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Over-expressed LOC101927196 suppressed oxidative stress levels and neuron cell proliferation in a rat model of autism through disrupting the Wnt signaling pathway by targeting FZD3

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ABSTRACT

Accumulating evidence indicates that long non-coding RNAs (lncRNAs) play an important role in autism. Herein, we delineated the functions of LOC101927196 and its potential mitigation effect on a rat model of autism. We retrieved various bioinformatics databases and websites to screen differentially expressed lncRNAs associated with autism. Next, a rat model of autism was established with the neuron cells extracted for transfection of different plasmids. The regulatory effect of LOC101927196 on neuron cell proliferation, apoptosis as well as oxidative stress was also investigated. Firstly, microarray dataset GSE18123 revealed that LOC101927196 was poorly expressed in a rat model of autism. Poor development and growth and oxidative stress disorder were also observed in a rat model of autism. In addition, LOC101927196 targeting FZD3 played a vital role in a rat model of autism through the Wnt signaling pathway. Furthermore, we further demonstrated that over-expressed LOC101927196 blocked neuron cell proliferation and reduced oxidative stress levels, while promoting apoptosis by suppressing the activation of the Wnt signaling pathway. Our findings illustrate that up-regulated LOC101927196 attenuated oxidative stress disorder in a rat model of autism through suppressing the activation of Wnt signaling pathway by targeting FZD3.

1. Introduction

Autism spectrum condition (ASC; namely autism) is a complex lifelong neurodevelopmental disorder associated with social and communication deficits alongside restricted and repetitive behaviors [1,2]. Autism is more prevalent in males, with a sex ratio of about 4:1 [3]. The risk factors contributing to the etiology of autism consist of both genetic and environmental factors [4]. Furthermore, the occurrence of autism can be attributed to oxidative stress or inflammation [5]. As one of the potential mechanisms that combine genes with the environment, oxidative stress is defined as an alteration in the balance between pro-oxidant and anti-oxidant molecules, which is also characterized by long-term damage [6]. A prior study revealed that featured by lipid peroxides and higher levels of free radicals, oxidative stress plays an important role in autism as shown in a mouse model of autism, suggesting that it leads to cell damage and even cell death, which in turn, is responsible for autism [7]. Long noncoding RNAs (lncRNAs) have been reported to function in the development, epigenetic and gene expression regulation, drug resistance and nutrition-related, and a vast amount of lncRNAs are found in the human brain and play a key role in neurodevelopment and neurodevelopmental disorders including ASC [8].

Aberrantly expressed lncRNAs are found in autistic brain, leading to the dysregulation of protein-coding loci in ASC [9]. With retrieval in bioinformatics databases and prediction on biological websites, we identified LOC101927196 was involved in the development of autism, and it might target frizzled 3 (FZD3) to play a vital role in autism *via* the Wnt signaling pathway. Strikingly, FZD3 is known to be involved in both cancer and neuropsychiatric disorders' biology as a putative mental illness susceptibility gene on chromosome 8p [10]. A growing body of evidence supports the importance of the Wnt signaling pathway and oxidative stress in the pathogenesis of autism [11]. The Wnt signaling pathway has also been reported to be regulated by the autism-linked UBE3A in a cell context-dependent manner and an autism-linked

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mutation exacerbates the signaling effects [12]. Since the data of bioinformatics indicated a potential role of LOC101927196 in the development of autism, in the current study, we aim to elucidate the mechanisms of the regulation of LOC101927196 on oxidative stress disorder in a rat model of autism and neuron proliferation and apoptosis through regulation of the Wnt signaling pathway.

2. Materials and methods

2.1. Ethics statement

The current study was carried out in strict accordance with the recommendations of experimental animal protection clause of the First Affiliated Hospital of Xi'an Jiaotong University. All efforts were made to minimize the number and suffering of the included animals.

2.2. Microarray-based gene expression analysis

Autism microarray expression profiles (GSE18123) and probe annotations were retrieved and downloaded from the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo). Background correction and normalization were carried out using the Affy package of the R language [13]. Next, with traditional *t*-test and linear model-empirical Bayes estimation in the Limma package, non-specific filtration of expression profiles was implemented. Subsequently, the differentially expressed genes were screened [14]. Additionally, differentially expressed lncRNAs were predicted using the Multi Experiment Matrix (MEM, http://biit.cs.ut.ee/mem/). Finally, the function of target gene was confirmed with Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/pathway.html).

2.3. Study subjects

Sprague-Dawley (SD) neonate rats aged < 2 days, healthy adult Wistar male rats (300–350 g) and female rats (200–250 g), were all purchased from Experimental Animal Department of the Chinese Academy of Sciences, Beijing, China, were housed in specific cages of Fudan University, with free access to food and water. The cage environment was set at around 25 °C and kept quiet, with light exposure modulated using a daylight lamp.

2.4. Model establishment

In order to allow the rats to adapt to the environment, the adult female and male rats were housed with periodic light (7: 00 am. - 7: 00 pm.), stable temperature at 25 °C and humidity of 55% for several days. After that, the rats were kept in the same cages with a ratio at 2: 1 (female: male rats) overnight. Conception was examined among female rats in the next morning, and then the pregnant rats were housed independently and assigned into two groups randomly: one group of rats was administered intraperitoneal injections with 600 mg/kg valproate sodium (VPA) after gestation for 12.5 d, and their neonate rats were regarded as the autism group; the other group was administered intraperitoneal injections with same amounts of saline on the same day, while their neonate rats were regarded as the normal group. All neonate rats were weaned after 23 d. A total of 80 neonate rats were selected separately from the autism group and the normal group for subsequent experimentation.

2.5. Examination of body weight and brain weight

Four filial male rats from the autism group and the normal group aged 7 d, 14 d, 23 d, 56 d and 90 d were weighed separately. After narcosis, the brains were removed and weighed with olfactory bulbs and brain tissues below medulla oblongata abandoned. The mean values of body weight and brain weight in the two groups were analyzed and recorded [15].

2.6. Eye-opening time test

Five filial rats were selected from the autism group and the normal group respectively, and their eye-opening time was recorded respectively 12–16 d after birth. The scoring standard was as follows: 0 points, with no eye open; 1 point, with 1 eye open; 2 points, with 2 eyes open. The scores of eye-opening in the two groups were recorded [16].

2.7. Direction ability determination

When filial rats grew to 7–10 d, 5 juvenile rats from the autism group and the normal group were selected respectively, and placed with their heads downwards on a smooth 25°-tilting plane. The activity of 180° rotation of rats was observed, and the time spent on the rotation in the two groups was recorded and compared. When rats were placed with their heads down on the tilting plane, the rats had the instinct to automatically turn 180° with heads up. The time of rotation reflected the development of vestibular sensation and motive function in rats [17].

2.8. Swimming test

The neonate rats aged 10 d, 12 d, 14 d and 16 d were placed in a thermostatic bath at 26 °C to perform the swimming test to test the coordination of rats. Five rats in the autism group and the normal group were placed in the middle of the thermostatic bath separately and observed for 10–15 s. Next, the rats were scored according to the relative position of their ears and vertices, and the water. The scoring standard was as follows: 0 points, vertices and noses were below water; 1 point, noses were below water, while vertices were above water; 2 points, noses and vertices were above water or leveled with water while ears were below water; 3 points, the positions of noses and vertices were the same as those required by 2 points, but the water level was between the ears; 4 points, the positions of noses and vertices were the same as those required by 2 points, but the water level was under the ears [18]. After testing, the rats were wiped and put back into the cages.

2.9. Repetitive and stereotyped behavior test

Five rats aged 30 d in the autism group and the normal group were selected respectively. Each rat was placed individually in a clean observation box for 10 min in a quiet and dark environment. The appearance frequency, persistence time and walking distance of repetitive and stereotyped behaviors in rats were observed and recorded. Here, repetitive and stereotyped behaviors referred to the incessant irregular motion of rats in a small ambit, such as circling, jumping, combing the fur on the body, running, nose picking, lip biting and sucking repeatedly [15].

2.10. Detection of 4-hydroxy-2-nonenal (4-HNE), reactive oxygen species (ROS) and reactive nitrogen species (RNS)

Intraperitoneal injections were administered for narcosis with 2% pentobarbital sodium at 40 mg/kg. After narcosis, the rats were sacrificed. The hearts of rats were cleaned and the superfluous moisture was sucked. Immersed in an ice bath, the same part of each heart was weighed, and a homogenate was made at the ratio of 1: 4 (weight: volume). Next, the homogenate was centrifuged at 2862 × *g* for 10 min with the supernatant placed in Eppendorf (EP) tubes and preserved at −20 °C. Cells were collected and mixed with a proper amount of phosphate buffered saline (PBS) at a concentration of about 1×10^9 cells/L. Then the cells were disrupted with ultrasonic cell disruptor at low temperature, and 0.2 mL homogenate was taken as the testing

sample. After that, 4-HNE, ROS, and RNS were detected according to the instructions of kits respectively (GMS13985, GMS26571, GMS26733, NanJing JianCheng Bioengineering Institute, Nanjing, China). The experiment was conducted 3 times to obtain the mean value.

2.11. Immunohistochemistry

Tissue sections were prepared as follows: 4 rats in the autism group and the normal group were euthanized after 90 days for brain collection which was fixed in 4% polyformaldehyde after distilled water rinsing. The volume ratio of brain tissues to formaldehyde was 1: 4. Then the tissues were preserved at 4 °C in a refrigerator. The rat brain tissues were rinsed with running water for 24 h, dehydrated with gradient alcohol, cleared with xylene and embedded with paraffin. Then paraffin sections were sliced to thickness of 4–6 μm.

The prepared sections were dried in an oven at 50–60 °C, dewaxed with regular xylene and dehydrated with gradient alcohol. Next, the sections were placed in 3% H_2O_2 for 10 min, and washed with distilled water for 3 times, with 3 min each time. With high pressure antigen retrieval for 1–3 min, the sections were bathed in cold water, cooled to room temperature, then rinsed with PBS (0.01 M pH 7.4) twice, with 3 min each time. Afterwards, the sections were added with 10% normal goat serum sealing solution (CWBio, Beijing, China), and kept at room temperature for 20 min. With excessive liquid dumped, the sections were added with appropriate amounts of frizzled class receptor 3 (FZD3) rabbit anti rat monoclonal antibody (dilution ratio of 1: 500, ZSGB-Bio, Beijing, China) and incubated at 4 °C overnight. After being taking out, the sections were rinsed with PBS 3 times, with 3 min each time. Then the sections were added with secondary antibody working solution goat against rabbit immunoglobulin G (IgG) labeled with biotin (dilution ratio of 1: 1000, ab6789, Abcam Inc., Cambridge, MA, UK) and incubated at 37 °C for 30 min. After incubation, the sections were rinsed with PBS 3 times, with 3 min each time. After that, the sections were added with streptomycin anti biotin protein-peroxydase solution (ZSGB-Bio, Beijing, China). Subsequently, the sections were incubated in oven at 37 °C for 20 min, rinsed with PBS 3 times, each time 5 min, and developed with Diaminobenzidine (DAB) (DA1010-3 mL, Beijing Solarbio Science & Technology Co. Ltd., Beijing, China) for 5–10 min. Staining degree was tested under a microscope. Sections were rinsed with distilled water for 10 min. After that, the slicer was immersed in hematoxylin for 4 min, and rinsed with running water. The slicer was removed, soaked for 10 s in 1% hydrochloric ethanol, soaked and rinsed with running water for 5 min, and allowed to turn blue with 1% ammonia water for 10 s. Sections were dehydrated with regular gradient alcohol, cleared with xylene and sealed with neutral balsam. A total of 5 visual fields under a high-power microscope were selected in each section. The area of positive staining, the percentage of positive staining area and the mean integral optical density (OD) were examined and recorded.

2.12. Cell treatment in vitro

Primary neuronal cells were cultured *in vitro* as follows: hippocampus primary neuronal cells were prepared from SD neonate rats and filial rats in the autism group were prepared with the above steps, and the operations were as follows: several rats were anaesthetized and immersed in ethyl alcohol for sterilization, and then sacrificed by cervical dislocation. With ablation of skin in brain and jawbone under aseptic conditions, the whole brain was carefully excised and washed with D-Hank's solution several times. With the supernatant abandoned, the brain was added with low-sugar Dulbecco's modified eagle's medium (DMEM) with 0.15% collagenase and moved into sterile centrifuge tubes. After that, the brain was treated by stable-temperature electromagnetic stirrer at 37 °C, and centrifuged at 87 × *g* for 5 min. With the supernatant removed, the brain was added with a proper amount of low-sugar DMEM [including 20% fetal bovine serum (FBS)] and centrifuged again at 87 × *g*. The precipitation was added to the culture medium for cell suspension preparation. After trituration, the cell suspension was moved into disposable culture medium. The sediment was distributed evenly, washed with D-Hank's solution, added with appropriate amount of 0.25% trypsin containing 0.02% elhylene diamine tetraacetic acid (EDTA) and placed in a 5% CO_2 incubator at 37 °C (ZXKR-1150, Shanghai ZHICHENG Analytical Instrument Manufacturing Co. ltd., Shanghai, China) for 7–9 min. Then, the sediment was added with culture medium with 20% FBS to terminate the detachment, and centrifuged at 257 × *g* for 5 min. With the supernatant discarded, the cells were re-suspended with DMEM containing 20% FBS and then seeded to a novel culture medium in a 5% CO_2 incubator at 37 °C. The medium was changed every 2–3 days.

Next, the cells were assigned into the following 7 groups: the normal group (normal neuronal cell), the blank group (autism neuronal cell without transfection), the negative control (NC) group (autism neuronal cell transfected with NC vector), the LOC101927196 vector group (autism neuronal cell transfected with over-expressed LOC101927196 vector), the siRNA-LOC101927196 group (autism neuronal cell transfected with siRNA LOC101927196 vector), the Sulindac group (autism neuronal cell transfected with Wnt signaling pathway inhibitor) and the siRNA-LOC101927196 + Sulindac group (autism neuronal cell transfected with siRNA LOC101927196 and Wnt signaling pathway inhibitor).

Cell transfection was carried out after culture for 24 h. Then 200 μL serum-free Opti-MEM (31,985,070, Gibco, Carlsbad, California, USA) was used to dilute 6 μL lipofectamine 2000 (11668-019, Invitrogen, Carlsbad, California, USA), and 2 μg of target vector was diluted with 100 μL serum-free Opti-MEM. Then, the two were mixed separately and placed at room temperature for 10 min. Then the two were mixed together and incubated at room temperature for 20 min. With the primary culture medium in the 6-well plate discarded, each well was added with 180 μL Opti-MEM. The transfection complex was added to the corresponding cell culture well. After being cultured in a incubator at 37 °C with 5% CO_2 in air for 18 h, the medium was replaced by a fresh and complete one. After 48-h transfection, the cells were collected.

2.13. Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted using an ultrapure RNA extract kit (D203–01, Gen Star Biosolutions Co., Ltd., Beijing, China). Primers of LOC101927196, Wnt2, FZD3, glycogen synthase kinase-3β (GSK-3β), β-catenin, thioredoxin-2 (Trx2), B-Cell CLL/Lymphoma 2 (Bcl-2), Bcl-2-associated X protein (Bax) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed and synthetized by TaKaRa (Tokyo, Japan) (Table 1). RNA template, Primer Mix, dNTP Mix, dithiothreitol (DTT), RT Buffer, HiFi-moloney muriiie leukemia virus (MMLV) and RNase-free water were dissolved on ice for further use. Reverse transcription was carried out with 20 μL solution, according to the instructions of TaqMan MicroRNA Assays Reverse Transcription Primer (4,366,596, Thermo scientific, Waltham, MA, USA). The reaction conditions were as follows: reverse transcription at 42 °C for 30–50 min and reverse transcriptase inactivation at 85 °C for 5 s. Fluorescence quantitative PCR was carried out with the reaction solution, according to the instructions of SYBR® Premix Ex Taq™ II kit (RR820A, Action-award Co., Ltd., Guangzhou, China). The 50 μL reaction system comprised of the following: 25 μL SYBR® Premix Ex Taq™ II (2 ×), 2 μL PCR forward primer, 2 μL PCR reverse primer, l μL ROX Reference Dye (50 ×), 4 μL DNA template and 16 μL ddH_2O. Fluorescence quantitative PCR was carried out in ABI PRISM®7300 system (Prism®7300, Kunke Equipment Co., Ltd., Shanghai, China), and the reaction conditions were as follows: pre-denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing at 60 °C for 30 s, finally extension at 72 °C for 1 min. GAPDH acted as internal reference of

Table 1
RT-qPCR primer sequence.

Genes	Sequences
LOC101927196	F: 5′- GGGAAAATCGGTCCGAGAGG −3′ R: 5′- TGGCTAAGGCGTGATGGTTT −3′
Wnt2	F: 5′- CAGGGCAACTGGATGTGGTT-3′ R: 5′- CTCGTGTCGGAACTGGCTTC-3′
FZD3	F: 5′- ATGGCTGTGAGCTGGATTGTC-3′ R: 5′- GGCACATCCTCAAGGTTATAGGT-3′
GSK-3β	F: 5′- ATGGCAGCAAGGTAACCACAG −3′ R: 5′- TCTCGGTTCTTAAATCGCTTGTC -3′
β-catenin	F: 5′- ATGGAGCCGGACAGAAAAGC-3′ R: 5′- TGGGAGGTGTCAACATCTTCTT-3′
Trx2	F: 5′- TTCCCTCACCTCTAAGACCCT-3′ R: 5′ CCTGGACGTTAAAGGTCGTCA −3′
Bax	F: 5′-AGACAGGGGCCTTTTTGCTAC-3′ R:5′-AATTCGCCGGAGACACTCG-3′
Bcl-2	F: 5′- GCTACCGTCGTGACTTCGC-3′ R: 5′-CCCCACCGAACTCAAAGAAGG-3′
U6	F: 5′- ATGGGTCGAAGTCGTAGCC -3′ R: 5′- TTCTCGGCGTCTTCTTTCTCG −3′
GAPDH	F: 5′- AATGGATTTGGACGCATTGGT −3′ R: 5′- TTTGCACTGGTACGTGTTGAT −3′

Notes: Wnt2, Wingless and INT-2; FZD3, Frizzled class receptor 3; GSK-3β, Glycogen Synthase Kinase-3; β-catenin, B-cell lymphoma-2; Trx2, Thioredoxin 2; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma-2; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

LOC101927196, Wnt2, FZD3, GSK-3β, β-catenin, Trx2, Bcl-2, and Bax. $\Delta\Delta Ct$ = (mean value of $Ct_{\text{(target gene in the experimental group)}}$ – mean value of $Ct_{\text{(house-keeping gene in the experimental group)}}$) – (mean value $Ct_{\text{(objective gene in the normal group)}}$ – mean value $Ct_{\text{(house-keeping gene in the normal group)}}$). The expression of LOC101927196, Wnt2, FZD3, GSK-3β, β-catenin, Bcl-2 and Bax in tissues was calculated and recorded. (This method was also applicable to RT-qPCR detection in cells).

2.14. Western blot analysis

Total cell lysates were obtained using the Total Protein Extraction Kit. The obtained proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Diluted primary antibodies rabbit polyclonal antibody against Wnt2 (ab27794, dilution ratio of 1:2000), FZD3 (ab217032, dilution ratio of 1:1000), GSK-3β (ab93926, dilution ratio of 1:300), β-catenin (ab32572, dilution ratio of 1:1000), 4-HNE (ab46545, dilution ratio of 1:500), Bax (ab32124, dilution ratio of 1: 400), Bcl-2 (ab119506, dilution ratio of 1: 400), p-Wnt (ab164825, dilution ratio of 1: 500), p-GSK-3β (ab139336, dilution ratio of 1: 500) and p-β-catenin ((ab27798, dilution ratio of 1: 500), which were bought from Abcam (Cambridge, MA, UK), were added for incubation overnight and rinsed with PBS at room temperature for 3 times, 5 min per time. The horseradish peroxidase labeled goat anti rabbit IgG (dilution ratio of 1: 1000, Boster Biological Technology Co. Ltd., Wuhan, China) secondary antibody was added to the tissues for incubation at 37 °C for 1 h. The membrane was immersed in electrochemiluminescence (ECL) reagents (Pierce, Waltham, MA, USA) at room temperature for 1 min. GAPDH was used as internal reference, and the ratio of gray value of target band and internal reference band was regarded as the relative expression of protein [19] (This method was also applicable for Western blot detection in cells).

2.15. 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay

The stained cells at the logarithmic phase of growth were seeded in a 96-well plate at 1×10^4 cells/well, with 8 parallel wells in each group. In addition, a blank well with culture medium without cells was also set. When cells reached 70% confluence, each well was added with 10 μL 5 mg/mL MTT solution (ST316, Beyotime Institute of Biotechnology, Shanghai, China) for incubation at 37 °C for 24 h, 48 h and 72 h separately. With the supernatant abandoned, the cells were rinsed with PBS 1 time. Each well was added with 100 μL dimethyl sulphoxide (DMSO, D5879, Sigma-Aldrich, St. Louis, MO, USA). After oscillation for 10 min on a rocking bed, the OD values of the cells were examined at 490 nm using a microplate reader (MK3, Thermo, Pittsburgh, PA, USA). Cell viability = (OD in the experimental well – OD in the blank well)/OD in the blank well. Experiment was conducted 3 times to obtain the mean value.

2.16. Flow cytometry

After culture for 48 h, the cells were rinsed with PBS once with culture medium abandoned. Next, the cells were treated with 0.25% trypsin and collected, and then centrifuged at 4 °C at 178 ×*g* for 5 min, with the supernatant abandoned. Cells were rinsed with precooled PBS twice, and centrifuged at 178 ×*g* for 5 min, with the supernatant abandoned. With the addition of precooled 70% ethanol, the cells were fixed at 4 °C overnight. After PBS rinsing, the cells were centrifuged at 178 ×*g* for 5 min. With 10 μL RNase, the cells were incubated at 37 °C for 5 min. Subsequently, the cells were added with 1% PI (40710ES03, Shanghai Qcbio Science and Technologies Co., Ltd., Shanghai, China), and stained avoiding exposure to light for 30 min. Then the samples were placed in a flow cytometer (FCM) (FACSCalibur, BD, FL, NJ, USA), and red fluorescence at excitation wavelength of 488 nm was recorded to detect cell cycle. Experiment was conducted 3 times to obtain the mean values.

Cells were treated with elhylene diamine tetraacetic acid (EDTA)-free trypsin and collected 48 h after transfection, and centrifuged at 4 °C at 178 ×*g* for 5 min, with the supernatant removed. After precooled PBS rinsing, the cells were centrifuged at 178 ×*g* for 5 min, with the supernatant abandoned. Then cell apoptosis was detected using a Annexin-V-FITC/PI kit (CA1020, Beijing Solarbio Science & Technology Co. Ltd., Beijing, China). Cells were washed with the binding buffer, and the incorporation liquid of Annexin-V-FITC and binding buffer was prepared at a ratio of 1: 40. Cells were re-suspended, oscillated and mixed, and incubated at room temperature for 30 min. Then above-mentioned incorporation liquid was added to the cells for incubation at room temperature for 15 min. Cell apoptosis was detected by FCM. This experiment was conducted 3 times to obtain the mean values.

2.17. Statistical analysis

Statistical analyses were performed using the SPSS 21.0 software (IBM Corp. Armonk, NY, USA). Measurement data were expressed as mean ± standard deviation. Multiple groups were compared by one-way analysis of variance (ANOVA), while differences between two groups were compared by *t*-test. Enumeration data were presented as rate or percentage, and the chi-square test was used for comparative analysis. A value of $p < .05$ was considered to be statistically significant.

3. Results

3.1. Brain growth was inhibited in neonate rats with autism

Firstly, we examined the body weights, brain weights, eye-opening time and direction ability of neonate rats in order to compare the growth and development of filial rats. There were no significant differences in the development of rats in the autism group (offspring of the VPA-treated rats) when compared with the normal group in the early stage ($p > .05$). However, when rats grew to 23 d, variances were noted in weight ($p < .05$). The weight of rats in the autism group was found to be significantly lower than that in the normal group, and the weight difference was relatively smaller upon reaching 56 d and 90 d

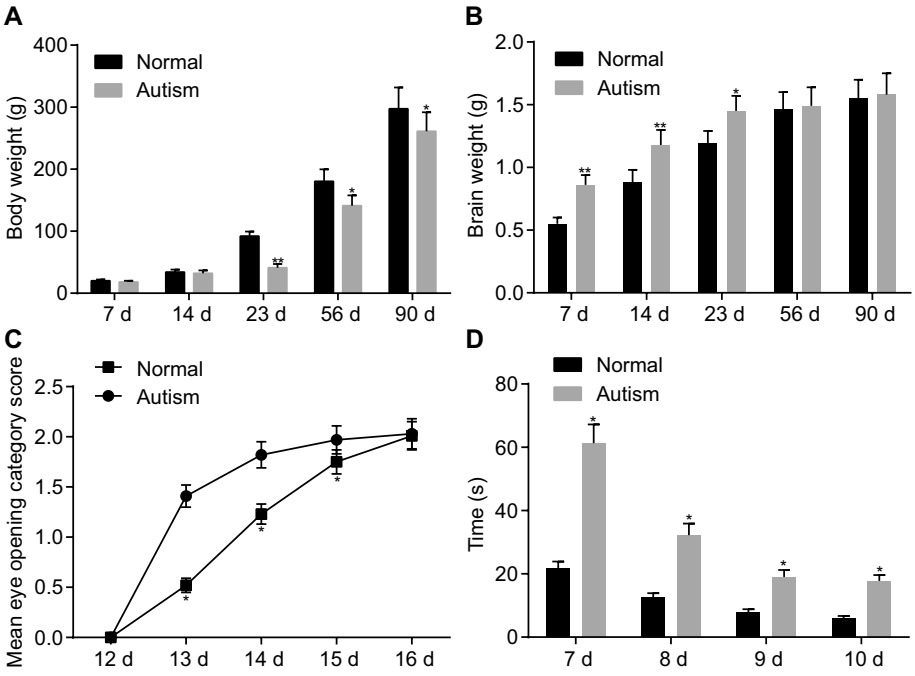

Fig. 1. Rat models of autism showed poor growth of vestibular sensation and motive function. A, the body weight of rats in the normal group and the autism group (n = 4); B, the brain weight of rats in the normal group and the autism group (n = 4); C, the eye-opening time of rats in the normal group and the autism group (n = 5); D, the time of automatic turning 180° in the normal group and the autism group (n = 5). Independent-sample t-test was used for analysis. * $p < .05$ *vs.* the normal group; ** $p < .01$ *vs.* the normal group.

(Fig. 1A) ($p < .05$). In the autism group, the brain weight of rats in early stage was noted to be significantly higher than that in the normal group ($p < .05$). When rats grew to 23 d, the variance decreased between the two groups ($p < .05$). When rats grew to 56 d and 90 d, there was no obvious variance between the two groups (Fig. 1B) ($p > .05$). The results of the eye-opening test revealed that compared with the normal group, the rats in the autism group opened their eyes later ($p < .05$). The specific manifestations were as follows: rats in the two groups did not open eyes at the 12th d ($p > .05$); at the 13th d, 14th d and 15th d, rats in the autism group opened eyes later ($p < .05$); at the 16th d, the two groups of rats opened eyes without significant difference ($p > .05$) (Fig. 1C). In each growth phase, the time used for turning of rats in the autism group was significantly higher than that of rats the normal group, which demonstrated poor vestibular sensation and motive function of rats in the autism group ($p < .05$) (Fig. 1D). Taken together, these findings indicated that neonate rats with autism exhibited poor development and growth.

3.2. Neonate rat models of autism showed poorer swimming coordination and increased repetitive and stereotyped behaviors

Next, the tests of ethology were performed to assess whether neonate rat model of autism was successfully established. The results of swimming test are shown in Fig. 2A. Compared with the normal group, the swimming test scores of the autism group (offspring of the VPA-treated rats) were found to be lower in addition to poorer swimming coordination ($p < .05$). At the 10th d and 12th d, the scores in the autism group were still lower than those in the normal group ($p < .05$). At the 14th d, the swimming ability of rats in the autism group was noted to improve significantly, but compared with the normal group, there was still a large variance ($p < .05$); At the 16th d, no significant differences were observed between the two groups. Repetitive and stereotyped behaviors are shown in Fig. 2B, C and D: The repetitive and stereotyped behaviors were significantly more common, the cumulative time of the repetitive and stereotyped behaviors was longer and the walking distance was longer in the autism group than those in the normal group ($p < .05$). The results demonstrated that neonate rat models of autism were successfully established.

3.3. LOC101927196 was poorly expressed and FZD3 was highly expressed in neonate rat models of autism

Differential analysis of GSE18123 demonstrated that LOC101927196 was decreased in a rat model of autism (Table 2). Prediction of the target gene for LOC101927196 on the MEM website revealed that LOC101927196 exerts effect on in a rat model of autism *via* the Wnt signaling pathway by targeting FZD3 (Table 3). Subsequently, RT-qPCR and Western blot analysis were employed in order to detect the expression of LOC101927196, Trx2, 4-HNE, GSK-3β, Bax, Wnt2, FZD3, β-catenin and Bcl-2. As shown in Fig. 3A-C, compared with the brain tissues in normal rats, LOC101927196 expression in the brain tissues of rats in a rat model of autism was noted to be decreased significantly, with evidently decreased mRNA expression of Trx2, and significantly elevated 4-HNE protein expression, while the mRNA and protein expression of GSK-3β and Bax significantly decreased and that of Wnt2, FZD3, β-catenin and Bcl-2 was significantly elevated. In addition, the phosphorylation protein expression of GSK-3β was decreased, while that of β-catenin was significantly increased (all $p < .05$). The above results suggested that LOC101927196 was underexpressed in neonate rat models of autism.

3.4. Higher positive rate of FZD3 protein was observed in neonate rats with autism

In order to elucidate the effect of FZD3 expression on neonate rat model of autism, we examined the positive expression of FZD3 using immunohistochemistry. It was observed that the positive expression of FZD3 was presented as brown staining, which primarily manifested as pale brown or tan cell membrane or/and endochylema with unequal positive expression, however, the positive expression in the cytoplasm was more intense. The positive rate of FZD3 protein expression in normal rats was (15.42 ± 2.12)%. The positive rate of FZD3 protein expression in rat models of autism (offspring of VPA-treated rats) was (78.47 ± 8.24)% ($p < .05$) (Fig. 4A-B). Compared with brain tissues of normal rats, the positive rate of FZD3 protein expression in brain tissues in the autism group was noted to be higher ($p < .05$). The above-mentioned results confirmed the significantly higher expressions of FZD3 in a rat model of autism.

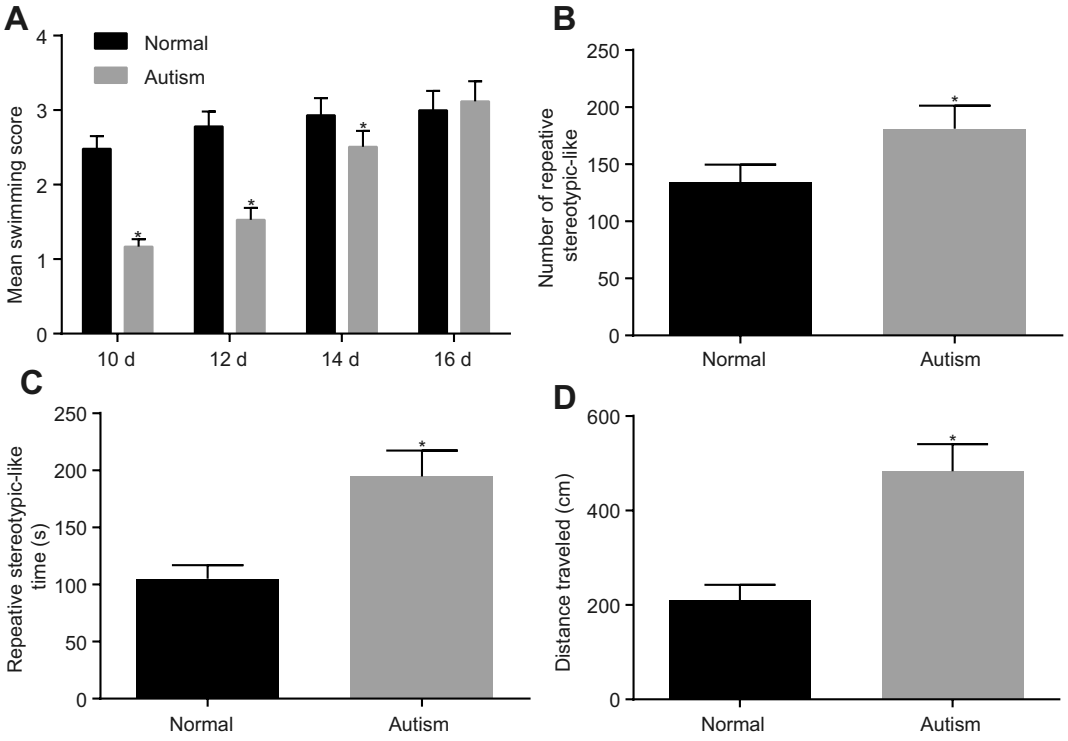

Fig. 2. Neonate rat models of autism showed up-regulated oxidative stress levels (n = 4). A, the swimming test scores of rats in the normal group and the autism group; B, the times of repetitive and stereotyped behaviors within 10 min; C, the cumulative time of repetitive and stereotyped behaviors within 10 min; D, walking distance of repetitive and stereotyped behaviors within 10 min; 4-HNE, 4-Hydroxynonenal; ROS, reactive oxygen species; RNS, reactive nitrogen species. Independent-sample *t*-test was applied for data analysis; * $p < .05$ *vs.* the normal group.

Table 2
Differentially expressed genes.

Gene	ID	Fold change	*p*. value	Adj. P. val
PAGE5	90,737	2.461999387	0.000640	0.055152902
HLA-DQA1	3117	4.034105900	0.002594	0.079882036
COL24A1	255,631	2.894212657	0.003836	0.091669960
MFSD6L	162,387	2.582191354	0.048504	0.247364980
ACY1	95	2.574918757	0.010707	0.134177346
PCDHGA10	56,106	2.645750391	0.006757	0.112774772
ZNF497	162,968	2.077508427	0.026901	0.190610317
CYP4F11	57,834	−2.694242022	0.030268	0.199638623
LOC101927196	101,927,196	−2.521005066	0.003633	0.089913275
SUN3	256,979	−2.034499416	0.001680	0.071845075

Notes: HLA-DQA1, major histocompatibility complex, class II, DQ alpha 1; COL24A1, collagen, type XXIV, alpha 1; MFSD6L, major facilitator superfamily domain containing 6-like; ACY1, aminoacylase 1; PCDHGA10, protocadherin gamma subfamily A, 10; ZNF497, zinc finger protein 497; CYP4F11, cytochrome P450 family 4 subfamily F member 11; SUN3, Sad1 and UNC84 domain containing 3.

Table 3
Prediction of target gene and function analysis of LOC101927196.

Pathway	Description	*p* value	ID	Gene
hsa04962	Vasopressin-regulated water reabsorption	0.042069	51,626	DYNC2LI1
hsa03460	Fanconi anemia pathway	0.04769	11,201	POLI
hsa05217	Basal cell carcinoma	0.048624	7976	FZD3
hsa00240	Pyrimidine metabolism	0.08627	221,264	AK9
hsa04916	Melanogenesis	0.087172	7976	FZD3
hsa04310	Wnt signaling pathway	0.122666	7976	FZD3
hsa04550	Signaling pathways regulating pluripotency of stem cells	0.12441	7976	FZD3
hsa05224	Breast cancer	0.128758	7976	FZD3
hsa04390	Hippo signaling pathway	0.131358	7976	FZD3
hsa04150	mTOR signaling pathway	0.133951	7976	FZD3

Notes: DYNC2LI1, dynein cytoplasmic 2 light intermediate chain 1; POLI, DNA polymerase iota; FZD3, frizzled class receptor 3; AK9, adenylate kinase 9; Wnt, Wingless and INT.

3.5. Over-expressed LOC101927196 inhibited the expression of FZD3 and the activation of the Wnt signaling pathway in neuron cells in a rat model of autism

To investigate the interaction of LOC101927196, FZD3 and the Wnt signaling pathway and their effect on hippocampus neuron cells in a rat model of autism, we adopted RT-qPCR and Western blot analysis to detect expression of related factors as well as the proliferation and apoptosis-related gene expressions. As shown in Fig. 5A-C, compared with the normal group, LOC101927196 was decreased, mRNA expression of Trx2 was decreased, protein expression of 4-HNE was increased, mRNA and protein expression of GSK-3β and Bax was decreased, while that of Wnt2, FZD3, β-catenin and Bcl-2 was increased and the extent of GSK-3β phosphorylation was decreased while the extent of β-catenin phosphorylation was increased in the other groups ($p < .05$). There were no obvious differences in the expression of LOC101927196, mRNA and protein expression of Wnt2, FZD3, GSK-3β, β-catenin, 4-HNE, Trx2, Bcl-2 and Bax, and the extent of GSK-3β and β-catenin phosphorylation between the blank group and the NC group ($p > .05$). Compared with the blank group and the NC group, LOC101927196 was found to be increased in the LOC101927196 vector group ($p < .05$), while there were no significant differences in LOC101927196 in the Sulindac group (the Wnt signaling pathway inhibitor) ($p > .05$). Compared with the blank and NC groups, the mRNA expression of Trx2 was increased, protein expression of 4-HNE was decreased, mRNA and protein expression of GSK-3β and Bax was increased, while that of Wnt2, FZD3, β-catenin and Bcl-2 was declined, the extent of GSK-3β phosphorylation was increased but the extent of β-catenin phosphorylation was decreased in the LOC101927196 vector group and the

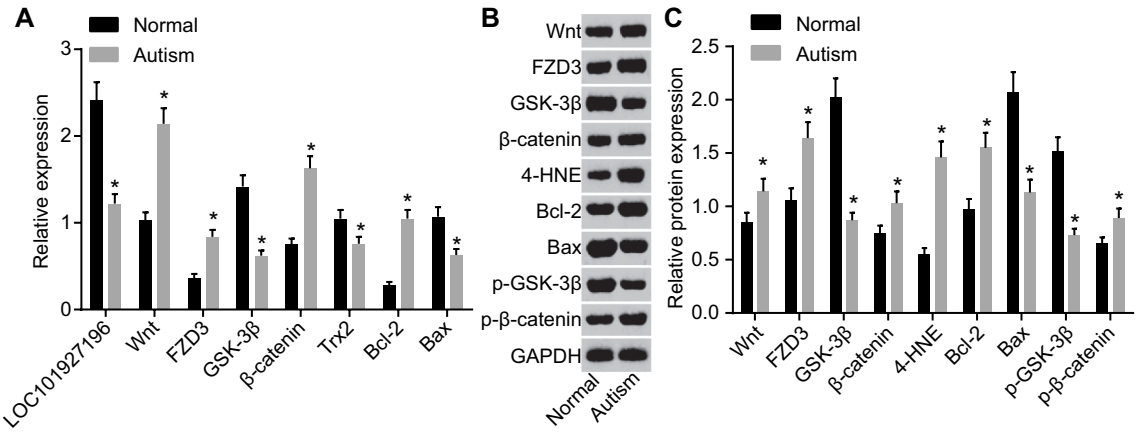

Fig. 3. LOC101927196 was decreased in a rat model of autism and LOC101927196 targeting FZD3 mediated autism in rats *via* the Wnt signaling pathway (n = 4). A, LOC101927196 expression and mRNA expressions of Wnt2, FZD3, GSK-3β, β-catenin, Trx-2, Bcl-2 and Bax in tissues detected by RT-qPCR; B, protein expression blotting graph of Wnt2, FZD3, GSK-3β, β-catenin, 4-HNE, Bcl-2, Bax, p-GSK-3β and p-β-catenin in tissues by Western blot analysis; C, statistical graph of protein expressions of Wnt2, FZD3, GSK-3β, β-catenin, 4-HNE, Bcl-2, Bax, p-GSK-3β and p-β-catenin in tissues; independent-sample *t*-test was used for analysis. * $p < .05$ *vs.* the normal group. FZD3, frizzled class receptor 3; GSK-3β, glycogen synthase kinase 3 beta; Trx2, thioredoxin 2; Bcl-2, B cell leukemia/lymphoma 2; Bax, BCL2 associated X; 4-HNE, 4-hydroxy-2-nonenal.

Sulindac group (all $p < .05$). Compared with the bank and NC groups, LOC101927196 expression was reduced, mRNA expression of Trx2 was reduced and protein expressions of 4-HNE was elevated, mRNA and protein expressions of GSK-3β and Bax were decreased, while that of Wnt2, FZD3, β-catenin and Bcl-2 was increased, the extent of GSK-3β phosphorylation was decreased but the extent of β-catenin phosphorylation was increased in the siRNA-LOC101927196 group ($p < .05$). Compared with the blank and NC groups, LOC101927196 was decreased in the siRNA-LOC101927196 + Sulindac group ($p < .05$), but no obvious variances were observed in the mRNA and protein expression of Wnt2, FZD3, GSK-3β, β-catenin, 4-HNE, Trx2, Bcl-2 and Bax, and the extent of phosphorylation of GSK-3β and β-catenin in the siRNA-LOC101927196 + Sulindac group ($p > .05$). The results suggested that neuron cells transfected with over-expressed LOC101927196 suppressed the Wnt signaling pathway by decreasing the expression of FZD3 in a rat model of autism.

3.6. Over-expressed LOC101927196 suppressed neuron cell proliferation in a rat model of autism

We examined the neuron viability with 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay in order to detect cell viability in all groups (Fig. 6). A total of 7 groups of cells were seeded in 96-well plates, and the viability was observed at 24 h, 48 h, and 72 h time intervals using MTT assay, and subsequently, the relative viability was calculated. There was no significant variance in cells after 24 h in all groups ($p > .05$). Compared with the rate of cell viability in all groups at 24 h, those at 48 h and 72 h presented with significant differences ($p < .05$). Compared with the normal group, the speeds of cell viability in other groups were significantly enhanced ($p < .05$). There was no significant variance between the blank group and the NC group ($p > .05$). Compared with the blank group and the NC group, cell viability was observed to be obviously slower in the LOC101927196 vector group and the Sulindac group ($p < .05$), while cell viability was enhanced in the siRNA-LOC101927196 group ($p < .05$); there was no significant variance in siRNA-LOC101927196 + Sulindac group ($p > .05$). The above results suggested that over-expression of LOC101927196 inhibited neuron proliferation in a rat model of autism.

3.7. Over-expressed LOC101927196 blocked cell cycle entry in a rat model of autism

We examined changes in cell cycle by means of PI single staining (Table 2). Compared with the normal group, the changes in cell cycles in the other groups were demonstrated as shortened G0/G1 phase (reduced cell proportion) and prolonged S phase (increased cell proportion) ($p < .05$). As shown in Fig. 7A-B, cell cycle in the blank group and the NC group exhibited no significant variance ($p > .05$). Compared with the blank group and the NC group, prolonged G0/G1 phase (increased cell proportion) and shortened S phase (reduced cell proportion) were observed in the LOV101927196 vector group and the Sulindac group ($p < .05$), while shortened G0/G1 phase (decreased cell proportion) and prolonged S phase (increased cell proportion) were observed in the siRNA-LOC101927196 group ($p < .05$); the siRNA-LOC101927196 + Sulindac group presented with no significant

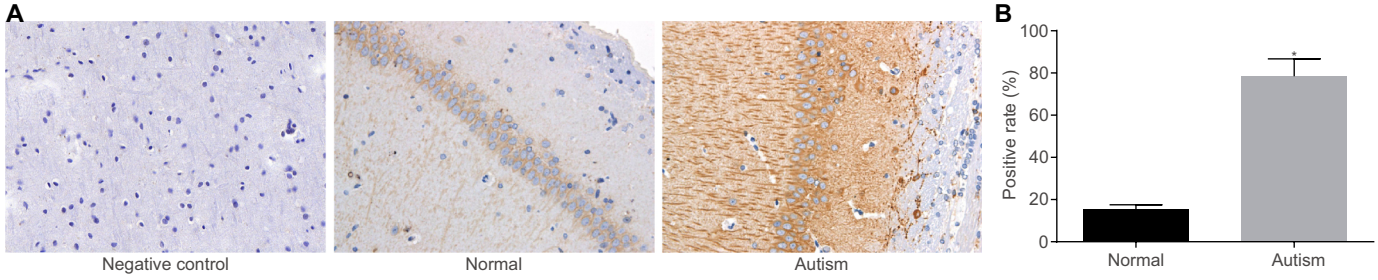

Fig. 4. Neonate rat models of autism showed high positive rate of FZD3 protein expressions. A, the brain tissues in the normal group and the autism group detected by immunohistochemistry (× 400); B, the positive rate of FZD3 in the two groups (n = 4); independent-sample *t*-test was used for date comparion. * $p < .05$ *vs.* the normal group. FZD3, frizzled class receptor 3.

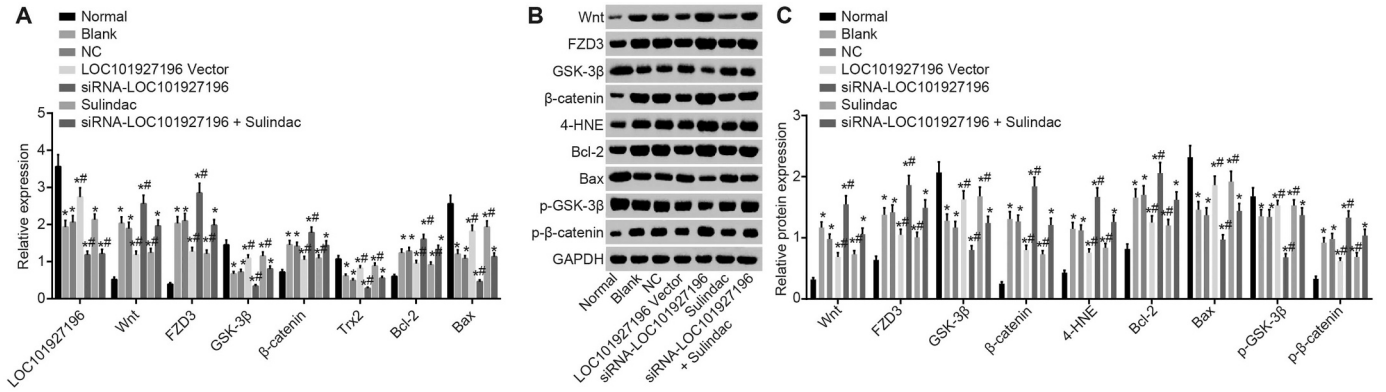

Fig. 5. Over-expressed LOC101927196 suppressed the expression of FZD3 *via* the Wnt signaling pathway to affect neuron cells in a rat model of autism. A, RT-qPCR analysis of LOC101927196 expression and the mRNA expressions of Wnt2, FZD3, GSK-3β, β-catenin, Trx-2, Bcl-2 and Bax in cells; B, Protein bands of Wnt2, FZD3, GSK-3β, β-catenin, 4-HNE, Bcl-2, Bax, p-GSK-3β and p-β-catenin in cells; C, statistical graph of protein expressions of Wnt2, FZD3, GSK-3β, β-catenin, 4-HNE, Bcl-2, Bax, p-GSK-3β and p-β-catenin in cells; the experiment was conducted 3 times. One-way ANOVA was used for data comparison. * $p < .05$ *vs.* the normal group; # $p < .05$ *vs.* the blank group and the NC group. FZD3, frizzled class receptor 3; GSK-3β, glycogen synthase kinase 3 beta; Trx2, thioredoxin 2; Bcl-2, B cell leukemia/lymphoma 2; Bax, BCL2 associated X; 4-HNE, 4-hydroxy-2-nonenal; NC, negative control; ANOVA, analysis of variance.

variance ($p > .05$). The aforementioned findings indicated that over-expression of LOC101927196 resulted in blocked cell cycle entry in a rat model of autism.

3.8. Over-expressed LOC101927196 promoted neuron cell apoptosis in a rat model of autism

We examined apoptosis in all groups by means of Annexin V/PI two-parametric analysis (Fig. 8A-B). The cell apoptotic rates of the normal group, the blank group, the NC group, the LOC101927196 vector group, the siRNA-LOC101927196 group, the Sulindac group and the siRNA-LOC101927196 + Sulindac group were (41.3 ± 3.74) %, (15.92 ± 1.48) %, (13.98 ± 1.29) %, (28.62 ± 3.15) %, (5.33 ± 0.81) %, (30.69 ± 2.87) %, (17.04 ± 1.50) %, respectively. Compared with the normal group, decreased cell apoptotic rates were noted in the other groups ($p < .05$). There was no significant variance in the cell apoptotic rates in the blank group and the NC group ($p > .05$). Compared with the blank group and the NC group, the cell apoptotic rate was found to be increased in the LOC101927196 vector group and the Sulindac group ($p < .05$), while it decreased in the siRNA-LOC101927196 group ($p < .05$), and there was no significant variance in the siRNA-LOC101927196 + Sulindac group ($p > .05$). The findings suggested that cell apoptosis was accelerated by over-expression of LOC101927196 in a rat model of autism.

3.9. Over-expressed LOC101927196 inhibited oxidative stress in a rat model of autism

The results of detection of concentration of 4-HNE, ROS and RNS were shown in Fig. 9. At first, colorimetry was employed to detect the concentration of 4-HNE, ROS and RNS of rats in the normal and autism groups (Fig. 9A). The results showed that compared with the normal group, the concentration of 4-HNE, ROS and RNS in serum of rats in the autism group was obviously elevated with disordered oxidative stress levels ($p < .05$).

Then the concentration of 4-HNE, ROS and RNS were determined in neurons of each group (Fig. 9B). Compared with the normal group, the concentrations of 4-HNE, ROS and RNS were found to be significantly increased in the other groups ($p > .05$). Compared with the blank group and the NC group, the concentrations of 4-HNE, ROS and RNS in the LOC101927196 vector group and the Sulindac group were evidently decreased ($p < .05$), while they were significantly increased in the siRNA-LOC101927196 group ($p < .05$), and there was no significant variance in the siRNA-LOC101927196 + Sulindac group ($p > .05$). The above-mentioned results indicated that over-expression of LOC101927196 suppressed oxidative stress in a rat model of autism.

4. Discussion

Autism is defined as a neurological and development disorder characterized by verbal and nonverbal communication, impairments in social interaction and restricted and repetitive behavior [20]. The common treatment of choice for autism is behavioral therapy, however it is proved to be partially effective in most cases [21]. Therefore, a new target for innovative therapy is warranted in order to raise the quality of life of patients plagued by autism. Interestingly, a previous research highlighted the importance of dysregulated primate-specific lncRNAs between frontal and temporal lobes in autism [22]. In the current study, we aimed to explore the effect of LOC101927196 on oxidative stress and neurons using a rat model of autism. Consequently, we observed that up-regulated LOC101927196 suppressed neuron cell proliferation while promoting apoptosis through inhibition of the Wnt signaling

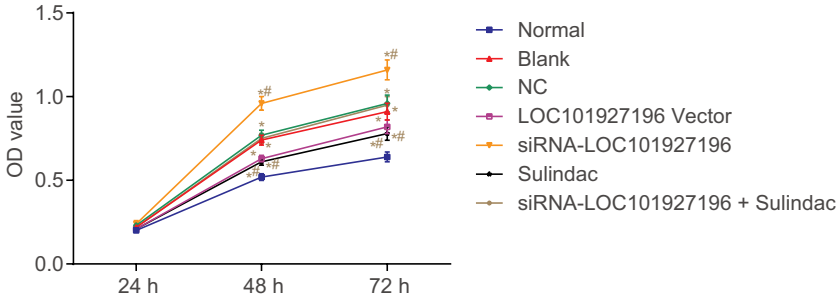

Fig. 6. Over-expressed LOC101927196 inhibited cell proliferation in a rat model of autism. The experiment was conducted 3 times, and two-way ANOVA was used for data comparison. * $p < .05$ *vs.* the normal group; # $p < .05$ *vs.* the blank group and the NC group. FZD3, frizzled class receptor 3; NC, negative control; OD, optical density; ANOVA, analysis of variance.

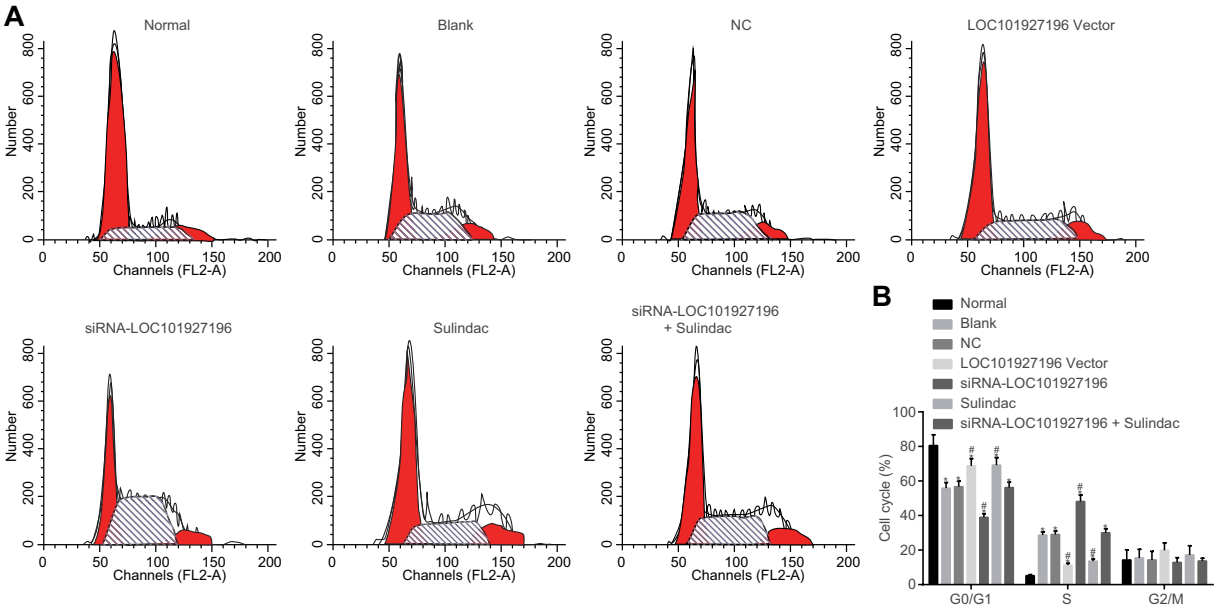

Fig. 7. Cell cycle entry was blocked by enhanced LOC101927196 in a rat model of autism. A, Cell cycle diagram of each group; B, the cell cycle distribution of each group; the experiment was conducted 3 times. One-way ANOVA was performed. * $p < .05$ *vs.* the normal group; # $p < .05$ *vs.* the blank group and the NC group. FZD3, frizzled class receptor 3; NC, negative control; ANOVA, analysis of variance.

pathway, and attenuated oxidative stress disorder in a rat model of autism.

Initially, our data showed that autism led to poor development and growth in neonate rats as evidenced by growth and development tests. In addition, autism patients are known to commonly exhibit limited physical activity and delayed motor skills and physical fitness [23]. With significantly elevated 4-HNE, ROS and RNS levels, we demonstrated the existence of a relationship between oxidative stress disorder and autism in neonate rats. Additionally, genetic, environmental and immunological risk factors induce oxidative and neuronal damage while reducing methylation activity, which is vital for the central nervous system; moreover, oxidative stress has been regarded as a potential target in the pathogenesis of autism [24]. Similarly, Kulbir Kaur reported that ROS was increased when lymphoblastoid cells from children with autism were exposed to bisphenol A (BPA), suggesting that BPA exposure results in increased oxidative stress and

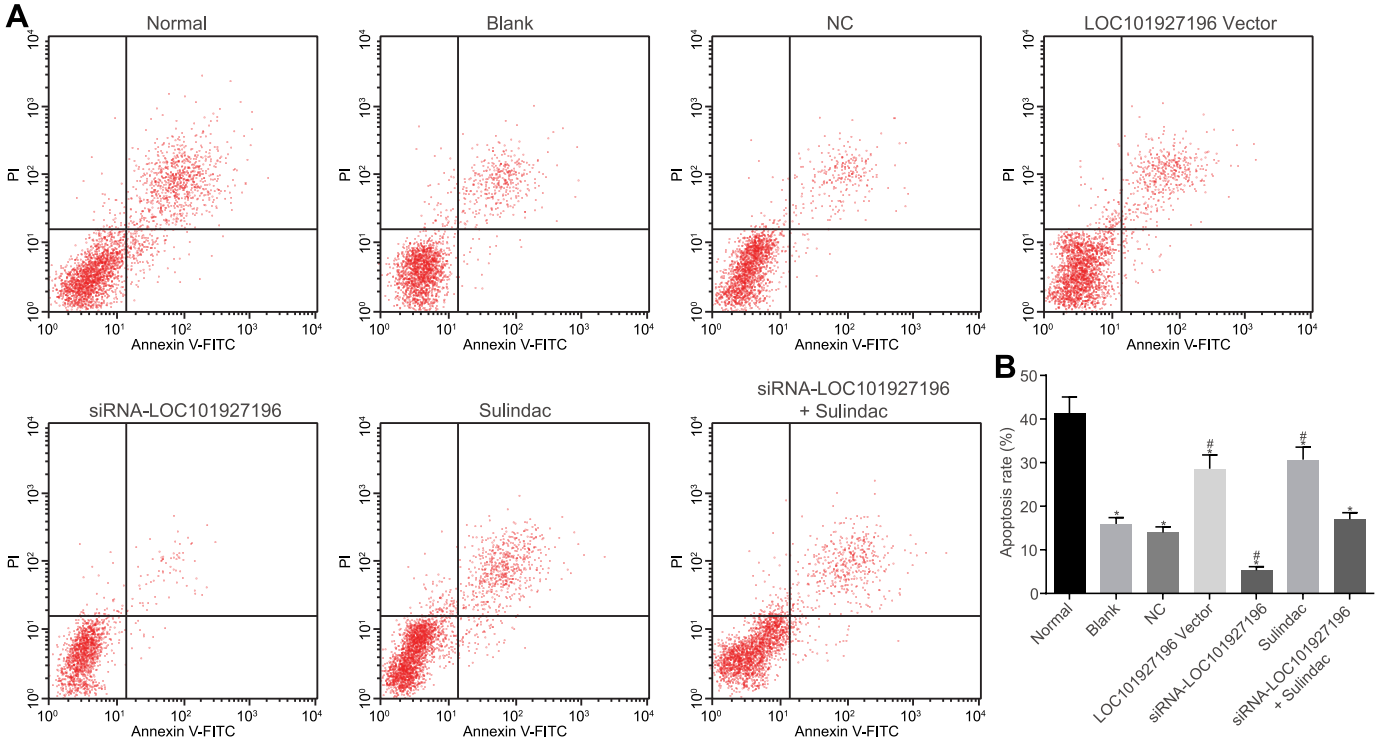

Fig. 8. Over-expressed LOC101927196 promoted cell apoptosis in a rat model of autism. A, cell apoptosis diagram of each group; B, the apoptosis rate in each group; The experiment was conducted 3 times. One-way ANOVA was performed. * $p < .05$ *vs.* the normal group; # $p < .05$ *vs.* the blank group and the NC group. FZD3, frizzled class receptor 3; NC, negative control; ANOVA, analysis of variance.

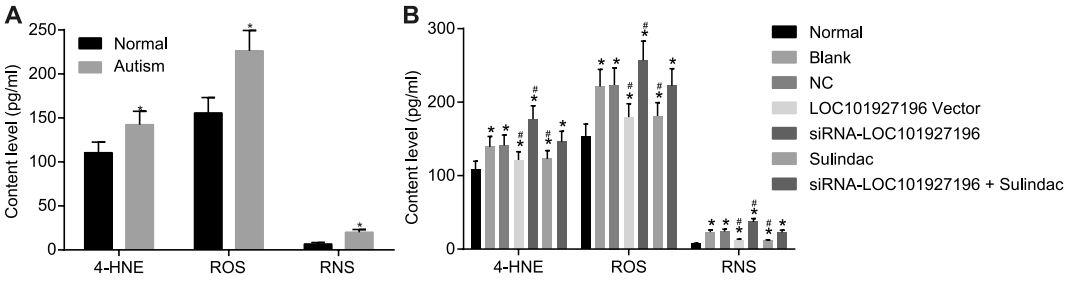

Fig. 9. Oxidative stress was suppressed in a rat model of autism after cells were transfected with the LOC101927196 vector. A, the concentration of 4-HNE, ROS and RNS of rats in the normal and autism groups; the data were analyzed by independent sample *t*-test. $^{\#}p < .05$ *vs.* the normal group. B, the concentration of 4-HNE, ROS and RNS of rats in the neurons of each group. The experiment was conducted 3 times. One-way ANOVA was performed for comparisons among multiple groups. $^{*}p < .05$ *vs.* the normal group; $^{\#}p < .05$ *vs.* the blank group and the NC group. FZD3, frizzled class receptor 3; 4-HNE, 4-hydroxy-2-nonenal; ROS, reactive oxygen species; RNS, reactive nitrogen species; NC, negative control; ANOVA, analysis of variance.

mitochondrial dysfunction in the autistic subjects [25].

In the subsequent experiments, we found that rat models of autism presented with decreased expression of LOC101927196, while that of FZD3 was increased. Furthermore, we revealed that LOC101927196 was indeed a target of FZD3. A prior study of Julieta Aprea showed that lncRNA-mediated alternative splicing of cell fate determinants controlled stem-cell neurogenic commitment during mouse brain development [26]. In another study, Tang et al. also reported that aberrant expressions of lncRNAs in both lymphoblastoid cell lines and postmortem brain tissues serve as future avenues to possibly apply lncRNAs in the treatment and prognosis for autism [8]. In addition, lncRNA FMR4 (Fragile X mental retardation 4) was vital in normal human neurobiology and also played a central role in the Fragile X repeat expansion-related disorders pathogenesis, while Fragile X syndrome usually contributed to inherited intellectual disability and autism as a famous genetic cause [27]. In addition, the over-expression of functional element autism-associated lncRNA MSNP1AS (moesin pseudogene 1, antisense) with genome-wide significance in human neuronal cells led to decreased protein expression of moesin, which was reported to be involved in neuronal process stability in a recent research [28]. Furthermore, Tabares-Seisdedos et al. reported the involvement of FZD3 in the nosogeny of cancer and neuropsychiatric disorders [10]. Besides, a prior study reported that FZD3 participates functionally or genetically in schizophrenia [29]. Meanwhile, the alteration of Wnt signaling pathway in the brains of patients with autism resulted in changes of astrocytes [30].

Additionally, the current study demonstrated that over-expressed LOC101927196 inhibits cell proliferation and oxidative stress but promotes cell apoptosis *via* the Wnt signaling pathway through inhibition of FZD3. As a receptor for Wnt glycoprotein that is implicated in the Wnt signal transduction cascades, alterations of FZD3 are associated with cyto-architectural defects production in schizophrenia [31]. In a recent study, the mutations in chromodomain helicase DNA-binding protein 8 (CHD8), *de novo* mutation significantly related to ASC, were found to regulate the Wnt signaling pathway [32]. Another study also found that the Wnt signaling pathway was activated and neural cell proliferation was increased in newborn rats with hypoxic ischemic encephalopathy [33]. Besides, the stimulation of the Wnt signaling pathway was proven to lead to the proliferation of neural progenitor cells during the early stage of neurogenesis [34]. Furthermore, the important role of the Wnt/β-catenin signaling pathway and oxidative stress in the pathogenesis of autism has been reported previously [11]. In the current study, a differential analysis of GSE18123 showed that LOC101927196 was expressed at low levels in autistic patients.

Overall, we confirmed that up-regulated LOC101927196 targeting FZD3 attenuated oxidative stress disorder in a rat model of autism, and promoted neuron cell apoptosis while suppressing proliferation through the Wnt signaling pathway, suggesting that LOC101927196 may serve as a new target for prevention and treatment for autism. In the future, the use of human cell lines will be required to draw a full picture of the research to deeply figure out the mechanisms of LOC101927196 on autistic subjects. Moreover, the effect of altered expression of other genes on autism models and on postmortem brain tissue from individuals with autism will be included in our future study.

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Author contributions

Wanxia Yao performed the research and prepared the figures; Junting Huang designed the research study; Hongling He analyzed the data and wrote the paper. All authors have revised this manuscript.

Disclosure of statement

No potential conflict of interest was reported by the authors.

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