Circular RNA circ_0032462 Enhances Osteosarcoma Cell Progression by Promoting KIF3B Expression

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Abstract
Circular RNAs are a recently discovered subclass of endogenous noncoding RNAs that have been confirmed to play an important role in various pathophysiological processes. However, the underlying function of circular RNAs in osteosarcoma still remains unclear. We aimed to comprehend the function of circ_0032462 in osteosarcoma, as it has been predicted to be highly expressed in osteosarcoma cells. Using real-time polymerase chain reaction, we verified the elevated expression of circ_0032462 in osteosarcoma cells than normal cells. Functional validation experiments revealed that circ_0032462 overexpression promoted proliferation, migration, and invasion in osteosarcoma cells, whereas circ_0032462 silencing was observed to inhibit cancer cell progression (proliferation, migration, and invasion). Furthermore, we found that circ_0032462 upregulated the messenger RNA and protein expression level of kinesin family member 3B. In addition, kinesin family member 3B inhibition was found to inhibit circ_0032462-induced enhanced osteosarcoma cell progression. circ_0032462 overexpression was observed to reverse circ_0032462 silencing-induced inhibitory effect on osteosarcoma cell progression. Overall, our research revealed the function of circ_0032462 in osteosarcoma progression, which might serve as a novel chemotherapeutic target for osteosarcoma.

Keywords
circRNA, KIF3B, proliferation, migration, invasion

Abbreviations
APC, adenomatous polyposis coli; CCK8, Cell Counting Kit-8; circRNA, circular RNA; FBS, fetal bovine serum; KAP3, kinesin-associated protein 3; KIF3B, kinesin family member 3B; mRNA, microRNA; mRNA, messenger RNA; OS, osteosarcoma; PCR, polymerase chain reaction; RNase R, ribonuclease R; siRNA, small interfering RNA

Introduction
Osteosarcoma (OS) is a primary bone tumor which arises from mesenchymal cells. Osteosarcoma has been reported to have the highest fatality rate among all cancers.¹ For the past several decades, cancer therapies have developed with the use of advanced surgical technology and multiple chemotherapies. Despite recent advanced developments, prognosis remains inadequate.²,³ Several patients have been reported to suffer from cancer recurrence and potential metastasis. Some patients with OS have been shown to benefit from certain molecular therapy due to the use of molecular-targeted drugs.⁴ As such targeted therapy can lead to severe side effects, it is essential to explore new therapy for treating OS.

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Circular RNAs (circRNAs) are a recently discovered subclass of endogenous noncoding RNAs. Circular RNAs have special structures, which are covalent loops with 3'-end and 5'-end connected to each other. The covalent loops are different from linear RNAs and have been shown to protect circRNAs from ribonuclease R (RNase R)-mediated digestion. Circular RNA was first discovered in RNA viruses during the 1970s. For a long time, researchers considered circRNAs as junk noncoding RNA. However, due to the development of sequencing and computational technology in recent years, an increasing number of researches have revealed the important roles of circRNAs in regulating gene expression at posttranscriptional level. Previous studies have favorably proved that circRNA can function through competing endogenous RNA mechanism. This proposes that circRNA can serve as microRNA (miRNA) sponges by competitively binding to target miRNA and suppress their expression and function. Interestingly, reports have demonstrated that circRNAs can code for proteins in eukaryotic cells. Recently, an increasing number of circRNAs have been discovered to play important roles in various cancers and potentially act as efficient biomarkers due to their special structure and prolonged half-life. However, little is known about the roles of circRNAs in OS.

It has been reported that circRNAs such as hsa_circ_0028173, hsa_circ_0032462, and hsa_circ_0005909 are highly expressed in human OS. Moreover, they have been predicted to promote cell adhesion molecule 1 expression by acting as miRNA sponge. Additionally, they analyzed OS-related circRNA profiles (GSE96964) and gene profiles (GSE36001, GSE33382, and GSE42352) from the Gene Expression Omnibus database (NCBI, http://www.ncbi.nlm.nih.gov/geo/) and predicted that hsa_circ_0032462, hsa_circ_0028173, and hsa_circ_0005909 played important roles in OS. However, the hypothesis needed further experimental proof. In this study, we designed experiments to confirm circRNA-related predictions in OS cell lines. This research will provide additional information on the functional role of circRNAs in OS.

**Materials and Methods**

**Cell Lines and Culture**

Human OS-derived cell lines, 143B, MG-63, U2OS, SOSP-9607, SJSA-1, and HOS, were purchased from Fu-Heng Cell Center. 143B and MG-63 cells were cultured in Dulbecco’s modified Eagle medium (HyClone) containing 10% fetal bovine serum (FBS; Gibco). All other OS cell lines were cultured in RPMI 1640 medium (Gibco) with 10% FBS (Gibco). Mycoplasma was tested using Venor GeM Mycoplasma Detection Kit (Minerva Biolabs). Normal osteoblastic cell line (hFOB1.19) was obtained from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences and cultured in Dulbecco’s Medium (Gibco). All cell lines were cultured at 37 °C with 5% CO₂.

**RNA Extraction and RNase R Digestion**

Total RNA was extracted from cell lines using TRIzol reagent (Invitrogen). Subsequently, NanoDrop 2000 (Thermo Fisher Scientific) was used to quantify RNA. Total RNA was treated with RNase R (Epicentre) for 30 minutes at 37 °C at a proportion of 3 units of RNase R for every 1 mg RNA.

**Quantitative Real-Time PCR Analysis and RNA Interference**

Complementary DNAs were synthesized from total RNA by reverse transcription using random priming method (PrimeScript RT Reagent Kit; TaKaRa). Quantitative real-time polymerase chain reaction (PCR) was performed using SYBR Green qPCR Master Mix (Thermo Fisher Scientific). Primers were shown in Table 1. Gene expressions were normalized to endogenous control of human glyceraldehyde-3-phosphate dehydrogenase expression. Relative expression was calculated using 2^(-ΔΔCT) method.13

**Cell Transfection**

Cell transfection was performed using small interfering RNAs (siRNAs), which were supplied by GenePharma. Transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

**Wound Healing Assay**

Osteosarcoma cells were seeded in 6-well plates and were grown to 95% confluency. Using 200 μL pipette tip, a wound was created. Cells were then washed thrice with phosphate-buffered saline. Images were captured 48 hours post-wound. Relative distance of cell migration was measured and the healing percentage was evaluated.

**Migration and Invasion Assay**

Osteosarcoma cells (3 × 10⁵ cells/mL) were suspended in FBS-free cell culture medium containing mitomycin C (2 μg/mL). Cells were seeded in the upper well of polycarbonate transwell insert (Millipore). The membrane was coated with Matrigel (Beyotime Institute of Biotechnology). Furthermore, we added 600 μL of medium with 10% FBS to the lower chamber. After 48 hours, noninvasive cells were removed using a cotton swab, while the outer membrane was fixed and stained.

**Statistical Analysis**

Statistical analyses were evaluated by GraphPad Prism version 6.0 (GraphPad Software Inc) and SPSS (IBM). One-way analysis of variance test and Student t test were used to evaluate the significant difference. P < .05 was considered to be statistically significant.
hsa_circ_0032462 Is Relatively Highly Expressed in OS Cell Lines

Earlier research has reported that hsa_circ_0032462, hsa_circ_0028173, and hsa_circ_0005909 are highly expressed in OS cells and might be playing important roles in OS progression. Thus, at first, we designed experiments to confirm the expression levels of these circRNAs in OS cell lines. To identify the expression of hsa_circ_0032462, hsa_circ_0028173, and hsa_circ_0005909 in OS cell lines, we cultured several OS cell lines and normal human osteoblastic cell line. Real-time PCR was performed to quantify the circRNA expression level in 143B, MG-63, U2OS, SOSP-9607, SJSA-1, HOS, and normal hFOB1.19 cells. Results showed that hsa_circ_0032462 was highly expressed in all OS cell lines than normal human osteoblastic cell line, hFOB1.19 (Figure 1A). On the other hand, hsa_circ_0028173 and hsa_circ_0005909 showed diversity in gene expression pattern. In conclusion, hsa_circ_0032462 was found to be relatively highly expressed in OS cell lines, suggesting its significance in OS.

hsa_circ_0032462 Promotes Proliferation in OS Cells

As results showed that hsa_circ_0032462 was highly expressed in OS cell lines, we investigated the function of circRNA in OS. We constructed hsa_circ_0032462 overexpressing and silenced OS cell lines to investigate the function of hsa_circ_0032462 in OS cells. The efficiency of hsa_circ_0032462 overexpression (Figure 2A and B) and silencing (Figure 2C and D) was assessed by real-time PCR. At first, we used Cell Counting Kit-8 (CCK8) assay to identify the influence of hsa_circ_0032462 on cell growth. Results showed that hsa_circ_0032462 overexpression promoted cell growth (Figure 3A and B), whereas hsa_circ_0032462 silencing was observed to decrease cell growth in OS cells (Figure 3C and D). Overall, these findings revealed the momentous role of hsa_circ_0032462 in proliferation of OS cells in vitro.

hsa_circ_0032462 Promotes Migration and Invasion in OS Cells

Although surgical techniques and chemotherapy have considerably developed in the past few decades, survival of patients with OS remains substandard. A majority of patients suffer from cancer recurrence due to distant metastasis. Thus, it is utmost urgent to investigate the specific mechanism underlying OS metastasis. We investigated the functional role of hsa_circ_0032462 in metastasis.

We examined metastasis-related functions of hsa_circ_0032462 by performing migration and invasion assay in hsa_circ_0032462 overexpressed or silenced OS cells. Data
showed that the overexpression of hsa_circ_0032462 enhanced the migration and invasion ability of 143B and MG-63 OS cells (Figure 4A and B). To further confirm the function of hsa_circ_0032462 in promoting metastasis in OS cells, we performed hsa_circ_0032462 knockout experiments. Figure 4C and D depicts that hsa_circ_0032462 silencing was found to weaken the migration and invasion ability of OS cells. In conclusion, results showed that hsa_circ_0032462 can promote OS cell progression including migration and invasion.

Kinesin Family Member 3B Is Upregulated by hsa_circ_0032462

We designed experiments to investigate the specific mechanism of hsa_circ_0032462-induced OS progression. It has been predicted that hsa_circ_0032462 can regulate various proteins such as protein argonaute-1, kinesin family member 3B (KIF3B), ataxin 7, charged multivesicular body protein 7, F-box/SPRY domain-containing protein 1, and apoptotic protease-activating factor 1. Kinesin family member 3B is one of the most commonly expressed KIFs. In addition, KIF3 is composed of KIF3A/3B heterodimer and kinesin-associated protein 3 (KAP3). As a member of KIF3 subfamily, KIF3B has been reported to play an important function in vesicular transport and membrane expansion. Moreover, adenomatous polyposis coli (APC) protein has been shown to be transported by KIF complex before regulating cell migration.

We investigated whether hsa_circ_0032462 can regulate KIF3B expression. At first, we transfected 143B and MG-63 OS cells with increasing dose of hsa_circ_0032462 and examined KIF3B expression level. Real-time PCR showed that in 143B cells, messenger RNA (mRNA) and protein expression level of KIF3B increased with increase in hsa_circ_0032462 expression (Figure 5A and C). Similar results were obtained...
Figure 3. hsa_circ_0032462 promotes cell growth in OS cells. A, The CCK8 assay was performed to evaluate cell growth of NC and hsa_circ_0032462-overexpressing 143B cells at day 0, day 2, day 4, and day 6. B, The CCK8 assay was performed to evaluate cell growth of NC and hsa_circ_0032462-overexpressing MG-63 cells at day 0, day 2, day 4, and day 6. C, The CCK8 assay was performed to evaluate cell growth of NC and hsa_circ_0032462-overexpressing SJSA-1 cells at day 0, day 2, day 4, and day 6. D, The CCK8 assay was performed to evaluate cell growth of NC and hsa_circ_0032462-overexpressing HOS cells at day 0, day 2, day 4, and day 6. CCK-8 indicates Cell Counting Kit-8; NC, negative control.

Figure 4. hsa_circ_0032462 promotes migration and invasion in OS cells. A, Wound healing assay and Transwell assay were performed in NC and hsa_circ_0032462-overexpressing 143B cells for 48 hours. B, Wound healing assay and Transwell assay were performed in NC and hsa_circ_0032462-overexpressing MG-63 cells for 48 hours. C, Wound healing assay and Transwell assay were performed in NC and siRNA targeting hsa_circ_0032462 (si-hsa_circ_0032462)-expressing SJSA-1 cells for 48 hours. D, Wound healing assay and Transwell assay were performed in NC and si-hsa_circ_0032462-expressing HOS cells for 48 hours. NC indicates negative control; OS, osteosarcoma; siRNA, small interfering RNA.
with MG-63 cells (Figure 5B and D). Furthermore, we found that hsa_circ_0032462-induced KIF3B upregulation at mRNA (Figure 6A) and protein (Figure 6C) level was hindered on hsa_circ_0032462 silencing. Subsequently, on reexpressing hsa_circ_0032462, KIF3B expression was observed to recover from hsa_circ_0032462 silencing-induced downregulation (Figure 6B and D). Overall, we concluded that hsa_circ_0032462 can enhance KIF3B expression at mRNA as well as protein level.

**Discussion**

In recent years, an increasing number of circRNAs have been discovered due to the development of next-generation sequencing methods and gene chip technology. Owing to special characteristics such as sturdy construction, cell-specific expression, and conservation across species, circRNAs have been reported to play important roles in various cellular functions. However, there are many questions, which are still unexplored. Our present study confirmed the predictions of previous report that hsa_circ_0032462 is highly expressed in OS and has specific roles.

In this study, we detected the expression of hsa_circ_0032462, hsa_circ_0028173, and hsa_circ_0005909 in 143B, MG-63, U2OS, SOSP-9607, SJSA-1, HOS, and
We found that hsa_circ_0032462 was highly expressed in OS cell lines compared to its expression in normal osteoblastic cells. To determine the functional role of hsa_circ_0032462 in OS cells, we overexpressed hsa_circ_0032462 in 143B and MG-63 cells, which were found to have relatively low hsa_circ_0032462 expression. In addition, we silenced hsa_circ_0032462 in SJSA-1 and HOB cells, which were observed to have high hsa_circ_0032462 expression. Furthermore, CCK8 assays were used to evaluate the growth rate of different OS cells. Results showed that the overexpression of hsa_circ_0032462 promoted cell growth in OS cells (Figure 3A and B), whereas hsa_circ_0032462 silencing reduced cellular growth rate (Figure 3C and D). Additionally, we performed migration and invasion assay to detect the metastatic ability of OS cells. hsa_circ_0032462 overexpression was found to enhance the migration and invasion ability of OS cells (Figure 4A and B), whereas hsa_circ_0032462 silencing was observed to deteriorate the metastasis ability of OS cells (Figure 4C and D).

Further study showed that hsa_circ_0032462 overexpression upregulated KIF3B expression at mRNA and protein level, whereas on silencing hsa_circ_0032462, KIF3B expression was downregulated (Figures 5 and 6). Kinesin family member 3B belongs to KIF superfamily, which is known to be one of the motor proteins dependent on microtubule. Kinesin family member 3B transports intracellular molecules along microtubules by consuming adenosine triphosphate. Kinesin family members have been reported to play important functions in a number of cellular processes such as transporting macromolecules, mitosis, chromosome translocation, gene deletion, and carcinogenesis.

Kinesin family member 3 is widely expressed, which composes of KAP3 and KIF3A/3B. As a member of KIF3 subfamily, KIF3B includes several molecular motors and plays critical roles in the expansion of membrane and transport of vesicular. It has been reported that KIF complex regulates cell migration by transporting APC protein. All relevant studies have associated the role of KIF3B in carcinogenesis. However, the function of KIF3B has not yet been elucidated in OS.

Subsequently, this study revealed that hsa_circ_0032462 can upregulate the mRNA and protein expression level of KIF3B. Additionally, we investigated whether KIF3B was
responsible for hsa_circ_0032462-induced OS progression by performing detailed experiments. Moreover, we found that KIF3B knockout can inhibit hsa_circ_0032462-induced enhanced OS progression, whereas KIF3B overexpression can revive inhibited OS progression, which is induced on hsa_circ_0032462 silencing.

In our present research, we first confirmed the elevated expression of hsa_circ_0032462 in OS cells. Subsequently, we revealed that hsa_circ_0032462 can promote OS progression by regulating KIF3B expression. In conclusion, our study proves that KIF3B plays a critical role in hsa_circ_0032462-induced OS carcinogenesis by regulating cell proliferation, migration, and invasion. We suggest that hsa_circ_0032462/KIF3B can serve as a novel biomarker and chemotherapeutic target for OS.

Our experiments did not involve patient and animal experiments.

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Table 1. Primers used in Real-Time PCR.

| Gene           | Forward primer                 | Reverse primer                 |
|----------------|--------------------------------|--------------------------------|
| β-actin        | 5'-CATGTACGTG>                 | 5'−CTCCTTAATG                  |
|                | CTATCCAGGC-3'                  | TACCGACGAT-3'                  |
| hsa_circ_0032462 | 5'-GAAACTGGAT                 | 5'-GCCGTCTGTG                  |
|                | GAAACAGG-3'                    | CCAACAAC-3'                    |
| KIF3B          | 5'-TGGAATGTTGGA                | 5'-TCGGAACGTCT                  |
|                | TGTTAAGCTG-3'                  | CATCGTACAG-3'                  |

Declaration of Conflicting Interests
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References
1. Liu YJ, Li W, Chang F, Liu JN, Lin JX, Chen DX. MicroRNA-505 is downregulated in human osteosarcoma and regulates cell proliferation, migration and invasion. Oncol Rep. 2017;39(2):491-500.
2. Cai H, Lin L, Cai H, Tang M, Wang Z. Combined MicroRNA-340 and ROCK1 mRNA profiling predicts tumor progression and prognosis in pediatric osteosarcoma. Molecular Sci. 2014;15(1):560-573.
3. Marulanda GA, Henderson ER, Johnson DA, Letson GD, Cheong D. Orthopedic surgery options for the treatment of primary osteosarcoma. Cancer Control. 2008;15(1):13-20.
4. Posthuma deboer J, Witlox MA, Kaspers GJL, Royen BJV. Molecular alterations as target for therapy in metastatic osteosarcoma: a review of literature. Clin Exp Metastas. 2011;28(5):493-503.
5. Sebastian M, Panagiotis P, Oliver P, Nikolaus R, Sebastien P. Identification and characterization of circular RNAs as a new...
class of putative biomarkers in human blood. *Plos One*. 2017;10(10):e141214.
6. Shen T, Han M, Wei G, Ni T. An intriguing RNA species perspectives of circularized RNA. *Protein Cell*. 2016;6(12):871-880.
7. Belousova EA, Filipenko ML, Kushlinskii NE. Circular RNA: new regulatory molecules. *Bull Exp Biol Med*. 2018;164(6):803-815.
8. Salmena L, Poliseno L, Tay Y, Kats L, Pandolfi PP. A ceRNA hypothesis: The Rosetta Stone of a hidden RNA language. *Cell*. 2011;146(3):358.
9. Chen L, Zhang S, Wu J, et al. circRNA_100290 plays a role in oral cancer by functioning as a sponge of the miR-29 family. *Oncogene*. 2017;36(32):4551-4561.
10. Gaia P, Francesca M, Maria B. What is new in the miRNA world regarding osteosarcoma and chondrosarcoma? *Molecules*. 2017;22(3):417.
11. Patop IL, Kadener S. circRNAs in cancer. *Curr Opin Genet Dev*. 2017;48:121.
12. Chen G, Wang Q, Yang Q, et al. Circular RNAs hsa_circ_0032462, hsa_circ_0028173, hsa_circ_0005909 are predicted to promote CADM1 expression by functioning as miRNAs sponge in human osteosarcoma. *Plos One*. 2018;13(8):e202896. doi:10.1371/journal.pone.0202896
13. Jin W, Liao X, Lv Y, et al. MUC1 induces acquired chemoresistance by upregulating ABCB1 in EGFR-dependent manner. *Cell Death Dis*. 2017;8(8):e2980. doi:10.1038/cddis.2017.378
14. Jin W, Xu H, Zhang S, et al. Tumor-Infiltrating NETs predict postsurgical survival in patients with pancreatic ductal adenocarcinoma. *Ann Surg Oncol*. 2019;26(2):635-643.
15. Yu X, Wen H, Cao J, et al. Temporal and spatial expression of KIF3B after acute spinal cord injury in adult rats. *J Mol Neurosci*. 2012;49(2):387-394.
16. Jimbo T, Kawasaki Y, Koyama R, et al. Identification of a link between the tumour suppressor APC and the kinesin superfamily. *Nat Cell Biol*. 2002;4(4):323-327.
17. Bonizzato A, Gaffo E, Te Kronnie G, Bortoluzzi S. CircRNAs in hematopoiesis and hematological malignancies. *Blood Cancer J*. 2016;6(10):e483.
18. Legnini I, Di Timoteo G, Rossi F, et al. Circ-ZNF609 is a circular RNA that can be translated and functions in myogenesis. *Mol Cell*. 2017;66(1):22-37.
19. Liang D, Tatomer DC, Luo Z, et al. The output of protein-coding genes shifts to circular RNAs when the Pre-mRNA processing machinery is limiting. *Mol Cell*. 2017;68(5):940-954. e3.
20. Vale R, Reese T, Sheetz M. Identification of a novel force-generating protein, kinesin, involved in microtubule-based motility. *Cell*. 1985;42(1):39-50.
21. Miki H, Okada Y, Hirokawa N. Analysis of the kinesin superfamily: insights into structure and function. *Trends Cell Biol*. 2005;15(9):467-476.
22. Wang Q, Wang L, Li D, et al. Kinesin family member 14 is a candidate prognostic marker for outcome of glioma patients. *Cancer Epidemiol*. 2013;37(1):79-84.
23. Fan J. A role for the spectrin superfamily member Syne-1 and kinesin II in cytokinesis. *J Cell Sci*. 2004;117(pt 4):619-629.
24. Keil R, Kiessling C, Hatzfeld M. Targeting of p0071 to the midbody depends on KIF3. *J Cell Sci*. 2009;122(pt 8):1174-1183.