NFIX Circular RNA Promotes Glioma Progression by Regulating miR-34a-5p via Notch Signaling Pathway

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Objective: The present study aimed to explore the association between NFIX circular RNA (circNFIX) and miR-34a-5p in glioma. Furthermore, this study investigated the influence that circNFIX has on glioma progression through the upregulation of NOTCH1 via the Notch signaling pathway by sponging miR-34a-5p.

Methods: We applied five methods, CIRCexplorer2, circRNA-finder, CIRI, find-circ and MapSplice2, to screen for circRNAs with differential expression between three glioma tissue samples and three paired normal tissue samples. The GSEA software was used to confirm whether significantly different pathways were activated or inactivated in glioma tissues. The binding sites between circNFIX and miR-34a-5p were confirmed by TargetScan. QRT-PCR and western blot were used to measure the relative expression levels of circNFIX, miR-34a-5p and NOTCH and identify their correlation in glioma. RNA immunoprecipitation (RIP) validated the binding relationship between circNFIX and miR-34a-5p, while the targeted relationship between NOTCH1 and miR-34a-5p was verified by a dual luciferase reporter assay. Cell viability and mobility were examined by a CCK-8 assay and wound healing assay, and a flow cytometry assay was employed to analyze cell apoptosis. The nude mouse transplantation tumor experiment verified that si-circNFIX exerted a suppressive effect on glioma progression in vivo.

Results: Twelve circRNAs were differentially expressed between the tissue types. Of those, circNFIX was the sole circRNA to be overexpressed in glioma among the five methods of finding circRNAs. In addition, the Notch signaling pathway was considerably upregulated in tumor tissues compared with the paired normal brain tissues. It was determined that circNFIX acted as a sponge of miR-34a-5p, a miRNA that targeted NOTCH1. Downregulation of circNFIX and upregulation of miR-34a-5p, while the targeted relationship between NOTCH1 and miR-34a-5p was verified by a dual luciferase reporter assay. Cell viability and mobility were examined by a CCK-8 assay and wound healing assay, and a flow cytometry assay was employed to analyze cell apoptosis. The nude mouse transplantation tumor experiment verified that si-circNFIX exerted a suppressive effect on glioma progression in vivo.

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**INTRODUCTION**

Glioma, a tumor in the central nervous system, has a poor survival rate and high mortality (Zhang Y. et al., 2017). The latest report suggested that the incidence of glioma has increased from 5.9/100,000 people to 6.61/100,000 people between 1973 and 2016 with the application of improved radiological diagnosis (Lu et al., 2017). Despite multiple treatment options, cancer remains one of the leading causes of death worldwide. It is therefore imperative that further research be completed to determine novel molecular targets for enhanced cancer therapy (Wang R. et al., 2017).

Circular RNA (circRNA), a non-coding RNA with considerable regulatory potency, has received increased attention from RNA researchers in recent years (Li et al., 2015). Recently, it has been found that many circRNAs are derived from protein-coding exons and are widely expressed in cancer cells (Salzman et al., 2013). Most circRNAs originate from exons and are located in the cytoplasm (Venø et al., 2015). CircRNAs are formed by back-splicing covalently joined 3'- and 5'-ends (Zhang X. et al., 2017) and play a role in several cellular functions, including protein binding, RNA transport, and the regulation of translation (Zhang X. et al., 2017). Recently, it has been suggested that circRNAs play a critical role in late-stage gastric cancer (Fang et al., 2017). However, the influence of circRNAs in glioma needs further investigation.

MicroRNAs (miRNAs) are small noncoding RNAs that play critical roles in regulating various cellular functions by transcriptional silencing (Bezerra and Latronico, 2014). For instance, Zhou et al. (2017) proposed that miR-224 could target SMAD4 to promote colorectal cell propagation. It has been reported that miR-203 downregulates RGS17 to inhibit cell growth in non-small cell lung cancer (Chi et al., 2017). A growing body of research indicates that miRNAs can play key regulatory roles in glioma, uncovering novel biomarkers for glioma therapy. For example, a study by Xu L. et al. (2017) also indicated that miR-543 acted as a tumor suppressor that could inhibit glioma development in vitro and in vivo. MiR-34a is an essential member of the miR-34 family (Xu H. et al., 2017). Increasing evidence suggested that miR-34a is important in the research of glioma. Silber et al. (2012) verified that miR-34a significantly affected the growth of proneural glioma cells in vitro and in vivo. Li et al. (2014) found that upregulation of miR-34a inhibited glioma cell viability and promoted apoptosis. Gao et al. (2013)’s study also provided sufficient evidence for miR-34a as a potentially useful factor for predicting the prognosis of glioma. The present study investigates in greater depth the role of miR-34a-5p in glioma.

**Conclusion:** CircNFIX was markedly upregulated in glioma cells. CircNFIX could regulate NOTCH1 and the Notch signaling pathway to promote glioma progression by sponging miR-34a-5p via the Notch signaling pathway. This finding provided a deeper insight into the function of circNFIX in human glioma cancer progression.

**Keywords:** glioma, NFIX circular RNA, miR-34a-5p, NOTCH1, Notch signaling pathway

**MATERIALS AND METHODS**

**RNA-Seq Data**

Five kinds of blob selector were used in the experiment, including CIRCexplorer2, circRNA-finder, CIRI, find-circ and MapSplice2. RNA-Seq data were analyzed through CIRCexplorer2, circRNA-finder, CIRI, find-circ and MapSplice2 to screen differentially expressed circRNAs in glioma tissues. Gene Set Enrichment Analysis (GSEA) was performed with data from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. By combining in vivo and in vitro experiments, we confirmed that the Notch signaling pathway was activated in glioma tissues. We further examined the expression levels of circNFIX and miR-34a-5p in glioma tissues and cells and verified their association with glioma progression.
normal brain tissues from three males were downloaded from the Gene Expression Omnibus (GEO) database1, a publicly available database. The series accession number was GSE86202, and the platform was GPL16791. Data quality control (QC) was assessed with FastQC. GENCODEv19 Gene Transfer Format file was used as a transcript reference (GENCODE annotation). We used RNA-Seq to match sequencing data to the genome and then extracted the undirected comparison of fragments, followed by recombination and comparison with the genome.

Gene Expression Profiles

The DEseq2 package was used to analyze the differentially expressed circRNAs and mRNAs with the threshold set as \(|\log_2 FD| > 1\) and adjusted \(P < 0.05\) (FD: fold change). Through the Venn intersection analysis, 12 common circRNAs with differential expression including circNFIX were identified in glioma tumor tissues. The 10 most significantly upregulated mRNAs including NOTCH1 and the 10 most significantly downregulated mRNAs were screened out using the pheatmap package.

Gene Set Enrichment Analysis

GSEA was performed using data from the KEGG database. The expression data of total normalized mRNAs were uploaded to GSEA v3.0 software. Based on the analysis of GSEA, we also employed the R language “GSEABase” package to perform data processing. We used the ggplot2, DOSE, ggjoy, and clusterProfiler packages to construct the dotplot and joyplot.

Cell Culture

All cell lines used in the experiment including normal astrocytes HA1800 and human glioma cell lines SF-539, SHG-44 and U87 were obtained from BeNa Culture Collection (Beijing, China). The cell lines were cultured in high-glucose Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA), 100 U/ml penicillin and 100 µg/ml streptomycin and then incubated in a humidified atmosphere containing 5% CO₂ at 37°C.

Cell Transfection

Si-circNFIX, miR-34a-5p mimics and miR-34a inhibitor were synthesized by GenePharma Co., Ltd. (Shanghai, China). The sequence of si-circNFIX is CACACTCGGGAGTACCTCCA. Glioma cells were placed into a 6-well plate at a concentration of \(1 \times 10^5\) cells in each well and cultured at 37°C until 90% confluence was reached. Transfections were performed using the Lipofectamine 2000 kit (Invitrogen) according to the manufacturer’s instructions, and the transfection efficiency of the cells was detected after 24 h of incubation.

RNA Immunoprecipitation (RIP)

A biotin-labeled circNFIX probe (5'-CACCCGGTTCATCGAGG CACTGCTG-3'-biotin) was generated by Sangon Biotech Inc. (Shanghai, China). We performed RNA immunoprecipitation (RIP) experiments using the Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (Millipore, USA) according to the manufacturer’s instructions. U87 cells were first fixed for 10 min using 1% formaldehyde and then lysed and sonicated. Following centrifugation, 50 µl of supernatant was retained and incubated with circNFIX-specific probes-streptavidin Dynabeads (Invitrogen) and then incubated at room temperature for 24 h. Having washed the Dynabeads-probes-circRNA mixture and incubated it with 200 µl of lysis buffer, we utilized proteinase K to reverse the formaldehyde crosslinking. Lastly, TRIzol was added to the mixture for RNA extraction.

Luciferase Reporter Assay

The fragment from NOTCH1 containing the putative binding sites for miR-34a-5p was amplified by PCR, cloned in the firefly luciferase expression vector pMIR-REPORT (Invitrogen) and named NOTCH1-WT. To mutate the putative binding sites for miR-34a-5p in NOTCH1, the sequence of the putative binding site was replaced as indicated and was named NOTCH1-MUT. Before being stably transfected with the pMIR-REPORT-NOTCH1-WT and pMIR-REPORT-NOTCH1-MUT reporter vectors, together with the Renilla luciferase-expressing vector pRL-TK (Promega, Madison, WI, USA) and the miR-34a-5p mimic or NC using Lipofectamine™ TM 2000 (Thermo Fisher, USA), U87 cells were placed into a 24-well plate at a density of \(5 \times 10^5\) cells in each well. The relative luciferase reporter activity was detected at 48 h post-transfection.

QRT-PCR

We used TRIzol (Invitrogen, USA) to isolate the total RNA from cells according to the instructions. Real-time quantitative PCR (qRT-PCR) was used to measure the expression of miR-34a-5p, circNFIX, and NOTCH1. We used a TaqMan™ Advanced miRNA cDNA Synthesis Kit (#A28007, Applied Biosystems) to amplify the miRNA. CircRNA and mRNA were amplified with SuperScript™ VILO™ cDNA Synthesis Kit (#11754250, Invitrogen). Total RNA was detected by qRT-PCR using DyNaamo ColorFlash SYBR Green qPCR Kit (#F416XL, Invitrogen). Relative quantification of mRNA expression was normalized by the \(2^{-\Delta\Delta C_t}\) method, and GAPDH was used for normalization. All reactions were carried out in triplicate by GeneAmp™ PCR System 9700 (Applied Biosystems). The primers are manifested in Supplementary Table S1.

Western Blot

Radioimmunoprecipitation assay (RIPA) buffer was utilized to prepare whole-cell lysates. Equal amounts of total protein (30 mg) from cell lysates were loaded on a 6% sodium dodecyl sulfate–polyacrylamide gel for electrophoresis, after which they were transferred to a polyvinylidene difluoride membrane (Millipore). We used an enhanced chemiluminescence western blotting detection system (Bio-Rad) for detection. Primary antibodies used were those against NOTCH1 (#ab52627, 1:1000, Abcam, Hong Kong, China), Jagged1 (#ab7771, 1:500, Abcam), Hes1 (#ab71559, 1:500, Abcam), Hes5 (#ab194111, 1:2000, Abcam), and HEY2 (#ab86010, 1:1000, Abcam). Goat

1https://www.ncbi.nlm.nih.gov/geo/
anti-rabbit IgG secondary antibodies (ab7090, 1:2000, Abcam) were employed at 37°C for 1.5 h. The full original images have been uploaded as Supplementary Figure S1.

**Statistical Analysis**

All data analyses were performed using GraphPad Prism 6.0. The above experiments were carried out at least three times. Continuous data were documented as the mean ± standard deviation. The difference between two groups was analyzed by Student’s t-test. A P value < 0.05 was indicative of statistical significance.

**RESULTS**

**CircNFIX Expression and KEGG Pathway Analysis**

Five kinds of Blob Selector were used to single out the circRNA that we were interested in, and we found there were 12 common circRNAs that were differentially expressed (Figure 1A). The results of the heat map showed that the 12 circRNAs presented significantly differential expression in tumor tissues, in which circNFIX was confirmed to be overexpressed by all five software packages (Figures 1B–F). The above results suggested that circNFIX was significantly upregulated in glioma tissues. Dotplot displayed 16 significantly different pathways in tumor tissues, of which the Notch signaling pathway was found to be activated in glioma tissues (Figure 2A). Furthermore, Joyplot also further confirmed that the Notch signaling pathway was upregulated in tumor tissues (P.adjust < 0.05, Figure 2B). The results of the heat map showed 20 differentially expressed genes (DEGs) including 10 upregulated genes and 10 downregulated genes, of which NOTCH1 was remarkably overexpressed in glioma tissues in comparison with adjacent normal tissues (Figure 2C). By intersecting the screened DEGs with genes in the Notch signaling pathway, we identified that NOTCH1 was notably upregulated (Figure 2D). Overall, the Notch signaling pathway was activated in glioma tissues compared with paired normal brain tissues.

**Validation of Relationships Among circNFIX, miR-34a-5p and NOTCH1**

It is well known that circRNAs function mainly as miRNA sponges to regulate gene expression. We next found the potential miRNAs associated with circNFIX. The result in Figure 3A shows that several miRNAs containing binding sites with circNFIX existed; these miRNAs included miR-34a-5p, miR-526b, miR-646, miR-502-5p, miR-769-5p, miR-620, miR-874, miR-758-3p and miR-145-5p. Of the above miRNAs, miR-34a-5p was the closest to the 5′-UTR, drawing our interest. The binding sites between circNFIX and miR-34a-5p were validated by TargetScan (Figure 3B). Furthermore, in order to verify the relationship between circNFIX and miR-34a-5p in glioma cells, a RIP experiment was performed and confirmed that there was a specific enrichment of circNFIX and miR-34a-5p compared to the controls (Figure 3C). The qRT-PCR results showed that circNFIX was highly expressed in glioma cell lines SF539, SHG-44 and especially U87 cells compared to the normal astrocytes HA1800 (P < 0.01, Figure 4A). Meanwhile, the qRT-PCR results indicated that miR-34a-5p
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FIGURE 1 | NFIX circular RNA (CircNFIX) expression was overexpressed in glioma tissues compared to paired normal brain tissues. (A) Venn intersection analysis selected 12 common differentially expressed circRNA in glioma tissues. (B) CIRCexplorer2 analyzed the expression of the 12 genes. (C) CircRNA-finder analyzed the expression of the 12 genes. (D) CIRI analyzed the expression of the 12 genes. (E) Find-circ analyzed the expression of the 12 genes. (F) MapSplice2 analyzed the expression of the 12 genes.

was significantly downregulated in glioma cell lines (P < 0.01, Figure 4B). Then, we selected the U87 cell line for the following experiments. After transfection with si-circNFIX, the expression of circNFIX was conspicuously decreased, while miR-34a-5p expression was considerably increased compared with the NC group (P < 0.01, Figures 4C,D). Additionally, TargetScan revealed that NOTCH1 3’-UTR WT had a binding site for hsa-miR-34a-5p, while NOTCH1 3’-UTR MUT could not bind to hsa-miR-34a-5p (Figure 4E). Moreover, the luciferase reporter assay also indicated that miR-34a-5p mimics remarkably repressed the relative luciferase activity of NOTCH1 WT but not NOTCH1 MUT in comparison with the miR-NC group (P < 0.01, Figures 4E–F). Taken together, these findings indicate that circNFIX was upregulated in glioma cells and the expression of miR-34a-5p was significantly increased by knockdown of circNFIX.

Si-circNFIX and miR-34a-5p Mimics Inhibited the Expression of NOTCH1 and the Downstream Protein Expression in the Notch Signaling Pathway

The qRT-PCR results suggested that NOTCH1 was overexpressed in glioma cells compared with the HA1800 cell line, especially in U87 cells (P < 0.01, Figure 5A). Si-circNFIX and miR-34a-5p mimics suppressed the expression of NOTCH1, while the miR-34a-5p inhibitor promoted the expression of NOTCH1. Si-circNFIX could reverse the facilitative effects of the miR-34a-5p inhibitor on NOTCH1 expression (P < 0.05, Figure 5B). Similarly, the western blot results suggested that miR-34a-5p mimics and si-circNFIX suppressed the expression of the NOTCH1 protein, while the miR-34a-5p inhibitor promoted the expression of the NOTCH1 protein (P < 0.05, Figure 5C).
The downstream proteins Jagged1, Hes1 and HEY2 in the Notch signaling pathway were significantly downregulated in U87 cells after knocking down circNFX1, while the protein level of Hes5 was increased. It is probable that Notch-Hes signaling may have different impacts, depending on the glioma cell type or differentiation stage of the precursor cell (Wu et al., 2003; \( P < 0.01 \), Figure 5D). These results suggested that circNFX1 could regulate NOTCH1 at both the mRNA and protein levels by acting as a sponge for miR-34a-5p.

Si-circNFX1 and miR-34a-5p Mimics Inhibited Cell Migration, Proliferation and Promoted Cell Apoptosis

The wound healing assay is often used to estimate the coordinated movement of a cell population (Rodriguez et al., 2005). The results of the wound healing suggested that si-circNFX1 and miR-34a-5p mimics could inhibit cell migration and propagation, the miR-34a-5p inhibitor could promote wound healing, and the miR-34a-5p inhibitor could alleviate the suppressive effect of si-circNFX1 on cell propagation (Supplementary Figure S2). The migration assay showed that si-circNFX1 and miR-34a-5p mimics suppressed cell migration compared with the NC condition. The miR-34a-5p inhibitor could alleviate the suppressive impact of si-circNFX1 (\( P < 0.05 \), Figures 6A,D). As the flow cytometry assay showed, si-circNFX1 and miR-34a-5p mimics could promote cell apoptosis compared with the NC condition. Similarly, miR-34a-5p could alleviate the suppressive impact of si-circNFX1 (\( P < 0.05 \), Figures 6B,E). The CCK8 assay results suggested that the si-circNFX1 and miR-34a-5p mimics suppressed cell proliferation and miR-34a-5p inhibitor promoted cell proliferation (\( P < 0.05 \), Figure 6C). All above results demonstrate that downregulation of circNFX1 and overexpression of miR-34a-5p could suppress cell propagation and promote apoptosis.
Si-circNFIX Inhibited the Tumor Growth of Glioma in Vivo

All above experiments demonstrated that si-circNFIX could regulate NOTCH1 to promote glioma progression by sponging miR-34a-5p in vitro. To confirm that knockdown of circNFIX could suppress the tumor growth of glioma in vivo, U87 cells transfected with si-circNFIX (2 × 10⁶ cells) were injected into the right limbs of mice. Then, we found that the tumor volume and tumor weight containing si-circNFIX was significantly lower than those containing NC (Figure 7A). Then, the tumor tissues taken out from mice were used to detect the expression of circNFIX, miR-34a-5p and NOTCH1. The qRT-PCR results showed that the expression of circNFIX and NOTCH1 was significantly reduced in the si-circNFIX group compared with NC group, while miR-34a-5p was remarkably upregulated after treatment with si-circNFIX. These results indicated that si-circNFIX could repress the expression of circNFIX and NOTCH1 but promoted the expression of miR-34a-5p (P < 0.01, Figures 7B–D). Western blotting showed that circNFIX suppressed the expression of the NOTCH1 protein relative to that in the NC group (P < 0.01, Figure 7E). The results suggested that si-circNFIX exerted an inhibitory influence on glioma progression in vivo.

DISCUSSION

RNA-Seq, as a revolutionary tool for transcriptomics, has been applied in many experiments. For example, Mortazavi et al. (2008) predicted fusion transcripts based on the gene fingerprint profiles of the RNA-Seq paired-end reads (Li et al., 2017). Trapnell et al. (2009) discovered splice junctions with RNA-Seq.

Until recently, circRNAs have largely been considered insignificant by the scientific community due to low expression levels (Chen et al., 2016). However, several research breakthroughs have provided profound evidence that they play major roles in biological functions. For example, evidence suggests that they play a key role in gene regulation, suggesting that they could make viable therapeutic targets in diseases such as cancer (Liu et al., 2016). Therefore, circRNAs have received significantly greater attention in recent years in the study of cancer. For example, Guarnerio et al. (2016) discovered that fusion-circRNAs could both allow cellular transformation and promote cell proliferation and had a tumor-promoting impact in cancer. Zhang H. et al. (2017) found that circRNA UBAP2 (circUBAP2) expression was increased in human osteosarcoma tissues. Li et al. (2018) identified that circ_0046701 was overexpressed in glioma. The study conducted by Yang et al. (2016) also clarified that knockdown of cZNF292 circular RNA could suppress tube formation by inhibiting glioma cell proliferation. Much is still unknown about the exact roles circRNAs may play in glioma development. Thus, we completed an RNA-Seq analysis comparing circRNA expression between glioma and matched adjacent normal tissue to better understand their role in glioma cells. Five methods, including CIRCexplorer2, circRNA-finder, CIRI,
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**FIGURE 4** CircNFIX and miR-34a-5p presented differential expression in glioma cells. (A) QRT-PCR analyzed the expression of circNFIX in the glioma cell lines SF539, SHG-44 and U87 and the normal cells HA1800. (B) QRT-PCR analyzed the expression of miR-34a-5p. (C) The expression of circNFIX after transfection with si-circNFIX. (D) The expression of miR-34a-5p after transfection with si-circNFIX. (E) TargetScan predicted that NOTCH1 3’-UTR WT had a binding site for hsa-miR-34a-5p. (F) The luciferase reporter assay analyzed the target relationship between NOTCH1 and miR-34a-5p. **P < 0.01 compared with the NC group.

find-circ and MapSplice2, were used to screen out differentially expressed circRNAs. Via a Venn diagram, we found circNFIX was upregulated in all five methods. The focus of our project is to determine the role circNFIX plays in glioma progression in addition to uncovering the underlying mechanisms that drive it.

As some of the noncoding RNAs, especially miRNAs, can be biomarkers for glioma diagnosis and treatment (Ahir et al., 2017), we also studied the function of a miRNA (miRNA-34a-5p) that played a role in glioma progression affected by circNFIX. Numerous studies have reported an important role of miR-34a in inhibiting various cancers, and hence, it is often deemed as a tumor suppressor gene (Chen et al., 2015). For instance, Guessous et al. (2010) confirmed that miR-34a was downregulated in glioma tumors. Gao et al. (2013) found that glioma tissues had lower miR-34a expression than normal brain tissues did. In our current study, the expression level of miR-34a-5p was also downregulated in glioma, which suppressed the expression of the genes downstream of the signaling pathway.
A better understanding of the mechanism of circRNAs and miRNAs acting on glioma is meaningful for the discovery of effective therapies against glioma. Specifically, a large number of circRNAs have been verified to sponge miRNAs, thereby functioning as miRNA inhibitors. Bezzi et al. (2017) proposed that circRNA could function as a competing endogenous RNA,
Si-circNFIX and miR-34a-5p mimics inhibited cell migration and proliferation and promoted cell apoptosis. (A) The migration assay analyzed cell migration after the transfections. (B) Flow cytometry analyzed cell apoptosis after the transfections. Within the ellipse are apoptotic cells. (C) The CCK8 assay analyzed cell proliferation. (D) The cell migration cartogram presents cell migration levels. (E) The cell apoptosis cartogram presents cell apoptosis levels. *P < 0.05 compared with the NC group, #P < 0.05 compared with the si-circNFIX group.

In which different RNAs could regulate each other by means of competing for miRNAs through miRNA recognition elements. As Han et al. (2017) reported that the circRNA circMTO1 acted as a sponge of microRNA-9 to repress liver cancer progression. Liang et al. (2017) confirmed the sponge effect of circ-ABCB10 on miR-1271 in breast cancer. Militello et al. (2017) also verified that in addition to lncRNAs, circRNAs could also function as miRNA sponges. These results were consistent with our findings. In our study, circNFIX could suppress glioma progression by sponging miR-34a-5p. However, recent studies had questioned the sponge role that circRNAs play on miRNAs and reported that circRNAs could be translated or take part in tumor progression through a variety of methods. For instance, Legnini et al. (2017) and Pamudurti et al. (2017) reported that circRNAs could function as a eukaryotic endogenous circular RNAs and encode proteins. This may be due to their property that limits the election of the target region for duplication and further restriction of the parameters commonly used for designing mRNA-targeting siRNAs. In this study, we investigated circNFIX expression and its potential role as a microRNA sponge in vitro and in vivo, further supporting the sponge role of circRNAs.

Furthermore, we discovered that miR-34a-5p suppressed glioma progression in vitro by targeting the Notch signaling pathway, which was consistent with results that Jin et al. (2017) reported. In addition, Ji et al. (2008, 2009) found that miR-34 played an important role in cancer stem cell maintenance and survival. The above results all demonstrated that miR-34 could effectively regulate the Notch signaling pathway.

However, this study has several limitations. First, our study was based on U87 cells. Though the U87 cell line is a representative glioma cell type, primary glioma cells derived from patients will be used in our future research. Second, additional mechanisms of circNFIX in regulating glioma progression require further study.

Our research into the oncogenic role of circNFIX in glioma progression has uncovered possible mechanisms through which circRNAs influence cancer growth. However, due to the limited...
number of samples used in this study, we fully acknowledge that there may be additional circRNAs that play a key role that were not discovered in our data. However, our results suggest that the study of dysregulated circRNAs in cancer can prove fruitful to better understanding how to enhance cancer therapies.

AUTHOR CONTRIBUTIONS

HX contributed to the study design. YZ and LQ contributed most to experiments practice and data collection. LD and HY helped to analyze data and HJ mainly wrote the manuscript. All authors contributed to the revision and checked the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnmol.2018.00225/full#supplementary-material

FIGURE S1 | Full original images of western blots. The expressions of NOTCH1, HEY2, Hes5, Hes1 and Jagged1 were accessed by western blot.

FIGURE S2 | The wound healing assay analyzed cell proliferation after the transfections. ∗P < 0.05 compared with the NC group, #P < 0.05 compared with the si-circNFI group.

TABLE S1 | Primers for qRT-PCR.
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**Conflict of Interest Statement**: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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