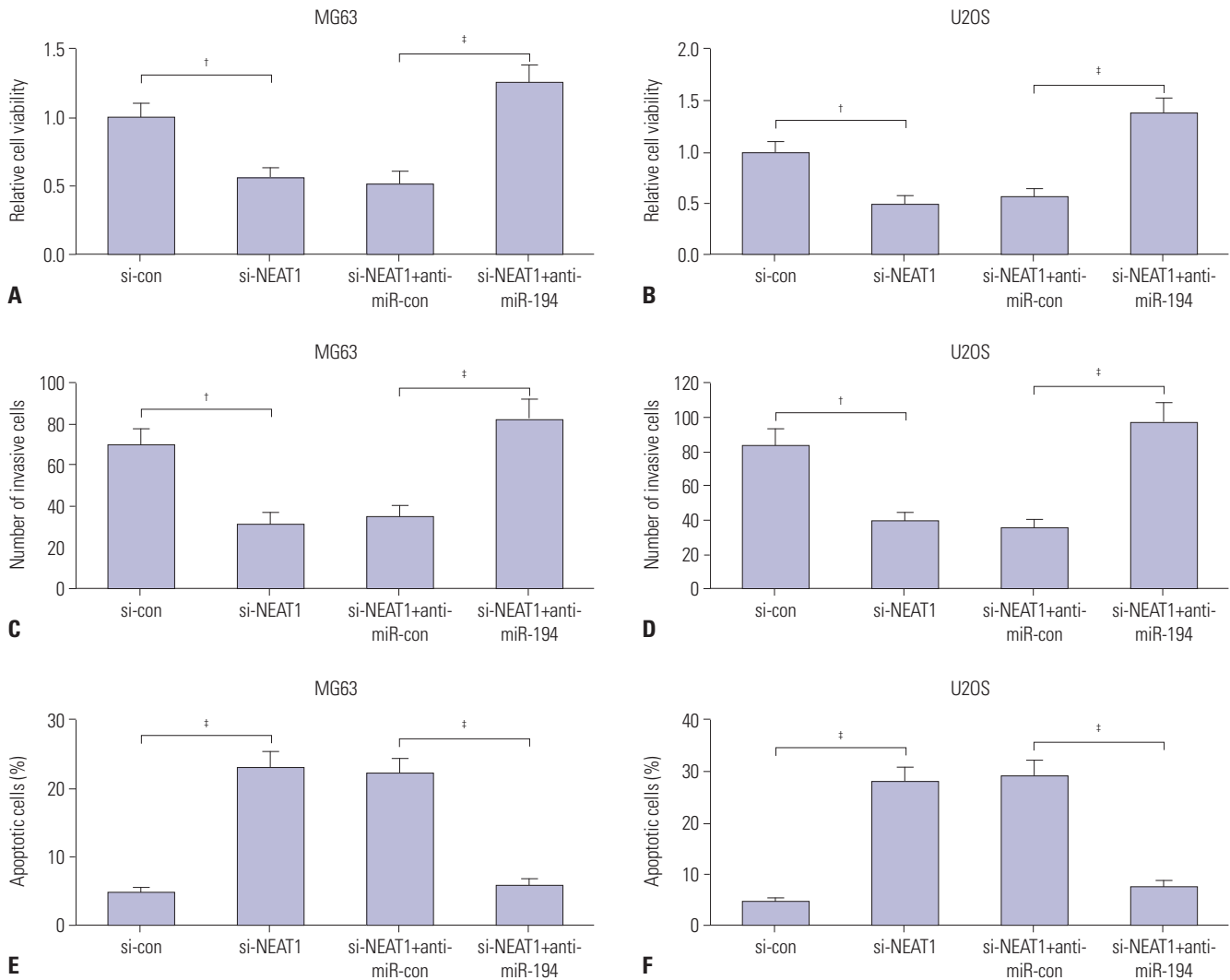
MG63 and U2OS cells were transfected with si-NEAT1 or co-transfected with si-NEAT1 and anti-miR-194. Then, MTT assay, Transwell invasion assay, and flow cytometry analysis were performed to determine the cell viability, invasive ability, and apoptosis of MG63 and U2OS cells, respectively. MTT assay revealed that NEAT1 knockdown resulted in prominent inhibition of cell viability in MG63 and U2OS cells, which was abrogated by miR-194 inhibition (Fig. 5A and B). Transwell invasion assay confirmed that downregulation of miR-194 reversed the inhibitory effect of NEAT1 silencing on the invasive ability of MG63 and U2OS cells (Fig. 5C and D). Flow cytometry analysis indicated that inhibition of miR-194 markedly attenuated NEAT1 depletion-induced apoptosis in MG63 and U2OS cells (Fig. 5E and F). Collectively, NEAT1 knockdown suppressed proliferation and invasion and the induced apoptosis of osteosarcoma cells through modulating miR-194 in osteosarcoma cells.

**DISCUSSION**

Growing reports have shown that many lncRNAs are dysregu-



**Fig. 4.** NEAT1 inhibited miR-194 expression in MG63 and U2OS cells. (A) The binding sequences of miR-194 in NEAT1 were marked. (B) Luciferase reporter assay was performed in MG63 and U2OS cells co-transfected with Wt- or Mut-NEAT1 and miR-194 mimic or miR-con. (C) qRT-PCR analysis of miR-194 expression in MG63 and U2OS cells transfected with si-NEAT1 or pcDNA-NEAT1. <sup>†</sup>*p*<0.01, <sup>‡</sup>*p*<0.001 vs. controls. Wt-NEAT1, wild type-nuclear paraspeckle assembly transcript 1; Mut-NEAT1, mutant-nuclear paraspeckle assembly transcript 1; NC, blank control; qRT-PCR, quantitative real-time PCR.



**Fig. 5.** miR-194 inhibition overturned suppression of proliferation and invasion and the promotion of apoptosis elicited by NEAT1 knockdown in MG63 and U2OS cells. MG63 and U2OS cells were transfected with si-NEAT1 or co-transfected with si-NEAT1 and anti-miR-194. (A and B) MTT assay was used to test the viability of transfected MG63 and U2OS cells. (C and D) Transwell invasive assay was applied to measure the invasive ability of transfected MG63 and U2OS cells. (E and F) Flow cytometry analysis was conducted to observe the apoptotic rate of transfected MG63 and U2OS cells. †*p*<0.01, ‡*p*<0.001 vs. controls. NEAT1, nuclear paraspeckle assembly transcript 1; MTT, 3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide.

lated in various cancers and exhibit important functions in cancer progression.<sup>14</sup> Recently, numerous lncRNAs have been reported to be involved in osteosarcoma. For instance, lncRNA MALAT1 accelerated the proliferation and metastasis of osteosarcoma cells via activation of the PI3K/Akt pathway.<sup>22</sup> LncRNA HOXA transcript at the distal tip enhanced chemoresistance of osteosarcoma cells by activating the Wnt/ $\beta$ -catenin pathway.<sup>23</sup> Sun, et al.<sup>24</sup> revealed that lncRNA highly upregulated in liver cancer reflected poor prognosis and promoted osteosarcoma cell metastasis. LncRNA HNF1A-AS1 was found to promote cell proliferation and metastasis in osteosarcoma by activating the Wnt/ $\beta$ -catenin signaling pathway.<sup>25</sup> The present study focused on lncRNA NEAT1.

NEAT1 is a novel lncRNA localized specifically to nuclear paraspeckles.<sup>26</sup> To date, many studies have reported the biological function of NEAT1 and its potential mechanism in var-

ious cancers. For example, NEAT1 was illuminated to function as a competing endogenous lncRNA by sponging miR-98-5p to alleviate its suppression on copper transporter 1, subsequently enhancing the sensitivity of non-small cell lung cancer cells to cisplatin.<sup>27</sup> NEAT1 knockdown suppressed proliferation and invasion and induced apoptosis in laryngeal squamous cell carcinoma through upregulating miR-107 to repress CDK6 (a cyclin-dependent kinase) expression.<sup>28</sup> NEAT1 promoted proliferation, migration, and invasion in esophageal squamous cell carcinoma.<sup>29</sup> All these studies demonstrated that NEAT1 could function as an oncogene in cancers. In the present study, its oncogenic activities were also confirmed in osteosarcoma. Our study revealed that NEAT1 was overexpressed in osteosarcoma tissues and cells, and knockdown of NEAT1 suppressed proliferation and invasion and promoted apoptosis of osteosarcoma cells. Consistent with our findings,

Zhao, et al.<sup>20</sup> confirmed that NEAT1 was upregulated in osteosarcoma tissues, and silencing of NEAT1 inhibited osteosarcoma cell proliferation, migration, and invasion.

Previous documents have implicated that miRNAs exert important functions in regulating biological processes, including cell proliferation, metastasis, and apoptosis, in various cancers.<sup>30</sup> Specifically, many miRNAs involved in osteosarcoma carcinogenesis could be regulated by lncRNAs. For instance, lncRNA plasmacytoma variant translocation 1 promoted proliferation, migration, and invasion and suppressed apoptosis and cell cycle arrest in osteosarcoma cells by acting as a molecular sponge to regulate miR-195.<sup>31</sup> LncRNA TUG1 knockdown suppressed cell proliferation and colony formation, and induced G0/G1 cell cycle arrest and apoptosis by sponging miR-9-5p to alleviate its repression on POU class 2 homeobox 1 (POU2F1) expression in osteosarcoma.<sup>32</sup> Previous studies discovered that miR-194 acted as a tumor suppressor through suppressing its targets, such as bone morphogenetic protein 1 and ras-related gene.<sup>33,34</sup> Additionally, miR-194 has been shown to inhibit osteosarcoma cell proliferation, migration, and invasion *in vitro* through targeting Cadherin 2 and type 1 insulin-like growth factor receptor and to suppress tumor growth and metastasis of osteosarcoma *in vivo*.<sup>12</sup> In agreement with the previous study, our findings suggested that miR-194 repressed proliferation and invasion and induced apoptosis of osteosarcoma cells. Next, we applied luciferase reporter analysis and qRT-PCR analysis to conclude that NEAT1 functioned as a miR-194 sponge to regulate its expression. Further rescue experiments indicated that miR-194 downregulation abolished the NEAT1 knockdown-induced suppression on proliferation and invasion, as well as promotion of apoptosis in osteosarcoma cells, indicating that NEAT1 suppresses proliferation and invasion and induces apoptosis of osteosarcoma by acting as a sponge to modulate miR-194 expression.

In summary, our study showed that NEAT1 is upregulated in osteosarcoma cells. Functionally, knockdown of NEAT1 suppressed proliferation and invasion and induced apoptosis of osteosarcoma cells. Mechanistically, NEAT1 promoted osteosarcoma progression by functioning as a miR-194 sponge to suppress its expression. Thus, NEAT1 could potentially serve as a novel therapy target for osteosarcoma.

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