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RESEARCH PAPER

miR-222-3p is involved in neural tube closure by directly targeting Ddit4 in RA induced NTDs mouse model

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ABSTRACT

Previously our results showed miR-222-3p was significantly downregulated in retinoic acid-induced neural tube defect (NTD) mouse model through transcriptome. Down-regulation of miR-222-3p may be a causative biomarker in NTDs. In this study, RNA was extracted from mouse embryos at E8.5, E9.5 and E10.5, and the expression level of miR-222-3p was measured by quantitative real-time PCR analysis. The preliminary mechanism of miR-222-3p in NTDs involved in cell proliferation, apoptosis and migration was investigated in mouse HT-22 cell line. The expression of miR-222-3p was significantly decreased at E8.5, E9.5 and E10.5 developed in mouse embryos which were consistent with our transcriptome sequencing. Suppression of miR-222-3p in HT-22 cells resulted in the inhibition of cell proliferation and migration, cell cycle and apoptosis. Moreover, DNA damage transcript 4 (Ddit4) was identified as a direct and functional target of miR-222-3p. miR-222-3p is negatively regulated by Ddit4. The mutation of binding site of Ddit4 3′UTR abrogated the responsiveness of luciferase reporters to miR-222-3p and showed that Ddit4 expression partially attenuated the function of miR-222-3p. We preliminatively confirmed that low expression of miR-222-3p has reduced the expression of β-catenin, TCF4 and other related genes in the Wnt/β-catenin signaling pathway.

Collectively, these results demonstrated that miR-222-3p regulates the Wnt/β-catenin signaling pathway through Ddit4 inhibition in HT-22 cells, resulted in cell proliferation and apoptosis imbalance, and thus led to neural tube defects.

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miR-222-3p; DNA damage transcript 4 (Ddit4); Neural Tube Defects (NTDS); wnt/β-catenin signaling pathway; apoptosis

1. Introduction

Neural tube defects (NTDs) are the most common (second in occurrence after congenital heart disease) and serious birth defects of central nervous system [1], due to failure of complete neural tube closure during embryonic development [2]. World-wide, it has been estimated that approximately 300,000 NTDs cases are reported each year [3]. According to the World Health Organization estimate, the incidence rate of birth defects in China is about 5.6%, which is close to the average level of middle-income countries in the world [4]. Li et al. has reported that prevalence of NTDs tends to be higher in northern China (18.7 per 10,000 births) than in southern China (9.7 per 10,000 births) [5]. The clinical manifestations of NTDs have two main sub-groups (open and closed defects) NTDs including anencephaly, spina bifida and myelomeningocele [5–8]. NTDs are multifactorial disorders induced both by genetic and environmental factors [9–11]. Despite many etiological studies in humans [11–15], pathogenesis of NTDs remains poorly understood.Table 1

Retinoic acid (RA) is a vitamin A metabolite and nutritionally derived small molecule involved in anterior-posterior and neural tube patterning [16,17]. Excess or insufficient RA levels can lead to neural tube abnormalities which have been extensively investigated in the developmental toxicity with retinoids in animal models, as well as in humans after taking RA in multivitamin preparations during pregnancy [18–21]. During early embryogenesis (E7-E10), excessive RA exposure

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Table 1. List of primers used.

Gene	forward primer (5′-3′)	reverse primer (5′-3′)
Ddit4	GATGCCTAGCCAGTTGGTAAG	CTAAACAGCCCCTGGATCTTG
β-actin	GCTCTTTTCCAGCCTTCCTT	AGGTCTTTACGGATGTCAAGG
miR-222-3p	GAAAGTTCGTCCAGCTACATCTG	TATGGTTGTTCTCGTCTCTGTGTC
U6	CAGCACATACTAAAATTGGAACG	ACGAATTTGCGTGTCATCC

can lead to NTDs, including anencephaly, exencephaly and spina bifida [16,22].

MicroRNAs (miRNAs) are non-coding RNAs, composed of approximately 22 nucleotides which regulate gene expression by complementarily binding to the 3′UTR sequence of their target mRNA and thus lead to mRNA degradation or translational repression. MiRNAs are widely involved in crucial life processes such as cell cycle, embryonic development, cell growth and differentiation, apoptosis, stress response, metabolism and morphogenesis [23–26]. It has been confirmed that miRNAs play key role in neurobiology of neuronal lineage, synapses and neurogenesis, and have significant effects on differentiation of neuronal cells [27]. An increasing number of studies have implied that miRNAs could be a biomarker for NTDs, but still the underlying mechanism involved is not clear. Recently, several miRNAs have been reported to be associated with NTDs. miR-129-1, miR-129, miR-9, miR-197, miR-124 and miR-27 have been shown to influence neural tube formation [28–33]. Our previous transcriptome sequencing showed that miR-222-3p has markedly down-regulated in NTDs mice. The expression of miR-222-3p has been reported to be up-regulated in human breast cancer [34], thyroid cancer [35] and glial cancers [36]. However, miR-222 is elevated in Human TK-6 and DKO cells under folate deficiency [37,38]. The function of miR-222-3p in neural tube closure and formation of defects (NTDs), as well as its possible target genes, were still unknown.

Further review of relevant literature showed that the closure of the neural tube requires coordination of various cellular mechanisms, including apical constriction, cell proliferation, and apoptosis [39]. Recent studies have shown that NTDs are mainly a result of excessive apoptosis of neural crest cells or ventral cells of neural tube [40,41]. Moreover, abnormal cell proliferation leads to NTDs in the process of neuronal furrow formation [42]. Previous results of our research group have shown that apoptosis is one of the main causes of NTDs [43]. Yang et al. [44] evaluated that miR-222 has blocked cell cycle by inhibiting the expression of p27 gene and proliferation of hepatoma cells. Terasawa et al. [45] studied that low expression of miR-222-3p inhibited cell growth, migration, and promoted apoptosis. The above research indicated that miR-222-3p may affect the proliferation, apoptosis and cycle of cells, thus affecting the normal cell function. However, the effect of miR-222-3p on neural tube defects remained largely unknown. Therefore, we speculated that miR-222-3p may cause NTDs by affecting cell proliferation and apoptosis.

In this study, we used a retinoic acid (RA)-induced mouse NTDs model to correlate the spatial and temporal expression pattern of miR-222-3p with NTDs. Surprisingly, miR-222-3p was differentially expressed in NTDs group as compared with the control group. We then investigated the role of miR-222-3p on cell proliferation, apoptosis and migration in HT-22 cell line. To explore the underlying mechanism of miR-222-3p in NTDs, we hope to explore a promising target in HT-22 cells. We identified potential target genes of miR-222-3p using bioinformatics algorithms Targetscan, PicTar, miRanda, PITA and miRDB. All resulted in one prediction that Ddit4 was an important target gene of miR-222-3p and is also an oxidative stress gene [46]. We identified Ddit4 as a target gene of miR-222-3p and demonstrated that it can partially mediate the function of miR-222-3p in HT-22 cells. Furthermore, we preliminarily confirmed that low expression of miR-222-3p inhibited the Wnt/β-catenin signaling pathways. Taken together, our results demonstrated a link between miR-222-3p expression andNTDs, which enhanced our understanding regarding abnormal epigenetic modification in NTDs.

2. Materials and methods

2.1 Animals

SPF C57BL/6 mice (8–12 weeks, 19–25 g) were purchased from the Experimental Animal Center of Shanxi Medical University (Taiyuan, China). Mature male and female mice were mated overnight and the vaginal plug was detected in the morning. Pregnant mice were randomly divided into control and NTDs group. At 7.5 day of pregnancy (E7.5), NTDs model group mice were gavaged with all-trans retinoic acid (ATRA) (Sigma, USA, dissolved in sesame oil) at a dose of 28 mg/kg, and the control group was treated with the same dose of sesame oil. At E8.5, E9.5 and E10.5, cervical dislocation was used to euthanize pregnant mice, and embryonic neural tissues were collected according to previous research method (Mukhopadhyay P, Brock G, Appana S, Webb C, Greene RM, Pisano MM. MicroRNA gene expression signatures in the developing neural tube. Birth Defects Res A. 2011;91:744–62.).

2.2 Cell culture and transfection

The mouse clonal hippocampal neuronal cell line HT-22 cells (Jennio Biotech Co, Guangzhou, China) were cultured in DMEM high glucose medium (Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and 1% penicillin/streptomycin (Sigma, USA). All cells were maintained in a humidified incubator with 5% CO_2 and 37 °C atmospheres. 5×10^5 cells/ml was plated in antibiotic-free medium for 24 h before transfections. miR-222-3p mimics (mim-222), miR-222-3p inhibitor (anti-222), miR mimics negative control (mim-NC), miR inhibitor negative control (anti-NC) and siRNA-Ddit4 plasmid were designed and synthesized by GenePharma Co (Shanghai, China). When the HT22 cells become 75–85% confluent, cells were transiently transfected with 100 nM miRNA or siRNA using Lipofectamine RNAiMax (Santa Cruz Biotechnology, USA), according to the manufacturer's instructions. After 6 hours of transfection, the culture medium was replaced with fresh medium containing 10% FBS. The transfections were performed for 24 or 48 h, and then harvested for further analysis. The sequences of the small molecules are as follows:

mim-222:
AGCUACAUCUGGCUACUGGGUCU;
ACCCAGUAGCCAGAUGUAGCUUU.
anti-222:
AGACCCAGUAGCCAGAUGUAGCU;
mim-NC: CAGUACUUUUGUGUAGUACAA.
siRNA-Ddit4:
UGAGAGUCAUCAAGAAGAATT;
UUCUUCUUGAUGACUCUGATT.
anti-NC:UUCUCCGAACGUGUCACGUTT;
ACGUGACACGUUCGGAGAATT.

2.3 RNA extraction and qRT-PCR

Total RNAs from E8.5, E9.5 and E10.5 embryonic brain tissues were extracted using TRIzol reagent (Invitrogen, Carlsbad, CA), the quality and concentration of total RNA were measured by SoftMax Pro7.1 (NanoDrop, Thermo, US). For mRNA detection, reverse-transcribed complementary DNA was synthesized using the RevertAidlst cDNA Synth Kit (Thermo, USA). Quantitative PCR was performed on a Real-Time PCR platform (ABI Stepone Real-Time PCR system, USA) with SYBR Premix EX Taq (Takara, Japan). The procedure was as follows: (95°C, 10 min)×1 cycle; (95°C, 15 s; 60°C, 1 min)×40 cycles. The results were normalized to β-actin expression. For miRNA detection, the reverse transcribed cDNA was synthesized with the Hairpin-it miRNAs qRT-PCR and quantification kit qRT-PCR detection of miR-222-3p (GenePharma, China), and normalized by U6 small nuclear RNA. The data was analyzed using $2^{-\Delta\Delta Ct}$ method and all qPCR reactions were performed in triplicate. Primer sequences were shown in Additional file 1: Table S1.

2.4 Cell proliferation assay

For the cell proliferation assay, the Cell Counting Kit-8 (CCK-8; Dojindo) was used to determine the viability of cells. After 24 h of transfection, HT22 cells at a density of 1×10^3 cells/well were plated in 96-well microplates and incubated in normal culture conditions. The cell viability assay was performed every 24 h for 96 h by adding 10 μl of

CCK-8 reagent to each well, incubated at 37°C for an additional 1 h and then measured the absorbance/optical density (OD) at a wavelength of 450 nm using a Bio-Rad imarkTM microplate absorbance reader (Bio-Rad Laboratories, Hercules, CA, USA).

2.5 Cell cycle analysis

The cell cycle was analyzed by flow cytometry using a cell cycle detection kit (KeyGEN, Suzhou, China). Transfected cells were seeded in 6-well plates and incubated for 48 h. Cells were washed twice with phosphate-buffered saline (PBS), trypsinized and then centrifuged at 1000 × g for 5 min. The harvested cells were re-washed twice with PBS, fixed in 70% cold ethanol at 4°C for 24 h and stained with 50 μg/ml propidium iodide (PI) for 30 min. Cell cycle distribution was analyzed by Coulter Epics XL flow cytometry (BD Biosciences, San Jose, CA, USA) and cells in distinct cell cycle phases were determined using ModFit LT3.2 (Verity Software House).

2.6 Cell apoptosis assay

The apoptosis of transfected cells was measured using Annexin V/Fluorescein isothiocyanate (FITC) apoptosis detection kit (KeyGEN, Suzhou, China). After 48 hours of transfection, the cells were digested with 0.25% trypsin without ethylenediaminetetraacetic acid (EDTA) and centrifuged at 1000 × g for 5 min. A mixture of 500 μL binding buffer, 5 μL PI and 5 μL Annexin V/FITC was added to the cells, and incubated for 15 minutes. The apoptotic rate was detected on flow cytometry (BD Biosciences, San Jose, CA, USA). All experiments were performed in triplicate for each group.

2.7 Acridine Orange/Ethidium Bromide staining (AO/EB)

Acridine Orange/Ethidium Bromide staining (AO/EB) (Solarbio, Beijing, China) was used to detect the cell apoptosis according to the manufacturer's instructions. Transfected cells were seeded in 24-well plates and incubated for 24 h. The cells were then washed thrice with 1x PBS and stained with 10 μg/ml Hoechst and 10 μg/ml Acridine Orange/Ethidium Bromide (AO/EB) for 15 min in a humidified chamber. The cells were washed again with 1x PBS and visualized by fluorescence inverted microscope (Nikon, Tokyo, Japan).

2.8 Cell migration assays

Transfected cells were seeded in 6-well plates, cultured for 16–18 h, and serum-starved overnight. A perpendicular line was drawn at the center of the cells monolayer with the help of p10 pipette tip to create the wound. The cells were washed thrice with PBS and incubated in serum-free medium at 37°C and 5% CO_2. The wound closure was photographed at specified time intervals (0, 24 h) by an inverted microscope with a microscope digital camera. Wound healing area was calculated using ImageJ with the following formula: Wound healing rate = (initial scratch width – final scratch width)/initial scratch width × 100%. These experiments were performed in triplicate.

2.9 Western blotting

After 72 h of transfection, the cells were lysed with RIPA lysis buffer (Solarbio, Beijing, China) containing 10 mM PMSF (Solarbio, Beijing, China). The concentration of protein was measured with BCA Protein Assay kit (Thermo Fisher Scientific, USA). The samples were subjected to SDS-PAGE and then electro-transferred into a PVDF membrane. The membrane was blocked from nonspecific first antibody in 5% skim milk with Tris-buffered saline containing 0.1% Tween 20 solution (TBST) at room temperature for 1 h. Subsequently, the membrane was incubated with primary antibodies (Rabbit anti- C-myc, β-catenin, GAPDH, PCNA, Cleaved Caspase-3, Cycin-D1, 1:3000, Abcam, United Kingdom; Mouse anti-TCF4, Ddit4, 1:3000, Abcam, United Kingdom) at 4°C overnight, followed by TBST washing and incubation with the corresponding secondary HRP-conjugated antibody (HRP-labeled goat anti-rabbit IgG and anti-mouse IgG, 1:10,000, ZXGB-BIO, Beijing, China). After washing with TBST, targeted proteins on the membrane were detected by using ECL chemiluminescent detection system (minibio, Shangxi, China). The blots were scanned and detected on the Gene Gnome Western blot

imaging system (Bio-
RAD, US). The band density was measured by ImageJ software.

2.10 Luciferase reporter gene assay

The target-binding site of miR-222-3p on Ddit4 was predicted by TargetScan (http://www.targetscan.org/). The sequence fragments of Ddit4 wild-type (Ddit4-WT) and mutant (Ddit4-MUT) were synthesized and cloned into GP-miRGLO luciferase vector (GenePharma, China). After transfection with miRNA mimics for 24 hours, GP-miRGLO-Ddit4-Wt/Mut (130 ng per well in 96-well plate) along with GP-miRGLO-control (130 ng per well) were transfected into the cells. After 24 h of incubation, the cells were harvested and lysed with passive lysis buffer (Promega, US). Luciferase activity was measured by a dual-luciferase reporter system (Promega, US). The luminescence intensity of Firefly luciferase was normalized to that of Renilla luciferase. All experiments were performed five times for each group.

2.11 Statistical analysis

All reactions were performed at least three times and each independent experiment was carried out in triplicate for each condition according to the manufacturer's instructions. Statistical analysis was performed using SPSS 17.0 software (SPSS, Chicago, IL, USA). The data were shown as mean ± SD. The Student's *t*-test was used to analyze the difference between two samples. *$p < 0.05$, **$p < 0.01$ or ***$p < 0.001$ was considered statistically significant.

3. Results

3.1 Down-regulation of miR-222-3p in NTDs mouse

In this study, NTDs mouse models were induced with 28 mg/kg dose of RA on 7.5 days of gestation, the failure of closure at the level of the forebrain at this stage leads to significant anencephaly [47,48]. As shown in Figure 1a, significant differences were shown in embryonic morphology between RA treatment group and control group on E10.5. The embryos of the normal group were intact with obvious outlines, clear sections and full brain. RA treated embryos showed a typical anencephalic phenotype with blurred outlines, small size and unclear body segments. Our previous sRNA sequencing in those brain tissues showed that the expression level of miR-222-3p was significantly decreased in the RA treated group on E8.5, E9.5 and E10.5 compared with the control group [49] (Figure 1c). Subsequently, we confirmed that miR-222-3p expression was down-regulated in RA treatment group on E8.5, E9.5 and E10.5 compared with the control group by qRT-PCR (Figure 1b). Results were consistent with Transcriptome sequencing. These results suggested that miR-222-3p may play a key role in the pathogenesis of NTDs.

3.2 miR-222-3p down-regulation inhibits the proliferation of HT-22 cells in vitro

To investigate the biological function of miR-222-3p in NTDs, we selected mouse hippocampal neuronal cell line (HT-22 cells) . According to the results (Figure 1), the expression of miR-222-3p in NTDs mice was decreased. Therefore, subsequent studies were performed on cell function by transfecting anti-222 to evaluate expression of miR-222-3p. Various types of miR with GFP were successfully transfected into HT-22 cells and the transfected efficacy is shown in Figure 2a. qRT-PCR showed that the expression level of miR-222-3p was significantly decreased compared with the anti-NC (miR inhibitor negative control) and the control groups (con, no transfection group) (Figure 2b). CCK-8 assay showed that low expression of miR-222-3p suppressed the viability of HT-22 cells (Figure 2c). Western blotting confirmed that knockdown of miR-222-3p significantly inhibited the expression level of proliferating cell nuclear antigen (PCNA), a marker of proliferation (Figure 2d).

3.3. Knockdown of miR-222-3p promotes HT-22 cells apoptosis in vitro

To further investigate the mechanism of miR-222-3p on HT-22 cell proliferation, cell cycle and

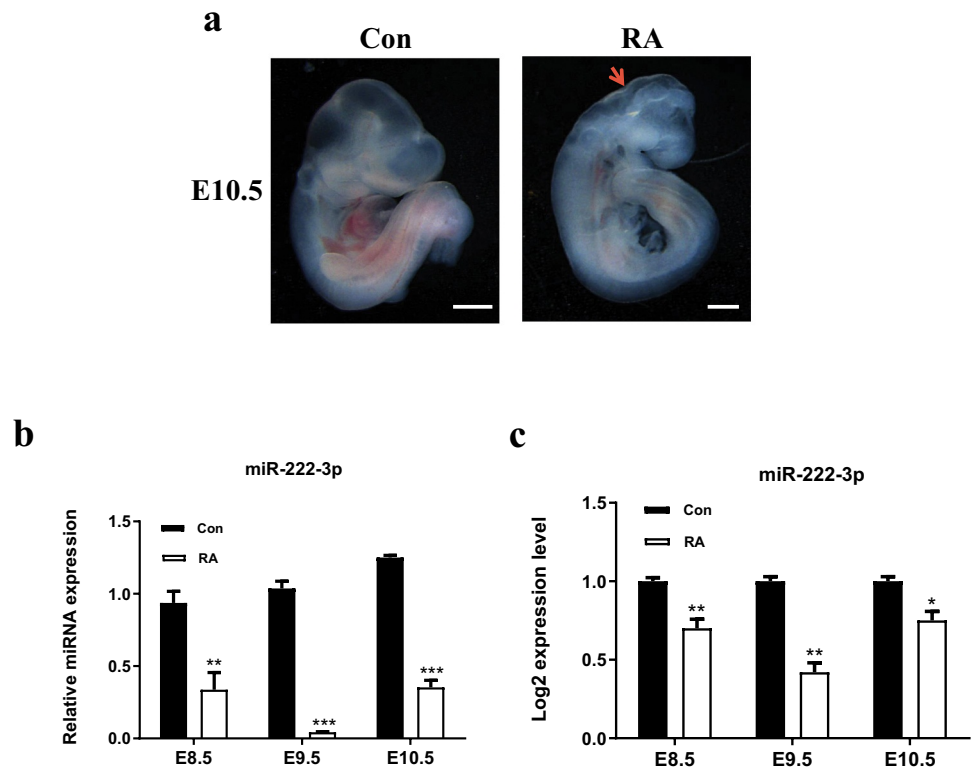

Figure 1. miR-222-3p expression was decreased in RA-treated mouse embryos. (a) Normal and RA-treated mouse embryos at E10.5. The red arrows indicate growth retardation in the forebrain and the posterior brain. (b) qRT-PCR was used to detect the mRNA level of miR-222-3p in normal and malformed brain tissue at E8.5-E10.5. (c) miR-222-3p miRNA-Seq results.

apoptosis, flow cytometry was used to show that the percentage of early apoptotic and late apoptotic cells were increased (the second quadrant represents late apoptotic cells, and the fourth quadrant represents early apoptotic cells) in the anti-222 transfected group compared with anti-NC and blank groups (Figure 3a). The expression of apoptosis-related proteins showed that cleaved caspase-3 was dramatically elevated in HT-22 cells post transfection with anti-222 (Figure 3b). AO/EB staining (Figure 3c) further confirmed that most cells in the control and anti-NC group have green fluorescence representing normal, viable and non-apoptotic cells with intact structure. However, anti-222 transfected cells showed orange and red fluorescence which increased significantly, representing apoptotic cells with condensed or fragmented chromatin. These results indicated that knockdown of miR-222-3p significantly promoted apoptosis in HT-22 cells. Flow cytometry assay showed that knockdown of miR-222-3p significantly increased the proportion of cells in the G_1 phase, while the proportion of cells in the S phase were decreased between two groups (Figure 3d). These results indicated that low expression of miR-222-3p affects cell proliferation by promoting apoptosis and cell cycle distribution of HT-22 cells.

3.4. Knockdown of miR-222-3p promotes migration of HT-22 cells in vitro

In view of the facts that occurrence of NTDs is related to neuronal migration, we further evaluated the effect of miR-222-3p on migration of HT-22 cells. The results showed that knockdown of miR-222-3p significantly inhibited migration of HT-22 cells (Figure 3e).

3.5. Ddit4 is a direct target of miR-222-3p in HT-22 cells

Real-time qPCR showed that mRNA expression of Ddit4 was up-regulated in RA treated group

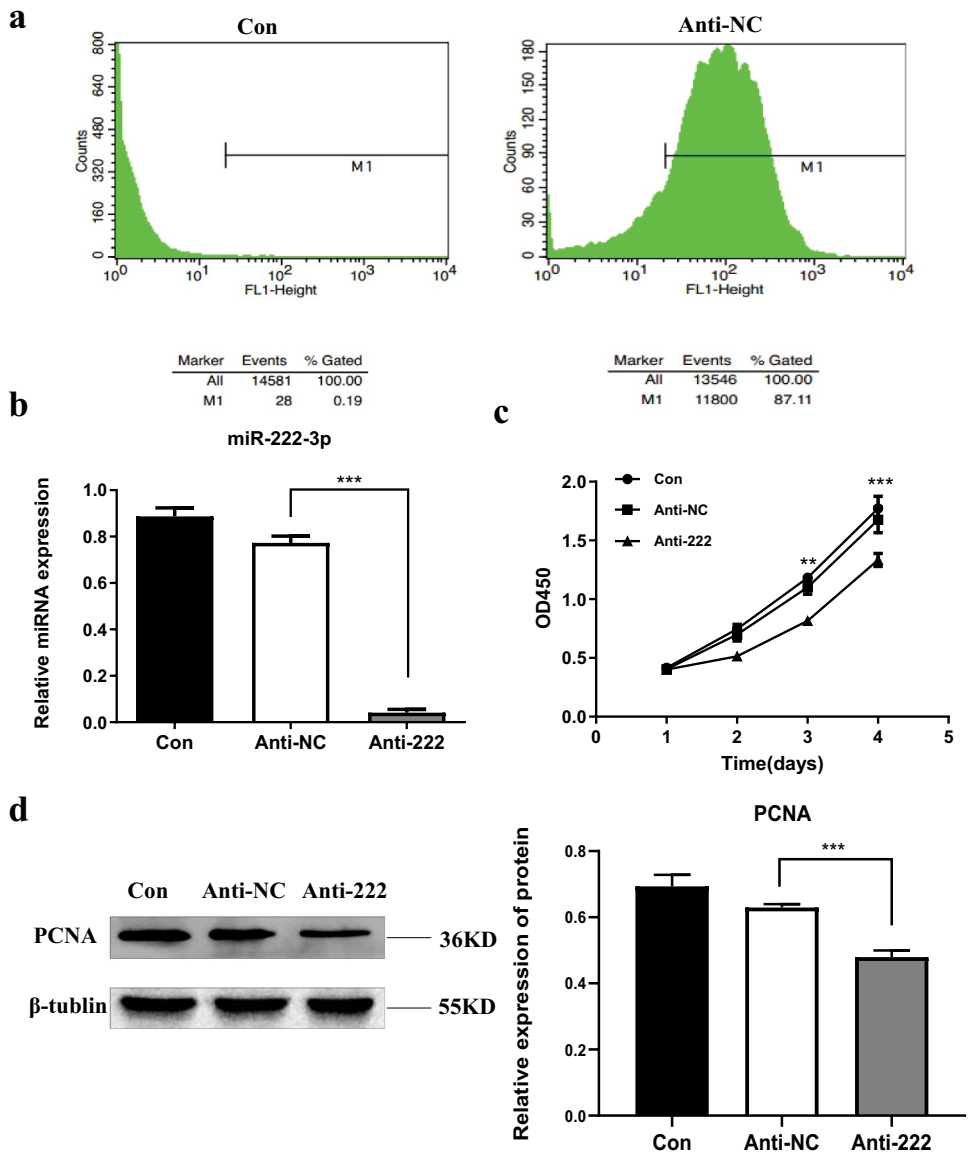

Figure 2. Inhibition of miR-222-3p affects the proliferation of HT-22 cells. (a) Transfection efficiency was detected by flow cytometry. (b) The mRNA level of miR-222-3p was analyzed by qRT-PCR in con (blank control group), anti-NC and anti-222 groups. The U6 was used as a control. (c) The proliferation of con, anti-NC and anti-222 groups cells were measured by CCK8. (d) The PCNA protein level in con, anti-NC and anti-222 groups were evaluated via western blot and the expression of β-tubulin was used for confirming equal loading.

compared with the normal group (Figure 4a), which was consistent with the sequencing. In order to determine whether miR-222-3p endogenously regulates Ddit4, miR-222-3p mimic (mim-222) and anti-222 were transfected into the cells to observe the expression of Ddit4. Figure 4c showed that the expression of miR-222-3p in HT22 cells could be well increased after transfection with mim-222. RT-qPCR showed that the mRNA expression level of Ddit4 was significantly increased after transfection with anti-222 (Figure 4b), while it was significantly decreased after transfection with mim-222 (Figure 4d). Similarly, WB showed that Ddit4 protein level was significantly increased in HT-22 cells after transfection with anti-222 while significantly decreased after transfection with mim-222 (Figure 4(e, f). Luciferase activity assay was used for validation of interaction between miR-222-3p and target gene Ddit4. In the empty

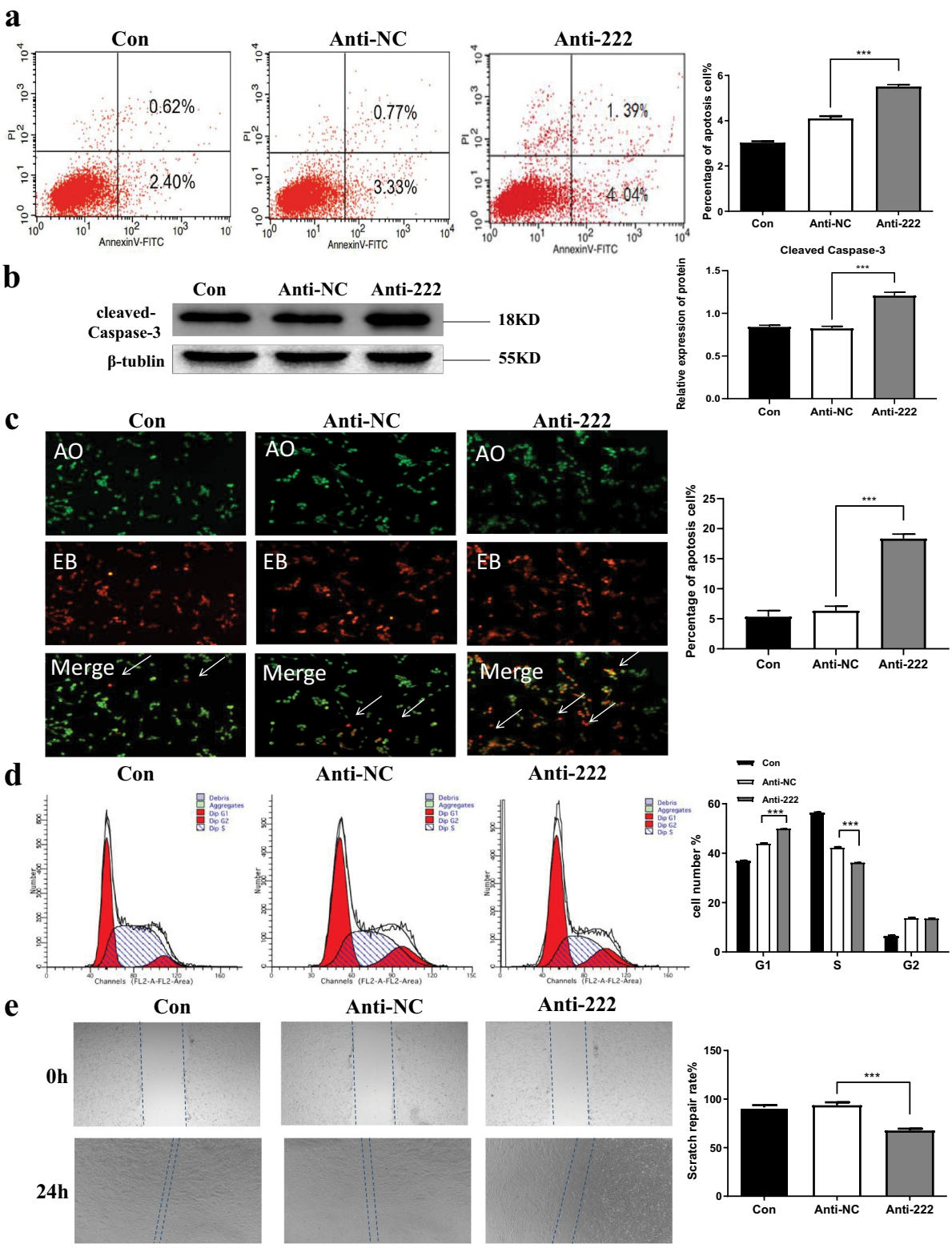

Figure 3. Inhibition of miR-222-3p affects the Cell function of HT-22 cells. (a) The cell apoptosis in con, anti-NC and anti-222 groups were analyzed by flow cytometry. (b) The Cleaved Caspase-3 protein level in con, anti-NC and anti-222 groups were evaluated via western blot and the expression of β-tubulin was used for confirming equal loading. (c) Fluorescence results of AO-EB double staining HT-22 cell nuclei. The arrows indicate apoptotic cells. (d) The cell cycle distribution in con, anti-NC and anti-222 groups was analyzed by flow cytometry. (e) The migration ability in con, anti-NC and anti-222 groups was analyzed by cell scratch experiment.

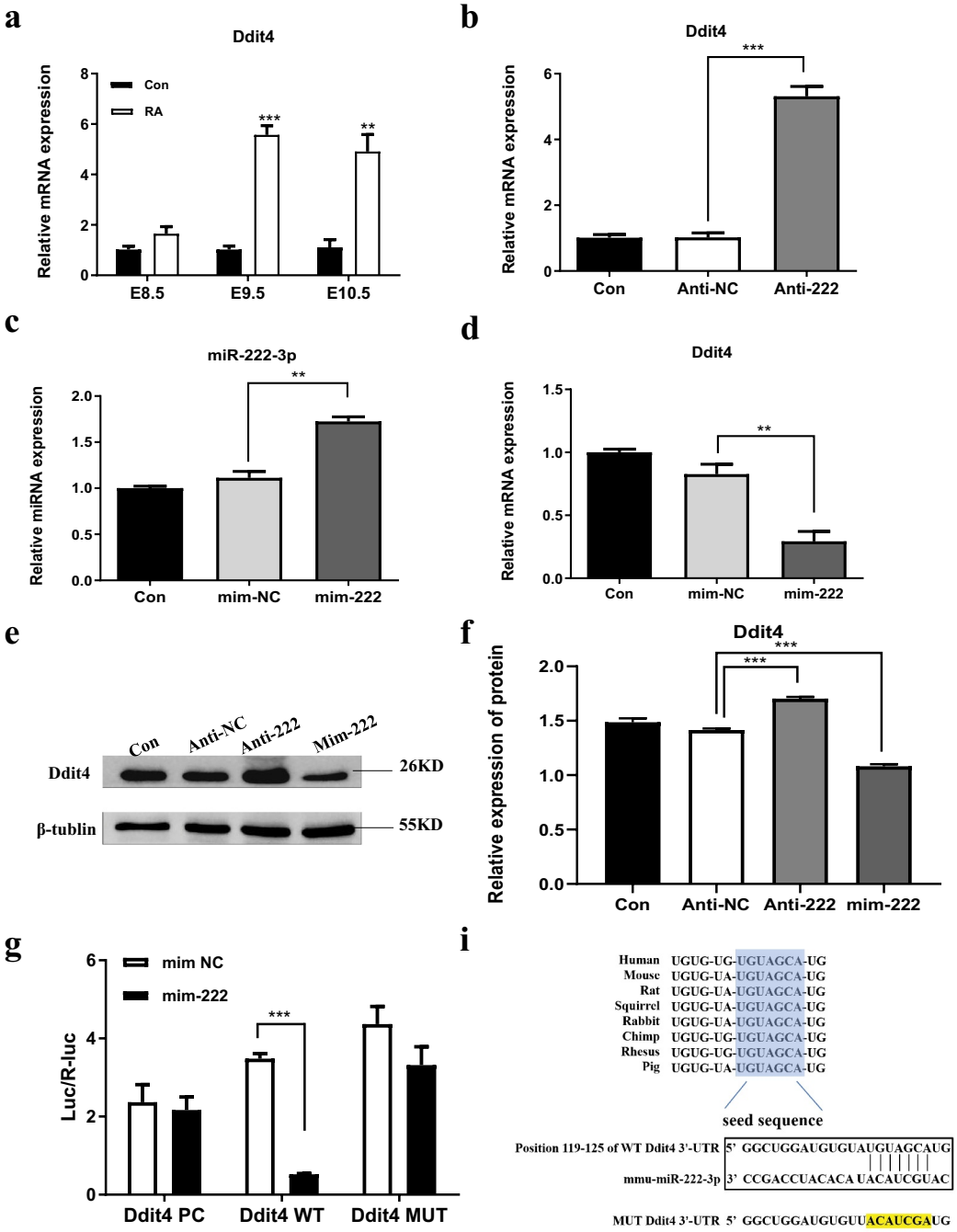

Figure 4. Ddit4 is a direct target of miR-222-3p in HT-22 cells. (a) qRT-PCR was used to detect the mRNA level of Ddit4 gene in normal and malformed brain tissue at E8.5-E10.5. (b) The mRNA level of Ddit4 gene was analyzed by qRT-PCR in con, anti-NC and anti-222 groups. The β-actin gene was used as a control. (c) The mRNA level of miR-222-3p was analyzed by qRT-PCR in con, mim-NC and mim-222 groups. The U6 was used as a control. (d) The mRNA level of Ddit4 gene was analyzed by qRT-PCR in con, mim-NC and mim-222 groups. The β-actin gene was used as a control. (e) The Ddit4 protein level in con, anti-NC and anti-222 and mim-222 groups was evaluated via western blot and the expression of β-tubulin was used for confirming equal loading. (f) Histogram analysis of graph E. (g) The fluorescence values of Ddit4 PC, Ddit4 WT and Ddit4 MUT combined with miR-222-3p mimis or NC were determined by double luciferase assay. (i) The seed sequence, mutated sequence, and complementary sequence of mir-222 and ddit4 3` UTR.

vector group without Ddit4 expression (Ddit4-PC) , mir-222 overexpression had no effect on Luc/R-LuC compared with miRNA simulated negative control. However, in the wild-type vector group (Ddit4-WT) with normal expression of Ddit4, the overexpression of miR-222-3p significantly decreased the luciferase activity of HT-22 cells transfected with GP-mirglo-DDIT4-WT. The deletion mutation of Ddit4 binding site (Ddit4-MUT) eliminated the effect of miR-222-3p on luciferase activity (Figure 4g). At the same time, the seed sequence, mutation sequence and complementary sequence diagram of Mir-222 and DDIT4 3′UTR are also shown in Figure 4i.

These results strongly suggested that miR-222-3p regulates the expression of Ddit4 by directly binding to the Ddit4 3′UTR sequence in HT-22 cells. These results indicated that Ddit4 is a potential target gene of miR-222-3p and the expression of Ddit4 was regulated by miR-222-3p.

3.6. Ddit4 reverses the effect of miR-222-3p on HT-22 cells

Previous studies have shown that Ddit4 is associated with cell apoptosis and cell proliferation [46]. Subsequently, Ddit4 was knocked down by a small interfering RNA (siRNA). As shown in Figure 5a, a significant reduction in Ddit4 was achieved in siR-Ddit4 compared with siRNA control (siR-NC). The CCK8 assay showed that cell proliferation capacity was reduced by miR-222 inhibitor, which was reversed by co-transfection of siR-Ddit4 (Figure 5b). Identically, PCNA was significantly decreased in HT-22 cells after transfected with miR-222 inhibitor and expression was reversed after Ddit4 was silenced (Figure 5c). Similarly, knock-down of Ddit4 protected anti-222-induced apoptosis in HT-22 cells (Figure 5(d, e)). In addition, down-regulation of Ddit4 ablated anti-222-mediated cell cycle arrest in HT-22 cells (figure 5f). Besides, low expression of Ddit4 also weakened the inhibitory effect of miR-222 on migration of HT-22 cells (Figure 5g). These results indicated that down-regulation of miR-222-3p in HT-22 cells affects proliferation, apoptosis and migration of HT-22 cells by binding and promoting expression of the target gene Ddit4 of miR-222-3p.

3.7 miR-222-3p is involved in regulation of Wnt signaling pathway

Ddit4 regulatesvWnt/β-catenin signaling pathway and affects the development of the dorsal-ventral axis of the embryo [50,51]. The present study confirmed that Ddit4 was a direct target of miR-222-3p. Wnt signaling pathway and its downstream indicators were investigated at mRNA and protein levels (Figure 6). RT-qPCR results showed that expression of β-catenin and TCF4 was significantly decreased in HT-22 cells after transfected with anti-222 (Figure 6a), and was greatly reversed by co-transfection of siR-Ddit4 (Figure 6b). WB showed that the protein levels of β-catenin, TCF4, c-Myc and cyclin D1 were significantly decreased in HT-22 cells after transfected with anti-222 compared with anti-NC (Figure 6c). This effect was enormously reversed by co-transfection of siR-Ddit4 (Figure 6d). These results suggested that down-regulation of miR-222-3p could restrain Wnt signaling pathway through Ddit4 inhibition.

4. Discussion

In the present study, we demonstrated that miR-222-3p was significantly down-regulated in NTDs mouse embryonic brain tissues. At the same time, an important link was observed between miR-222-3p and Ddit4 that we found miR-222-3p is negatively correlated with Ddit4 in HT-22 cells. Previous studies have demonstrated that aberrant expression of Ddit4 is associated with the occurrence progression and recurrence of various cancers, such as gastric and ovarian cancer, but not with neural tube malformation [52–54]. Collectively, these data supported the present study that Ddit4 is a direct target gene of miR-222-3p in NTDs.

Ddit4 (DNA damage-inducing transcript 4) belongs to the DDIT (DNA Damage Inducible Transcript) or GADD (Growth Arrest DNA Damage) protein family. This family member is involved in both physiological and pathological processes such as growth, development, DNA damage repair, apoptosis, inflammatory response,

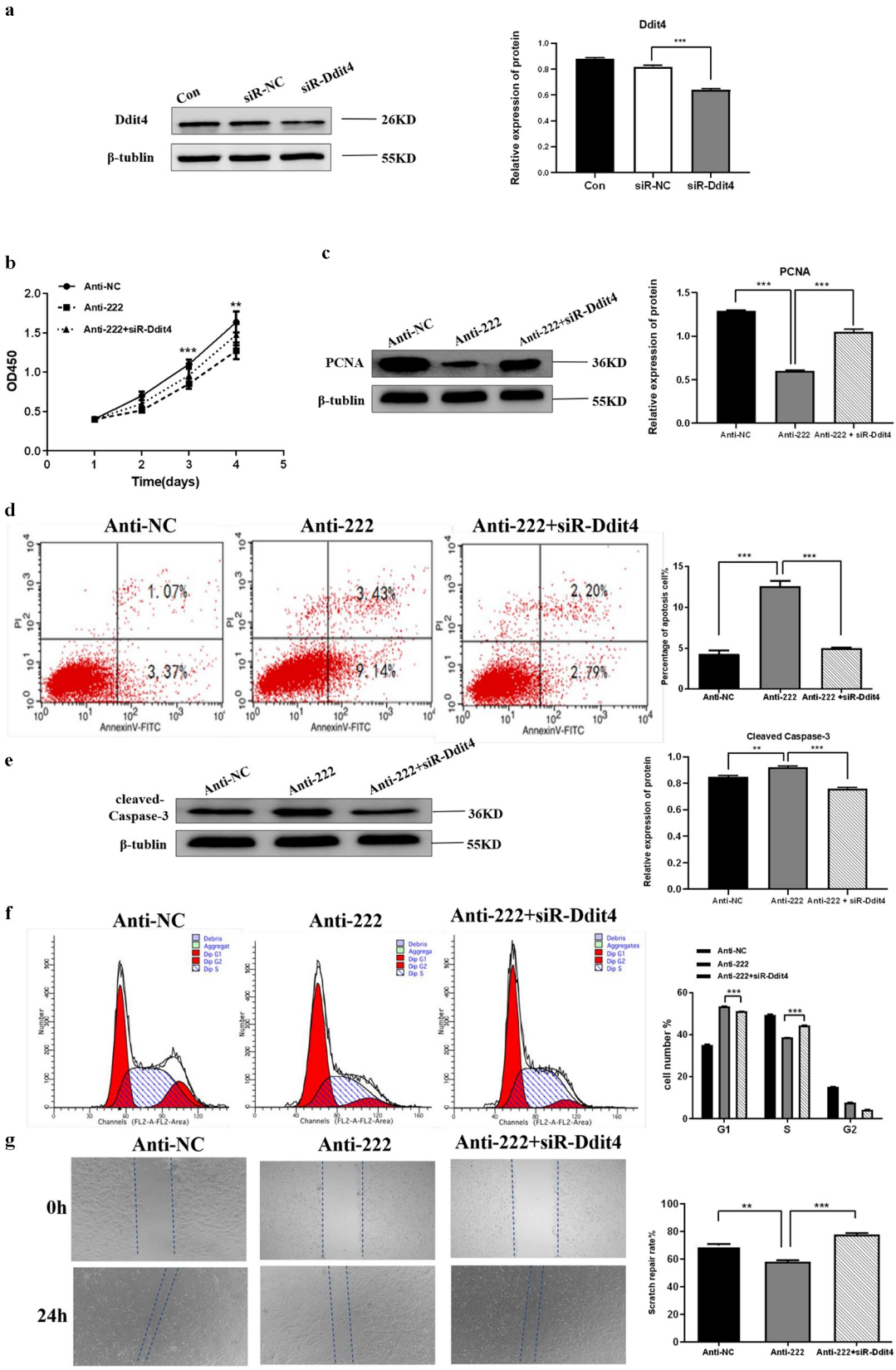

Figure 5. Ddit4 reverses the effect of miR-222-3p on HT-22 cells function. (a) The Ddit4 protein level in Con, siR-NC and siR-Ddit4 groups was evaluated via western blot and the expression of β-tubulin was used for confirming equal loading.(b)The proliferation of anti-NC, anti-222 and anti-222 + siR-Ddit4 groups cells were measured by CCK8. (c) The PCNA protein level in anti-NC, anti-222 and anti-222 + siR-Ddit4 groups was evaluated via western blot and the expression of β-tubulin was used for confirming equal loading.

(d) The cell apoptosis in anti-NC, anti-222 and anti-222 + siR-Ddit4 was analyzed by flow cytometry. (e) The Cleaved Caspase-3 protein level in anti-NC, anti-222 and anti-222 + siR-Ddit4groups was evaluated via western blot and the expression of β-tubulin was used for confirming equal loading. (f)The cell cycle distribution in anti-NC, anti-222 and anti-222 + siR-Ddit4 groups was analyzed by flow cytometry. (g) The migration ability in anti-NC, anti-222 and anti-222 + siR-Ddit4 groups was analyzed by cell scratch experiment.

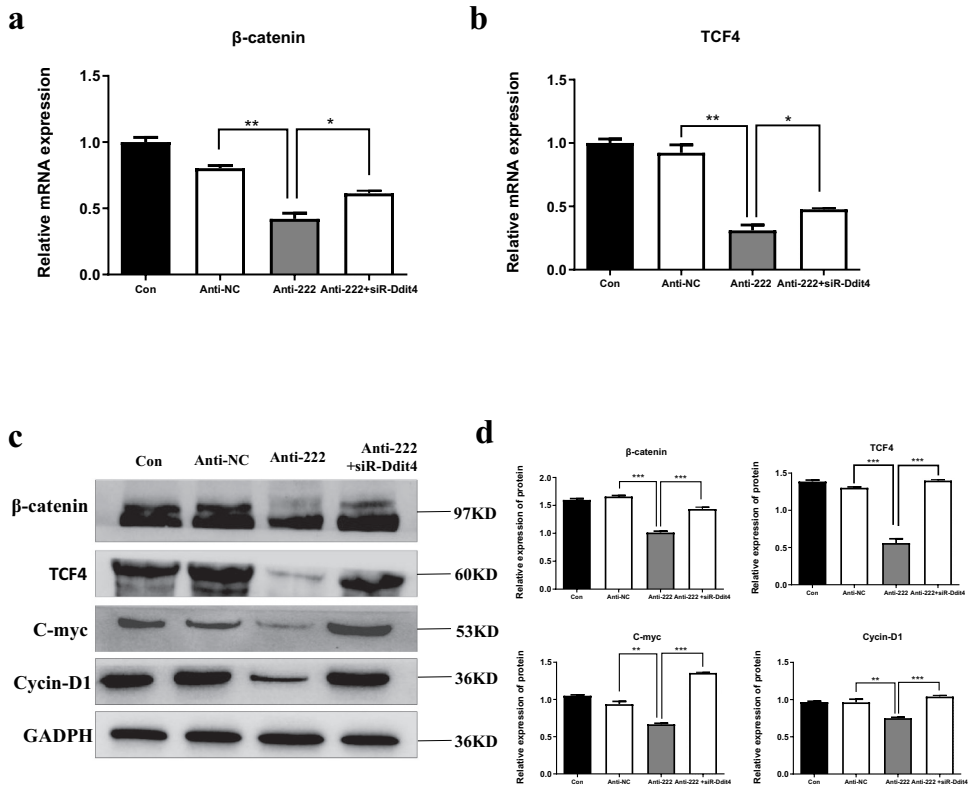

Figure 6. Inhibition of miR-222-3p regulates Wnt signaling pathway. (a) The mRNA level of β-catenin gene was analyzed by qRT-PCR in con, anti-NC, anti-222 and anti-222+ siR-Ddit4 groups. The β-actin gene was used as a control. (b) The mRNA level of TCF4 gene was analyzed by qRT-PCR in con, anti-NC, anti-222 and anti-222+ siR-Ddit4 groups. The β-actin gene was used as a control. (c) The β-catenin, TCF4, C-myc, Cycin D1 protein level in con, anti-NC, anti-222 and anti-222+ siR-Ddit4 groups was evaluated via western blot and the expression of GADPH was used for confirming equal loading.

stress and tumor formation [55–57]. The Ddit4 proteins in human and mouse are conserved at its C-terminus, and both contain RTP801-C protein domain with a sequence identity of 61%, and its homologous genes scylla and charybde are involved in the formation of the head of fruit fly [58]. During mouse embryonic development, Ddit4 is specifically expressed in the ectodermal axis and participates in the development and migration of cerebral cortical neurons during early embryonic development [22]. Studies have shown that Ddit4 gene is associated with a variety of neurodegenerative diseases, and its overexpression promotes apoptosis and inhibits cell proliferation [59,60].

Therefore, it may be a new breakthrough to understand the pathogenesis of NTDs through the study of miR-222-3p targeting Ddit4. Slawny and O'Shea [61] showed that Ddit4 regulates the Wnt/β-catenin signaling pathway, the imbalance of which leads to abnormal neural embryonic formation during embryonic development, and thus cause abnormal closure of neural tube [62]. To investigate whether Ddit4 was implicated in miR-222-mediated NTDs progression, HT-22 cells were transfected with anti-NC, anti-222 and co-transfection of anti-222 and siR-Ddit4 simultaneously. The decreased expression of β-catenin, TCF4, c-Myc and Cyclin D1 in HT-22 cells transfected with miR-222 inhibitor was

reversed by co-transfection of siR-Ddit4, suggesting that Ddit4 was involved in the regulation of Wnt/β-catenin pathway. Therefore, the present study has concluded an association between miR-222-3p and Wnt/β-catenin pathway, and the results suggested that knockdown of miR-222-3p suppressed the expression of Wnt signaling-related genes. Furthermore, the present study indicated that miR-222-3p inhibited HT-22 cell progression by targeting Ddit4 and further regulates the Wnt/β-catenin signaling pathway.

5. Conclusion

In conclusion, this study suggested a crucial role of miR-222-3p in the occurrence of NTDs, indicating a potential clinical value of miR-222-3p as an effective biomarker for diagnosis and prognosis of NTDs. In addition, our study demonstrated that miR-222-3p exerted its role by targeting Ddit4 through Wnt/β-catenin signaling pathway inhibition. Therefore, miR-222-3p may be a novel therapeutic target for the treatment of NTDs.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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