Hsa_circ_0002468 Regulates the Neuronal Differentiation of SH-SY5Y Cells by Modulating the MiR-561/E2F8 Axis

Background: It has been shown that circular RNAs (circRNAs) play a vital role in the regulation of neuronal differentiation; however, the precise role of circRNAs in human neuronal differentiation remains largely unexplored.

Material/Methods: A dual-luciferase reporter assay was carried out to confirm the targets of hsa_circ_0002468, miR-561, E2F8 (E2F transcription factor 8, a protein coding gene), and miR-561. We detected the expression of hsa_circ_0002468, miR-561, and E2F8 by using quantitative real-time polymerase chain reaction (qRT-PCR) analyses. In addition, we performed the functional experiments by using a BrdU (5-bromo-2'-deoxyuridine) assay and qRT-PCR analyses.

Results: In this study, we showed that hsa_circ_0002468 can act as a sponge of miR-561 to regulate SH-SY5Y proliferation and differentiation. A bioinformatics analysis showed that hsa_circ_0002468 had a binding site that corresponded to miR-561, which was verified by dual-luciferase reporter assay. The expression of hsa_circ_0002468 was increased during SH-SY5Y differentiation and was inversely correlated with miR-561 expression. Using qRT-PCR analysis, we showed that hsa_circ_0002468 negatively regulated miR-561 in SH-SY5Y cells. Intriguingly, the overexpression of hsa_circ_0002468 increased SH-SY5Y differentiation and reduced SH-SY5Y proliferation; the suppression of hsa_circ_0002468 led to decreased SH-SY5Y differentiation levels and increased SH-SY5Y proliferation levels. Additionally, overexpression of miR-561 rescued the SH-SY5Y proliferation deficiency induced by hsa_circ_0002468 overexpression and abolished the SH-SY5Y differentiation promoted by hsa_circ_0002468. Furthermore, E2F8 was validated as a direct target of miR-561.

Conclusions: Our data suggested that hsa_circ_0002468 was a novel circRNA that regulated SH-SY5Y cell proliferation and differentiation via targeting the miR-561/E2F8 axis. Therefore, manipulating hsa_circ_0002468 in SH-SY5Y cells could be a novel strategy to develop novel interventions for the treatment of relevant neurological disorders.

MeSH Keywords: E2F6 Transcription Factor • Neuregulins • RNA, Untranslated

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Background

Neural stem cells (NSCs) belong to a group of special types of cells that are self-renewing and proliferating; in addition, they have the ability to differentiate into neurons, astrocytes, and oligodendrites in the nervous system [1,2]. Recently, studies have indicated that NSCs have a therapeutic effect for neurological disorders, such as Alzheimer’s disease, Parkinson’s disease, and Huntington’s disease, and for spinal injuries [3–6]. In addition, NSCs are emerging as a promising new therapeutic strategy for cancer therapy [7]. Multiple pathways are involved in the process of NSC proliferation and differentiation. Previous studies have suggested that circular RNAs (circRNAs) play an important role in regulating NSC proliferation and differentiation. However, the precise role of circRNAs in NSCs remains largely unexplored.

Competing endogenous RNAs (ceRNAs) play important roles in post-transcriptional regulation. Dysregulation of the ceRNA networks is related to the development of human disease [8,9]. CircRNAs are also a class of ceRNAs and are a special type of endogenous noncoding RNA; they are formed by back-splicing events through exon or intron circularization and play important roles in post-transcriptional regulation [10]. More and more evidence has demonstrated that circRNAs play vital roles in many varieties of biological and pathological processes, including cell proliferation, differentiation, metastasis, cell cycle progression, and oncogenesis [11–15]. However, the precise role of circRNAs in NSCs is largely unknown.

Here, we showed that the overexpression of hsa_circ_0002468 increased cell differentiation and reduced cell proliferation in the human neuroblastoma differentiation model SH-SY5Y cells. In addition, the suppression of hsa_circ_0002468 resulted in the opposite effect. Moreover, we found that hsa_circ_0002468 regulated the proliferation and differentiation of SH-SY5Y cells by sponging miR-561. Furthermore, E2F8 (E2F transcription factor 8, a protein coding gene) was validated as a direct target of miR-561. Taken together, our data suggested that hsa_circ_0002468 was a novel circRNA that regulated the proliferation and differentiation of the human neuroblastoma cell line SH-SY5Y via targeting the miR-561/E2F8 axis.

Material and Methods

Cell culture, differentiation and transfection

SH-SY5Y cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) (Invitrogen) with 10% fetal bovine serum (FBS) at 37°C in an atmosphere of 5% CO₂. For differentiation, the culture medium was replaced with DMEM containing 10% FBS and 10 μM retinoic acid (RA). The cells were transfected with the Lipofectamine 2000 reagent (Invitrogen) for 6 hours, and then the culture medium was replaced with DMEM containing 10% FBS and RA (10 μM). Mouse NSCs were cultured in laminin-coated plates in DMEM-F12 (1: 1) medium (Invitrogen) supplemented with 10% B27 medium (Invitrogen), 10 g/mL gentamicin (Gibco), 10 units/mL heparin (Sigma), 20 ng/mL epidermal growth factor (EGF), and 10 ng/mL basic fibroblast growth factor (bFGF) (Invitrogen). For differentiation, the growth medium was replaced with neurobasal medium (Invitrogen) supplemented with 10% B27 medium (Invitrogen), 10 g/mL gentamicin (Gibco), and 10 units/mL heparin (Sigma). Our study was approved by the ethical board of the institute of the First Hospital of Harbin Medical University and complied with the Declaration of Helsinki.

RNA isolation, real-time polymerase chain reaction (RT-PCR) assays and western blot assays

The total RNA was isolated from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Real-time polymerase chain reaction (RT-PCR) assays were performed as described in previous studies [16,17]. The experimental procedures of the protein extractions and the western blots were performed as described by Wan et al. [18]. The anti-E2F8 and anti-actin antibodies were from Abcam (Cambridge, UK).

Luciferase reporter assays and cell biology assays

The NSCs were transfected with miR-561 mimics, NC mimics (miRNA mimic negative control), anti-miR-561, anti-NC, pLuc-E2F8-3′ UTR, and pLuc-E2F8-3′ UTR-Mut in a 48-well plate. Two luciferase reporters containing wild-type (WT) E2F8 or mutant (Mut) E2F8 were generated to analyze the interaction between E2F8 and miR-561. The details can be found in the references [19,20]. The procedures for BrdU (5-bromo-2’-deoxyuridine), cell apoptosis, and cell cycle assays can be found as described previously [16,21].

Biotin pulldown assay

This experimental procedure was performed as previously described in the references [22,23]. Briefly, the cells were transfected with 50 μM of the biotinylated miRNA mimic or the vector control and were lysed in 500 μL of lysis buffer. Subsequently, 50 μL of blocked streptavidin magnetic beads was added to each reaction and incubated for 4 hours at 4°C. Finally, TRIzol was used to extract the ncRNAs specifically interacting with the miRNAs.

Statistical analysis

All statistical analyses were performed in triplicate with Student’s t-test was used to calculate the significance of the comparison. P≤0.05 was considered to be significant.
Figure 1. Hsa_circ_0002468 was formed from CEP70 exons. (A) Hsa_circ_0002468, a novel circular RNA, was located in chr3: 138,289,159-138,290,198, and formed from the circular exons 4–6 of the human CEP70 gene. The photo shows the UCSC Genome Browser data. (B) The mouse homologue of hsa_circ_0002468 is mmu_circ_0016034, which is located in chr9: 99,162,964-99,164,228, and forms from the circular exons 2–3 of the mouse CEP70 gene, as seen by the USCS Genome Browser data.
Results

CEP70 genomic exons formed a novel circRNA

Hsa_circ_0002468, a novel circRNA, was located in chr3: 138289159-138290198 and formed from the circular exons 4–6 of the human CEP70 gene. The genomic length was 1039 bp and the spliced sequence length was 396 bp (Figure 1A). To check whether this circRNA was conserved among species, we searched its homologous gene in mice and found mmu_circ_0016034, which was located in chr9: 99162964–99164228 and formed from the circular exons 2–3 of the mouse CEP70 gene. The genomic length was 1264 bp and the spliced sequence length was 396 bp (Figure 1B).

Hsa_circ_0002468 directly targeted miR-561 during neuronal cell differentiation

To identify whether the expression levels of hsa_circ_0002468 and miR-561 were altered during the differentiation of SH-SY5Y cells, the cells were cultured in DMEM (10% FBS) containing retinoic acid (RA, 10 μM) to induce SH-SY5Y differentiation for 8 days, which showed the formation and elongation of neurites (Figure 2A). Figure 2B and 2C show that the expression of hsa_circ_0002468 was upregulated, while the expression of miR-561 was downregulated during the SH-SY5Y cell differentiation for 8 days. In addition, there was a negative correlation between the expression level of miR-561 and hsa_circ_0002468 (Figure 2D).
miR-561 and hsa_circ_0002468 have a regulatory relationship, a bioinformatics analysis was used and found that hsa_circ_0002468 contained a potential binding site of miR-561 (Figure 2E). A luciferase reporter assay was then carried out, and it was determined that the overexpression of miR-561 reduced the hsa_circ_0002468-WT fluorescence intensity in SH-SY5Y cells; however, the fluorescence intensity of hsa_circ_0002468-Mut was not influenced by miR-561 (Figure 2F).

Moreover, we performed biotin pulldown assays and confirmed that hsa_circ_0002468-Mut was not influenced by miR-561 (Figure 2F). SH-SY5Y cells; however, the fluorescence intensity of hsa_circ_0002468-WT was reduced in cells transfected with hsa_circ_0002468, si-circ_0002468 or the control vector, and it was determined that the overexpression of miR-561 inhibited hsa_circ_0002468 markedly enhanced cell proliferation (Figure 2A). The detection of Ki-67 mRNA (B), cyclinD1 mRNA (C), GFAP mRNA (D), Tuj1 mRNA (E), Nestin mRNA (F) as determined by real-time qPCR. SH-SY5Y cells were transfected with circ_0002468, si-circ_0002468 or the control vector, and BrdU assays were carried out to analyze the cell proliferation (A).

Hsa_circ_0002468 reduces cell proliferation and promotes neuronal differentiation of SH-SY5Y cells

As seen in Figure 2A, the cell numbers were decreased when the cells were differentiated for 8 days; we therefore speculated that the altered expression of hsa_circ_0002468 may affect SH-SY5Y cell proliferation during cell differentiation. A BrdU assay showed that the overexpression of hsa_circ_0002468 repressed the proliferation of SH-SY5Y cells, whereas the inhibition of hsa_circ_0002468 markedly enhanced cell proliferation (Figure 3A). Moreover, hsa_circ_0002468 overexpression significantly suppressed Ki-67 and cyclin D1 mRNA levels, while hsa_circ_0002468 repression promoted their mRNA levels (Figure 3B, 3C). Furthermore, hsa_circ_0002468 overexpression significantly promoted the expression of the astrocyte marker, glial fibrillary acidic protein (GFAP), in SH-SY5Y cells.
cells (Figure 3D). Meanwhile, the ectopic expression of hsa_circ_0002468 resulted in an increase in the neuronal marker class III β-tubulin (Tuj1), whereas the ectopic expression caused a decrease in the marker of neuroepithelial stem cell protein (Nestin), which is an NSC-specific marker (Figure 3E, 3F). These results indicate that hsa_circ_0002468 reduced SH-SY5Y cell proliferation and promoted the neuronal differentiation of SH-SY5Y cells. To further explore the mechanism of the inhibitory effect of hsa_circ_0002468 on SH-SY5Y cell proliferation, a cell apoptosis assay and a cell cycle assay were performed. The results demonstrated that hsa_circ_0002468 overexpression promoted cell apoptosis, whereas hsa_circ_0002468 inhibition repressed the cell cycle in SH-SY5Y cells (Figure 3G). In addition, hsa_circ_0002468 overexpression induced G1 phase arrest, whereas hsa_circ_0002468 inhibition promoted G1 transition (Figure 3H).

**MiR-561 promoted cell proliferation and reduced cell differentiation of SH-SY5Y cells**

To study the function of miR-561 in SH-SY5Y cells, we performed a BrdU assay. The results showed that the overexpression of miR-561 promoted cell proliferation of SH-SY5Y cells, whereas miR-561 inhibition markedly suppressed cell proliferation (Figure 4A). Furthermore, miR-561 overexpression significantly promoted Ki-67 and cyclin D1 mRNA levels, while miR-561 repression suppressed their mRNA levels (Figure 4B, 4C). Meanwhile, miR-561 overexpression significantly promoted the expression of GFAP mRNA in SH-SY5Y cells (Figure 4D); the ectopic expression of miR-561 reduced the mRNA level of Tuj1, whereas the ectopic expression of miR-561 promoted the Nestin mRNA level (Figure 4E, 4F). These results indicated that miR-561 promoted cell proliferation and reduced neuronal differentiation of SH-SY5Y cells.

**E2F8 was a direct target of miR-561**

Using bioinformatics analyses, we identified the putative binding sites for miR-561 within the 3’UTR of E2F8 mRNA (Figure 5A). The SH-SY5Y cells were co-transfected with miR-561 mimics, anti-miR-561 or a vector control with fluorescent reporter vectors (E2F8 3’UTR-WT or Mut) to perform the luciferase assay. The result showed that compared with the negative control, the luciferase intensity of E2F8 3’UTR-Mut was not altered by the transfection with anti-miR-561 in SH-SY5Y cells (Figure 5B). However, the luciferase intensity of E2F8 3’UTR-WT was reduced by miR-561 mimics, whereas the intensity increased by transfection with anti-miR-561 in SH-SY5Y cells (Figure 5B). Moreover, a correlation analysis indicated that miR-561 and E2F8 had a negative correlation in RA-exposed SH-SY5Y cells (Figure 5C). These data indicated that E2F8 was the direct target of miR-561 in SH-SY5Y cells during neuronal differentiation.
Figure 5. E2F8 is a direct target of miR-561. (A) The bioinformatics predictions of the matches between the mRNAs of E2F8 and miR-561. (B, C) SH-SY5Y cells were co-transfected with miR-561 mimics, anti-miR-561 or the vector control with the fluorescent reporter vectors (E2F8 3’UTR-WT or Mut) to perform the luciferase assays. (D, E) The detection of the E2F8 mRNA and protein levels using qRT-PCR in SH-SY5Y cells treated with RA for 0, 2, 4, and 8 days. (F) Pearson’s correlation analysis indicated a negative correlation between the expression levels of miR-561 and E2F8.

The miR-561/E2F8 axis regulated SH-SY5Y cell proliferation and differentiation

Furthermore, to confirm that the effect of miR-561 on cell proliferation and cell differentiation of SH-SY5Y cells was due to its regulation of E2F8, we performed functional rescue experiments. First, SH-SY5Y cells were co-transfected with miR-561 and either E2F8 or the control vector, and the mRNA and protein levels of E2F8 were examined by quantitative RT-PCR and western blot assays. The results showed that the overexpression of miR-561 could reduce E2F8 mRNA and protein levels in SH-SY5Y cells, and a rescue of the expression of E2F8 overexpression plasmid was effective in restoring E2F8 mRNA and protein levels (Figure 6A, 6B). Furthermore, the ectopic expression of E2F8 could reverse the inhibition effect of miR-561 on cell proliferation; also, the miR-561-mediated suppression of the GFAP and Tuj1 mRNA levels or the promotion of the Nestin mRNA level was restored by the overexpression of E2F8 (Figure 6C–6F). These results demonstrated that E2F8 was the mediator of the miR-561-induced repression of cell proliferation and of the promotion of cell differentiation in SH-SY5Y cells.

Discussion

CircRNAs are a class of endogenous noncoding RNAs that are formed by covalently closing the loop structures, and circRNAs regulate the expression of genes by interacting with miRNAs [10,24,25]. In this study, we found that hsa_circ_0002468 promoted the neuronal differentiation of SH-SY5Y cells and reduced cell proliferation. Moreover, our data showed that hsa_circ_0002468 promoted cell apoptosis but repressed the cell cycle of SH-SY5Y cells. Recent studies have shown that the main role of circRNAs is to bind to functional miRNAs to regulate gene expression [26,27]. We studied the potential mechanism by which hsa_circ_0002468 plays a role in SH-SY5Y cells. Bioinformatic analyses and luciferase reporter assays indicated that hsa_circ_0002468 can act as a sponge of miR-561 to regulate SH-SY5Y cell proliferation and differentiation. In addition, we found that miR-561 promoted cell proliferation and reduced cell differentiation of SH-SY5Y cells, and that miR-561 expression was inversely correlated with hsa_circ_0002468 expression. The study results showed that hsa_circ_0002468 could bind to miR-561 to regulate the proliferation and differentiation of SH-SY5Y cells.
Moreover, miRNAs have been shown to play their role by regulating the expression of their target genes [28]. In our study, we used bioinformatic analyses and luciferase reporter assays to demonstrate that E2F8 was a target of miR-561. In addition, we found there was an inverse relationship between miR-561 and E2F8 in RA-exposed SH-SY5Y cells. In our study, our results indicated that E2F8 was an important effector in the hsa_circ_0002468-induced neuronal differentiation of SH-SY5Y cells. To further validate that the role of hsa_circ_0002468 in SH-SY5Y cells was dependent on the axis of miR-561/E2F8, a series of functional rescue tests were performed. The results showed that the miR-561-mediated suppression of cell proliferation was reverse by the overexpression of E2F8. Additionally, the restoration of E2F8 expression abolished the effect on the suppression of the GFAP and Tuj1 mRNA levels or on the promotion of the Nestin mRNA level caused by miR-561.

**Conclusion**

In conclusion, our study indicated that hsa_circ_0002468 repressed cell proliferation and promoted the neuronal differentiation of SH-SY5Y cells by the miR-561/E2F8 axis. However, future studies are needed to better understand the detailed roles of the identified circRNAs in neuronal development and disease and to ascertain the contribution of hsa_circ_0002468 in the maintenance and establishment of neuronal differentiation.

**Conflict of interest**

None.

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