Circular RNA hsa_circ_0001658 Inhibits Intervertebral Disc Degeneration Development by Regulating hsa-miR-181c-5p/FAS

Ge-dong Meng
Tianjin Medical University; The Second Affiliated Hospital of Inner Mongolia Medical University

Baoshan Xu (✉ baoshanxu99@tmu.edu.cn)
Tianjin Hospital https://orcid.org/0000-0002-4407-8745

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Abstract

**Background and purpose:**

Intervertebral disc degeneration (IDD) is the main cause of low back pain, but its pathogenesis has not been studied clearly. Circular RNA is a type of non-coding RNA (ncRNA). In this study, we aim to study the potential role of circular RNA in the pathogenesis of IDD.

**Methods:**

We obtained microarray data (GSE116726, GSE67566) from Gene Expression Omnibus database, and differentially analyzed ncRNA in nucleus pulposus (NP) tissues of IDD patients. The potential circRNAs-miRNAs-mRNAs regulatory network was analyzed by starBase. The effect of the interaction between hsa_circ_0001658, hsa-miR-181c-5p and FAS on the proliferation and apoptosis of human neural progenitor cells (hNPCs) were studied.

**Results:**

Hsa_circ_0001658 was significantly up-regulated (logFC>2.0 and adj.P.Val<0.01) in the NP tissues of IDD patients, and hsa-miR-181c-5p expression was down-regulated (logFC<-2.0 and adj.P.Val<0.01). Silencing of hsa-miR-181c-5p or overexpression of hsa_circ_0001658 inhibited the proliferation of hNPCs and promoted their apoptosis. Hsa_circ_0001658 acted as a sponge of hsa-miR-181c-5p. Hsa-miR-181c-5p down-regulated the expression of FAS, promoted the proliferation and inhibited the apoptosis of hNPCs. Hsa_circ_0001658 functioned in hNPCs through targeting hsa-miR-181c-5p/FAS.

**Conclusion:**

Circular RNA hsa_circ_0001658 inhibits intervertebral disc degeneration development by regulating hsa-miR-181c-5p/FAS. It is expected to be a potential target for the therapy of IDD.

Introduction

IDD is the pathological basis of many intervertebral disc-related diseases, including lumbar disc herniation, lumbar spinal stenosis, cervical spondylosis, degenerative scoliosis, degenerative spinal instability, etc.[1–4]. The onset of IDD is related to a variety of environmental factors, such as smoking, age, diabetes, gender, etc.[5–7]. In recent years, with the change of lifestyle and the aging of the population, the incidence of IDD has increased significantly[8–10], studying the mechanism of IDD is of great significance to the prevention and treatment of IDD.

CircRNAs are a special type of non-coding RNA molecules, which are in a closed circular structure and not affected by RNA exonuclease. Compared with linear RNA molecules, circRNAs are more stable and not easily degraded[11]. Functional studies have shown that circRNAs contain a large number of microRNAs (miRNAs) binding sites, which act as miRNAs sponges in cells to eliminate or weaken miRNAs inhibition.
of their target genes, increase the expression level of target genes, and act as a competitive endogenous RNA (ceRNA)\[12, 13\].

The current methods of IDD treatment include medication, physical therapy, and surgery, but these are not optimal\[14\]. Considering that there is a potential ceRNA relationship between circRNAs and mRNA in IDD, we can accurately and efficiently predict and treat IDD by adjusting the competitive relationship between these RNAs.

Therefore, in this study, we studied the differential expression of circRNAs and miRNAs in degraded NP tissue, and constructed a circRNAs-miRNAs-mRNAs axis by bioinformatics techniques. Through in vitro experiments to verify the potential relationship between the circRNAs-miRNAs-mRNAs axis and IDD, and provide a potential basis for the prevention and treatment of IDD.

**Materials And Methods**

**Dataset**

We obtained the information of the GSE116726 dataset in the GEO database and analyzed the expression level of miRNAs in NP tissues of IDD patients and fresh traumatic lumbar fracture patients\[15\]. We obtained the information of the GSE67566 dataset and analyzed the expression level of circRNAs in the NP tissues of IDD patients and normal NP tissues\[16\].

**Cell culture**

The hNPCs (ScienCell™, Carlsbad, CA, USA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA). The hNPCs were cultured in a 37°C constant temperature incubator containing 5% CO₂. When the cells are 80% fused, they are digested with trypsin (Hy-Clone, Logan, UT, USA) and subcultured at a ratio of 3:1. The hNPCs from passage 3 to 5 were using for all of the experiments. In some assays, hNPCs were treated by 5 ng/ml of TNF-α+IL-1β (Sigma-Aldrich, St. Louis, MO, USA) for 12 h, with untreated hNPCs as controls.

**Luciferase Reporter Assay**

Binding sites of hsa-miR-181c-5p on hsa_circ_0001658 or FAS were verified by the dual luciferase reporter assay. HEK293 cells were co-transfected with hsa_circ_0001658 or FAS recombinant plasmids (hsa_circ_0001658 WT1/Mut1/WT2/Mut2 and FAS Wt/Mut) and hsa-miR-181c-5p mimic or miRNA mimic negative control (NC) using lipofectamine 2000 (Invitrogen, Carlsbad, CA). After transfection for 48h, the luciferase activity was determined by the dual luciferase assay kit (Promega, Madison, WI).

**Quantitative real-time PCR (qRT-PCR)**
Total RNA was isolated from hNPCs with Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). Then the extracted RNA was reverse transcribed into cDNA using PrimeScript RT Master Mix (TaKaRa, Dalian, China). The SYBR Premix Ex Taq II Kit (TaKaRa, Dalian, China) was used to detect the expression of hsa_circ_0001658 and hsa-miR-181c-5p by qRT-PCR assays. The primers of hsa_circ_0001658 is F: 5'-GAGGATGCAGCCTTTGGACT-3', R: 5'-GTCTGAAGCGGGGACGTT TA-3'. The primers of hsa-miR-181c-5p is F: 5'-GGGAACATTCAACCTGTCG-3', R: 5'-GTGCGTGTCGTGGAGTCG-3', the primers sequence of β-actin is F: 5'-GGACTCGTCATACTCCTGCTTG-3', R: 5'-GGAAATCGTGCGTGACATTAAG-3'. The expression levels of hsa_circ_0001658 and hsa-miR-181c-5p were normalized to the expression of β-actin by the $2^{-\Delta\Delta C_t}$ methods.

**Cell transfections**

Hsa-miR-181c-5p mimic, hsa-miR-181c-5p inhibitor, hsa_circ_0001658, hsa_circ_0001658 small interfering RNA (si-hsa_circ_0001658), hsa_circ_0001658+hsa-miR-181c-5p mimic, FAS overexpression plasmid (pc-FAS), FAS short hairpin RNA (sh-FAS), pc-FAS+hsa-miR-181c-5p mimic, blank plasmid (NC) were transfected into 5 ng/ml of TNF-α+IL-1β treated hNPCs respectively by lipofectamine 2000. Cells were collected and stored at -80°C when transfected for 24h.

**Cell Counting Kit-8 (CCK-8) assay**

The cells of each group were inoculated into 96-well plates at a density of $2\times10^5$ cells/well, and single-cell suspensions were made with DMEM medium containing 10%FBS and cultured at 37°C and 5% CO₂. There are 3 replicate wells in each group. When the cells grow to 80% confluence, add 20μL of CCK-8 solution 72 h after transfection. Incubate for 24h, and detect the absorbance (A) value at 490 nm in the microplate reader. Take the average of the A values of 3 wells and calculate the relative cell viability according to the following formula: Relative cell viability (%) = treatment group A/control group A×100%.

**Western blot**

The bicinchoninic acid (BCA) assay method was used for protein quantification. A total of 30μL of each sample was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and then the protein was transferred to Poly vinylidene fluoride (PVDF) membrane, then soaked the PVDF membrane in Tween-Tris buffered saline (TTBS) containing 5% non-fat milk at room temperature for 2 hours, and then incubated with the primary antibody as follows: mouse anti-human matrix metalloproteinase-3 (MMP-3), MMP-13, collagen II, aggrecan, FAS and GAPDH (dilution 1:1000, Abcam, Berlin, Germany) overnight at 4°C. Washed the PVDF membranes 3 times with TTBS and then incubated with HRP-labeled rabbit anti-mouse secondary antibody (dilution 1:10000, Abcam, Berlin, Germany) for 2h at room temperature. The enhanced chemiluminescence (ECL) kit (Amersham Pharmacia Biotech,
Chandler, AZ, US) was used to visualize and the relative integrated density values of the proteins were calculated based on GAPDH as an internal control.

**Flow cytometry**

The cells of each group were seeded into a 6-well plate at a density of $4 \times 10^5$ cells/well, washed with pre-cooled PBS, centrifuged and discarded the supernatant, and added 200μL Binding Buffer to the cell suspension. Add 5μL Annexin V-FITC and incubate at room temperature for 10 min, wash with 190ul Binding Buffer and resuspend in 190ul Binding Buffer, then add 10μL propidium iodide (PI) and mix well. Detect cell apoptosis on the BD Fortessa flow cytometer (Becton Dickinson, Mountain View, CA, USA).

**Statistical analysis**

In this study, SPSS 26.0 (SPSS, Chicago, IL, USA) was used for statistical analysis. The experimental data was represented by mean±standard deviation (SD). Statistical analysis used 2-tailed t test or analysis of variance (ANOVA). Correlation analysis used Pearson correlation analysis. P<0.05 meant significant difference.

**Result**

**Differential analysis of non-coding RNA in NP tissues of IDD patients**

Among the 2549 miRNAs detected in the miRNAs microarray (GSE116726 dataset), 56 miRNAs in degenerated NP tissue were up-regulated (logFC>2.0 and adj.P.Val<0.01), and 8 miRNAs were down-regulated (logFC< -2.0 and adj.P.Val<0.01) (Figure 1A). In addition, we found that these 8 miRNAs were not only down-regulated in the NP tissues of IDD patients (Figure 1B), but also significantly down-regulated in hNPCs induced by 5ng/ml TNF-α+IL-1β (Figure 1C).

Among the 2894 circRNAs detected in the circRNAs microarray (GSE67566 dataset), 48 circRNAs in the degenerated NP tissue were up-regulated (logFC>2.0 and adj.P.Val<0.01) and 58 circRNAs were down-regulated (logFC<2.0 and adj.P.Val<0.01) (Figure 1D).

**Hsa-miR-181c-5p silence or hsa_circ_0001658 over-expression inhibited the proliferation and promoted apoptosis of hNPCs**

The results of starBase (version 2.0) analysis showed that among the down-regulated miRNAs, hsa-miR-181c-5p has the binding site of hsa_circ_0001658 (Figure 2A). Hsa_circ_0001658 was significantly up-
regulated in the NP tissues of IDD patients (logFC>2.0 and adj.P.Val<0.01), so we chose hsa-miR-181c-5p and hsa_circ_0001658 for research.

In 5ng/ml TNF-α+IL-1β treated hNPCs, the expression of hsa-miR-181c-5p was significantly down-regulated, while hsa-miR-181c-5p mimic or si-hsa_circ_0001658 significantly reversed the expression of hsa-miR-181c-5p (Figure 2B). In addition, we found that hsa-miR-181c-5p inhibitor or hsa_circ_0001658 significantly inhibited the proliferation of hNPCs (Figure 2C) and promoted the apoptosis of hNPCs (Figure 2D), up-regulated the expression of MMP-3 and MMP-13, inhibited the expression of collagen II and aggrecan, and all these effects could be reversed by hsa-miR-181c-5p mimic or hsa_circ_0001658 (Figure 2E).

These results indicated that hsa-miR-181c-5p silence or hsa_circ_0001658 over-expression inhibited the proliferation of hNPCs and the metabolic function of extra-cellular matrix (ECM), but promoted the apoptosis of hNPCs.

**Hsa_circ_0001658 acted as a sponge of hsa-miR-181c-5p**

We tried to study the mechanism of hsa_circ_0001658 and hsa-miR-181c-5p in the development of IDD. According to research reports, the function of circRNAs related to IDD were mainly as a sponge of miRNAs to combine with functional miRNAs[17, 18], and then regulated the proliferation and apoptosis of NP cells. Therefore, starBase was used to predict the relationship between hsa_circ_0001658 and hsa-miR-181c-5p. The results showed that there was a binding site of hsa_circ_0001658 on hsa-miR-181c-5p (Figure 2A). We found that the expression level of hsa-miR-181c-5p in the NP tissue cells of IDD patients was low, but hsa_circ_0001658 was highly expressed. In 5ng/ml TNF-α+IL-1β treated hNPCs, hsa-miR-181c-5p was low expression, while hsa_circ_0001658 was high, and there was a negative correlation between them (R²=0.8967, P=0.0146, Figure 3A). The results suggested that hsa_circ_0001658 may regulate the expression of hsa-miR-181c-5p by directly sponging hsa-miR-181c-5p.

To verify this hypothesis, we performed luciferase reporter assay. Considering that there were two hsa-miR-181c-5p binding sites on hsa_circ_0001658, we set mutation sequences for these two binding sites respectively. Hsa_circ_0001658 WT1, hsa_circ_0001658 WT2, hsa_circ_0001658 Mut1 and hsa_circ_0001658 Mut2 sequences were inserted to the downstream of the luciferase reporter molecule. Then the hsa-miR-181c-5p mimic and luciferase reporter gene were co-transfected into hNPCs. Compared with the control group (NC), hsa-miR-181c-5p mimic significantly reduced the luciferase activity in the hsa_circ_0001658 WT1 and hsa_circ_0001658 WT2 groups (P<0.05), but not in the hsa_circ_0001658 Mut2 and hsa_circ_0001658 Mut2 groups (P>0.05) (Figure 3B, 3C). These results indicated that hsa-miR-181c-5p bound to two predicted sites on hsa_circ_0001658.

To determine whether hsa_circ_0001658 affected the proliferation and apoptosis of hNPCs by sponging hsa-miR-181c-5p, we used hsa_circ_0001658 overexpression vector to transfet hNPCs, and qRT-PCR results showed that hsa_circ_0001658 overexpressed hNPCs were successfully constructed (Figure 3D).
The expression level of hsa_circ_0001658 in the NP tissues of IDD patients was significantly increased (Figure 3E). In this study, we found that, compared with the control, hsa_circ_0001658 over-expression inhibited the proliferation and promoted the apoptosis of hNPCs, and these effects were reversed by hsa-miR-181c-5p mimic (Figure 3F, 3G).

These results showed that hsa_circ_0001658 acted as a sponge of hsa-miR-181c-5p.

Hsa-miR-181c-5p down-regulated the expression of FAS, promoted the proliferation and inhibited the apoptosis of hNPCs

As a member of the TNF receptor super-family, the protein encoded by FAS plays a central role in the physiological regulation of programmed cell death, and has been involved in the onset of various malignant tumors and immune system diseases[19-21]. The bioinformatics prediction results showed that FAS was a potential target of hsa-miR-181c-5p (Figure 4A). The results of dual luciferase reporter gene detection showed that the luciferase signal of wild-type FAS reporter gene was significantly inhibited by hsa-miR-181c-5p, however, the luciferase signal of the mutant FAS reporter gene was not significantly affected by hsa-miR-181c-5p (Figure 4A). The results of loss-of-function and gain-of-function experiments showed that hsa-miR-181c-5p mimic significantly inhibited the expression of FAS in hNPCs, while the expression of FAS in hNPCs increased significantly after treatment with hsa-miR-181c-5p inhibitor (Figure 4B). After treated with 5ng/ml TNF-α and IL-1β, the FAS expression level in hNPCs was significantly increased, and hsa-miR-181c-5p mimic treatment could significantly reverse the decrease in FAS expression level (Figure 4C). In addition, after overexpression of FAS, the proliferation ability of hNPCs was significantly decreased, the apoptosis rate was significantly increased, the expression levels of MMP-3 and MMP-13 proteins were up-regulated, the expressions of collagen II and aggrecan were down-regulated, while the hsa-miR-181c-5p mimic transfection reversed the effects of FAS overexpression on hNPCs proliferation, apoptosis and expression of MMP-3, MMP-13, collagen II and aggrecan (Figure 4D, 4E, 4F).

These results demonstrated that hsa-miR-181c-5p down-regulated the expression of FAS, promoted the proliferation and inhibited the apoptosis of hNPCs, and at the same time inhibited catabolic reactions and promoted the expression of ECM compositions.

Hsa_circ_0001658 functioned in hNPCs through targeting hsa-miR-181c-5p/FAS

Adenovirus carrying hsa_circ_0001658, hsa_circ_0001658 siRNA or blank control (si-NC) was transfected into hNPCs, the results of qRT-PCR showed that when the exogenous hsa_circ_0001658 overexpression vector was transfected, the hsa_circ_0001658 level in hNPCs was significantly increased. On the contrary,
the hsa_circ_0001658 expression level was significantly inhibited after hsa_circ_0001658 siRNA transfection (Figure 5A). The results of western blot analysis showed that overexpression of hsa_circ_0001658 caused an increase in the expression level of FAS protein in hNPCs, and the change in FAS expression level was reversed by hsa-miR-181c-5p mimic (Figure 5B). When hNPCs were treated with 5ng/ml TNF-α and IL-1β, the expression levels of hsa_circ_0001658 and FAS increased significantly, while the expression of hsa-miR-181c-5p decreased, these effects were reversed after the transfection with si-hsa_circ_0001658 (Figure 5C, 5D). Next, we studied whether FAS acted as a downstream mediator of hsa_circ_0001658 in 5ng/ml TNF-α and IL-1β treated hNPCs. The results showed that si-hsa_circ_0001658 and FAS knockdown significantly promoted the proliferation and inhibited the apoptosis of 5ng/ml TNF-α and IL-1β treated hNPCs (Figure 5E, 5F).

Based on the above results, we confirmed that hsa_circ_0001658 functioned in hNPCs through targeting hsa-miR-181c-5p/FAS.

**Discussion**

In this study, we obtained microarray data from the GEO database, and combined with bioinformatics analysis, we found that hsa_circ_0001658/hsa-miR-181c-5p/FAS had a potential interaction. Through *in vitro* research experiments, we confirmed that hsa_circ_0001658 inhibited the proliferation of hNPCs and promoted their apoptosis by regulating hsa-miR-181c-5p/FAS axis.

Previous studies on post-transcriptional regulation mainly focused on the inhibitory effect of miRNAs on mRNAs. Studies have confirmed that miR-133a inhibited the degradation of type II collagen by targeting MMP-9, and inhibited the occurrence and development of IDD[22]. The function mechanism of ceRNA as a brand-new RNA post-transcriptional regulation method has attracted the attention of researchers. It is known that miRNA can inhibit the translation of the target gene or degrade it by binding to the response element of the 3’untranslated region of them[23, 24]. Various types of RNA can use miRNA as a bridge to achieve mutual regulation. This regulation mode can form a ceRNA regulatory network in the cell. The discovery of this mechanism provides a new idea for the study of post-transcriptional regulation[25, 26]. Recent studies have shown that circRNAs, as miRNAs sponges, can participate in the occurrence and development of various diseases[27-29].

In this study, we selected and analyzed the data of GSE116726 dataset and GSE67566 dataset from the GEO database. Combined with bioinformatics technology, we found that there was a binding site of hsa_circ_0001658 on hsa-miR-181c-5p. At the same time, we found that the expression level of hsa-miR-181c-5p was low in NP tissue of IDD patients, but hsa_circ_0001658 was highly expressed. And after treatment with 5ng/ml TNF-α+IL-1β, hsa-miR-181c-5p was low expression in hNPCs, while hsa_circ_0001658 was high expression, and they are negatively correlated. It suggested that there was a ceRNA regulatory network between hsa_circ_0001658 and hsa-miR-181c-5p.

The results of *in vitro* experiments showed that hsa_circ_0001658 acted as a sponge of hsa-miR-181c-5p, and hsa-miR-181c-5p silence or hsa_circ_0001658 over-expression inhibited proliferation and promoted
apoptosis of hNPCs. The results of this study confirmed that hsa_circ_0001658 was the ceRNA of hsa-miR-181c-5p. Further research found that hsa-miR-181c-5p promoted the proliferation and inhibited the apoptosis of hNPCs by down-regulating the expression of FAS. Therefore, we inferred that hsa_circ_0001658 played a role in the occurrence of IDD by targeting hsa-miR-181c-5p/FAS.

We know that human NP cells are diverse in morphology, can synthesize extra-cellular matrix components, phagocytose substances through phagocytosis or autophagy, mitochondrial vacuolation indicates dysfunction, and express FAS and FASL as important immune privilege sites[30]. The characteristic of IDD is that the dysfunctional FASL reduce the expression level of FASL, unbalance the interaction between NP cells and immune cells, leading to certain regulatory factors that may play a role in this process[30], such as hsa_circ_0001658 and hsa-miR-181c-5p in this study. Targeting FAS to regulate its expression leads to an imbalance in the interaction between the FAS-FASL network of NP cells and immune cells, which may be one of the reasons leading to IDD. This is also a possible clinical application direction for IDD stem cell therapy.

There are some shortcomings in this study. First of all, the genetic background and age differences between the source of the obtained degenerated nucleus pulposus tissue and the control group may cause us to bias the ncRNA differences analyzed in this study. Secondly, in addition to ceRNA, whether hsa_circ_0001658 has other mechanisms to regulate the production of IDD is still unclear. At present, we lack more understanding of hsa_circ_0001658. Thirdly, we believe that in vivo experiments are one of the key ways to solve our current confusion, and we need to supplement the results of in vivo research.

In summary, circular RNA hsa_circ_0001658 inhibited IDD development by regulating hsa-miR-181c-5p/FAS. It is expected to be a potential target for the therapy of IDD.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets during and/or analysed during the current study available from the corresponding author on reasonable request.
Competing interests

The authors declare that they have no competing interests.

Funding

Not applicable

Authors' contributions

Baoshan Xu designed the research study and contributed essential reagents or tools; Gedong Meng did the experiment and analysed the data; Baoshan Xu and Gedong Meng wrote the paper.

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**Figures**

![Figure 1](image-url)
IDD-related non-coding RNA differentiation analysis. A, Volcano Plot of the differential expression of microRNAs in NP tissues of IDD patients. B, qRT-PCR analysis of down-regulated microRNAs expression levels in NP tissues of IDD patients. C, qRT-PCR analysis of the expression levels of down-regulated microRNAs in hNPCs (Control) not treated with 5ng/ml TNF-α and IL-1β and hNPCs treated with 5ng/ml TNF-α and IL-1β. D, Volcano Plot of differential expression of circRNAs in the GSE67566 dataset. *P<0.05, compared with Control.
hsa-miR-181c-5p silence or hsa_circ_0001658 overexpression inhibited the proliferation and promoted apoptosis of hNPCs. A, the binding sites in hsa_circ_0001658 and hsa-miR-181c-5p predicted by starBase. B, the hsa-miR-181c-5p level in hNPCs (Control), 5ng/ml TNF-α and IL-1β treated hNPCs, 5ng/ml TNF-α and IL-1β treated hNPCs combined with hsa-miR-181c-5p mimic transfection, 5ng/ml TNF-α and IL-1β treated hNPCs combined with si-hsa_circ_0001658 transfection were detected by qRT-PCR. C, the proliferation ability of 5ng/ml TNF-α and IL-1β treated hNPCs, 5ng/ml TNF-α and IL-1β treated hNPCs transfected with hsa-miR-181c-5p inhibitor, and 5ng/ml TNF-α and IL-1β treated hNPCs combined with hsa_circ_0001658 transfection were detected by Cell Counting Kit-8 (CCK-8). D, the apoptosis rates of 5ng/ml TNF-α and IL-1β treated hNPCs, 5ng/ml TNF-α and IL-1β treated hNPCs transfected with hsa-miR-181c-5p inhibitor, and 5ng/ml TNF-α and IL-1β treated hNPCs combined with hsa_circ_0001658 transfection were detected by Flow cytometry. E, the expression level of MMP-3, MMP-13, collagen II, and aggrecan in 5ng/ml TNF-α and IL-1β treated hNPCs, 5ng/ml TNF-α and IL-1β treated hNPCs transfected with hsa-miR-181c-5p inhibitor, and 5ng/ml TNF-α and IL-1β treated hNPCs combined with hsa_circ_0001658 transfection were detected by western blot. *P<0.05, **P<0.01, ***P<0.001 compared with Control.

Figure 3
Hsa_circ_0001658 acted as a sponge of hsa-miR-181c-5p. A, The correlation between the expression levels of hsa-miR-181c-5p and hsa_circ_0001658 in NP tissue cells of IDD patients. B and C, relative luciferase activity was conducted after hsa_circ_0001658 WT1, hsa_circ_0001658 WT2, hsa_circ_0001658 Mut1, hsa_circ_0001658 Mut2 and hsa-miR-181c-5p mimic co-transfection into hNPCs. D, the relative expression level of hsa_circ_0001658 in hNPCs with no template control (NC), hsa_circ_0001658 overexpression vector transfection was detected by qRT-PCR. E, the relative expression level of hsa_circ_0001658 in NP tissues of IDD patients (data from GSE67566 dataset). F, the cell proliferation of NC, hsa_circ_0001658, hsa_circ_0001658+hsa-miR-181c-5p mimic co-transfected hNPCs were detected by Cell Counting Kit-8 (CCK-8). G, the apoptotic rate of NC, hsa_circ_0001658, hsa_circ_0001658+hsa-miR-181c-5p mimic co-transfected hNPCs were detected by flow cytometry. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Figure 4

hsa-miR-181c-5p down-regulated the expression of FAS, promoted the proliferation and inhibited the apoptosis of hNPCs. A, FAS and hsa-miR-181c-5p targeting binding site prediction and comparison of luciferase activity in hNPCs for no template control (NC), hsa-miR-181c-5p mimic+FAS Wt, hsa-miR-181c-5p mimic+FAS Mut transfection. B, the relative expression level of FAS protein in hNPCs for NC, hsa-miR-
181c-5p mimic, hsa-miR-181c-5p inhibitor transfection were detected by western blot. C, the relative expression level of FAS protein in hNPCs for control group (Control), 5ng/ml TNF-α and IL-1β treated hNPCs, 5ng/ml TNF-α and IL-1β treated and transfected with hsa-miR-181c-5p mimic were detected by western blot. D, the cell proliferation of hNPCs for the NC group, FAS overexpression (pc-FAS) and pc-FAS+hsa-miR-181c-5p mimic transfection were detected by Cell Counting Kit-8 (CCK-8). E, the apoptosis rate of hNPCs for the NC group, pc-FAS and pc-FAS+hsa-miR-181c-5p mimic transfection were detected by flow cytometry. F, the protein expression levels of MMP-3, MMP-13, collagen II, aggrecan in hNPCs for the NC group, pc-FAS and pc-FAS+hsa-miR-181c-5p mimic transfection were detected western blot. *P<0.05, **P<0.01, ***P<0.001.
hsa_circ_0001658 functioned in hNPCs through targeting hsa-miR-181c-5p/FAS. A, hsa_circ_0001658 relative expression level in hNPCs for no template control (si-NC), hsa_circ_0001658 overexpression vector, hsa_circ_0001658 siRNA transfection was detected by qRT-PCR. B, the relative expression level of FAS protein in hNPCs for NC, hsa_circ_0001658 overexpression vector, hsa_circ_0001658+ hsa-miR-181c-5p mimic transfection were detected by western blot. C, the relative expression levels of
hsa_circ_0001658 in hNPCs (Control), 5ng/ml TNF-α and IL-1β treated hNPCs (NC), 5ng/ml TNF-α and IL-1β treated hNPCs for hsa_circ_0001658 siRNA transfection were detected by qRT-PCR. D, the relative FAS protein expression level of Control, NC, 5ng/ml TNF-α and IL-1β treated hNPCs for hsa_circ_0001658 siRNA transfection was detected by western blot. E, the cell proliferation of 5ng/ml TNF-α and IL-1β treated hNPCs for no temple control (NC), si-hsa_circ_0001658, FAS short hairpin RNA (sh-FAS) transfection was detected by Cell Counting Kit-8 (CCK-8) assay. F, the cell apoptosis rate of 5ng/ml TNF-α and IL-1β treated hNPCs for NC, si-hsa_circ_0001658, sh-FAS transfection was detected by flow cytometry. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.