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Short Communication

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Nkx2.1 downregulation is involved in brain abnormality induced by excess retinoic acid

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Abstract

Abnormal development of central nervous system (CNS) caused by neural tube defects is not only a major contributor in the prevalence of stillbirths and neonatal deaths but also causes lifelong physical disability in surviving infants. Due to insufficient known investigated causes, CNS developmental abnormality has brought sever burden on health around the world. From previous results of high throughput transcriptome sequencing, we selected transcription factor *Nkx2.1* as a candidate to investigate its role on brain abnormalities induced by excessive retinoic acid. The result of *in situ* hybridization showed that *Nkx2.1* was mainly expressed in mouse brain. After the *Nkx2.1* gene was silenced, retarded proliferation and accelerated apoptosis were found in mouse Neuro-2a (N2a) cells. Furthermore, our results indicated that the main components of sonic hedgehog (Shh) signaling pathway were affected in *Nkx2.1*-silenced cells, implying that *Nkx2.1* plays an important role in the development of mouse brain by regulating Shh signaling pathway.

Key words: brain abnormality, Nkx2.1, retinoic acid, apoptosis, proliferation

Introduction

Neural tube defects (NTDs) are common and serious birth defects of central nervous system (CNS) due to unclosed or partially closed neural tube. Its phenotypes mainly include anencephaly, spina bifida and encephalocele [1]. NTDs incidence leads to congenital heart diseases with a global prevalence ranging from 1 to 10 per 1000 births, which has posed a heavy burden on the development of society [2]. Most studies showed that deficiency of folic acid in the diet is a significant risk contributor to NTDs [3]. At present, pathogenesis of NTDs is considered to be associated with both genetic and environmental factors. The underlying genetic risks and mechanisms involved in the developmental abnormalities of CNS are still need to be further investigated.

Neural tube closure (NTC) is a crucial check point of CNS development and is regulated by multiple relevant signaling pathways, including sonic hedgehog (Shh), Notch, wnt/β-catenin signaling pathways and so on [4–7]. Retinoic acid (RA) is an indispensable morphogen in CNS development, but excessive RA leads to brain abnormalities including NTDs and its other phenotypes [8]. Currently, NTDs induced by excessive RA is widely used as a reliable model for investigating the molecular mechanisms of CNS development abnormality [9–12]. Our previous study has showed that expression of *Nkx2.1* is significantly reduced in the brain tissue of RA-induced

NTDs [13]. Therefore, we proposed a possibility that *Nkx2.1* is associated with brain abnormality induced by excessive RA. *Nkx2.1* is a member of vertebrate *Nkx* family, which is one of the earliest known genes to be expressed in the forebrain [14,15]. Several studies have proved that *Nkx2.1* plays a crucial role in multiple aspects of brain development including specification of cortical neurons, glia, telencephalon, oligodendrocytes, commissure development, *etc.* [16–18]. Recent studies have shown that Pax6 controls the expression of *Nkx2.1*, sonic hedgehog (Shh) and LIM homeobox domain 6 (Lhx6) in the prechordal neural tube [16]. There is a correlation between *Nkx2.1* and NTDs, but the detailed mechanisms and its role in N2a cells still needs to be investigated.

Shh signaling pathway has an important function in various embryonic developmental processes [19–21]. Shh binds to Patched (Ptch) and enhances Smoothened (Smo) expression, which further increases the expression of Glioblastoma-1 (Gli1), responsible for the regulation of cell proliferation, migration and differentiation [22,23]. Moreover, studies have also shown that down-regulation of Shh signaling pathway leads to abnormal cardiac and other embryonic developmental diseases [18,19]. In some cases, the Shh signaling pathway is correlated with *Nkx2.1* expression. An early study has provided evidence that expression of NKX2.1 in the neural domains requires Shh signals [24]. Recent investigations have confirmed that Shh signaling pathway maintains cortical interneuron progenitor identity and composition through its regulation of *Nkx2.1* expression [25].

In this study, we tested a hypothesis that *Nkx2.1* may promote cell proliferation and inhibit apoptosis through the Shh signaling pathway in brain abnormality induced by excessive RA. Specifically, we silenced *Nkx2.1* in N2a cells and results indicated a possible role of *Nkx2.1* in the CNS development by regulating the Shh signaling pathway. This study precisely highlights the important role of *Nkx2.1* in RA-induced brain abnormality.

Materials and Methods

Animals

C57BL/6 mice (9–10 weeks, 19–25 g) were provided by Shanxi Medical University (Taiyuan, China) and housed in specific pathogen-free (SPF) cages with approved facilities and 12 h light/dark cycle. Mature male and female C57BL/6 mice were mated overnight and were checked for vaginal plug in the morning. After 7.5 days (E7.5) of pregnancy, RA (Sigma, St Louis, USA) was intragastrically administered at a dose of 28 mg/kg to establish brain abnormal embryo model. In the control group, the same dose of sesame oil was administered by same route. The brain tissues and complete embryos were separated at indicated time points for whole mount *in situ* hybridization or quantitative RT-PCR (qRT-PCR). Three different pregnant mice were used in each experiment.

Cell culture and liposome transfection

N2a cells were obtained from (ATCC, Manassas, USA) and cultured in Dulbecco's Modified Eagle's Medium (DMEM; Hyclone, Logan, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, USA). All cells were incubated in a humidified atmosphere with 5% CO_2 at 37°C. The siRNA-Nkx2.1 plasmid was synthesized by Santa Cruz Biotechnology (Santa Cruz, USA) and transfected into the cells using Lipofectamine RNAiMax (Santa Cruz) according to the manufacturer's instruction. The sequence of siNkx2.1 was forward: 5′-GCGGCATGAATATGAGTGGC-3′, reversed: 5′-CGCCGACAGGTACTTCTGTT-3′, and that of the negative control (siCon) was forward: 5′-UUCUCCGAACGUGUCACGUTT-3′, reversed: 5′-ACGUGACACGUUCGGAGAATT-3′.

Whole mount *in situ* hybridization

The whole mount *in situ* hybridization was carried out basically as described by Chowanadisai *et al.* [28]. Hybridization with specific RNA probes (0.25–1 μg/ml) was performed overnight at 65°C. BM Purple (Roche, Basel, Switzerland) was used as substrate for the AP-enzyme. In some embryos, near the standard dark blue precipitate that marks high levels of RNA in some embryo tissues, a turquoise color appears. It marks sites with low specific RNA concentrations because it does not appear in any meaningful hybridization control experiments or in embryos with only low levels of RNA. Dioxygenin-labeled probes were generated from embryo brain tissues-derived cDNA using 5′-TACAAGAAAGTGGGCATGGA-3′ as forward primer and 5′-AGGGTTTGCCGTCTTTGACT-3′ as reverse primer for *Nkx2.1*. This cDNA fragment was subsequently cloned into the pGEM-T Easy vector (Promega, Madison, USA). Digoxigenin-labeled probes were from Boehringer Mannheim (Mannheim, Germany).

Quantitative RT-PCR

Total RNA was isolated from brain tissue using TRIzol reagent (Invitrogen, Carlsbad, USA) and cDNA was synthesized using Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, USA). Quantitative PCR was performed on a Real-Time PCR platform (Applied Biosystems, Foster City, USA) with 20 μl reaction mixtures containing 10 μl 2 × SYBRPremix Ex Taq (Takara, Shiga, Japan), 0.5 μl primers (2.5 μM), 2 μl cDNA, and 7 μl ddH_2O. The cycling conditions were as follows: initial denaturation at 95°C for 10 min and 45 cycles of 15 s denaturation at 95°C and 1 min annealing–extension at 60°C. The data was analyzed with $2^{-\Delta\Delta Ct}$ method and mRNA level was normalized to that of *β-actin*. The qRT-PCR primers for *Nkx2.1* were as follows: 5′-GTCCTCGGAAAGACAGCATC-3′ (forward) and 5′-GTGCTTTGGACTCATCGACA-3′ (reverse). The qRT-PCR primers for *β-actin* were 5′-GCTCTTTTCCAGCCTTCCTT-3′ (forward) and 5′-AGGTCTTTACGGATGTCAAGG-3′ (reverse).

Western blot analysis

The cells or embryo brain tissues were lysed with RIPA lysis buffer (Solarbio, Beijing, China) supplemented with a protease inhibitor cocktail (Sigma) and 10 mM PMSF (Solarbio). Following centrifugation at 12,000 *g* for 20 min at 4°C, the concentration of protein was measured using BCA Protein Assay kit (Thermo Scientific). Subsequently, equal amount of protein was subject to 12% SDS-PAGE and transferred to nitrocellulose membranes using semi-dry electrophoretic transfer method (Bio-Rad, Hercules, USA). After being blocked with 5% skim milk in PBST (PBS with 0.05% Tween-20) for 1 h at room temperature, membranes were incubated with primary antibodies overnight at 4°C, followed by incubation with the corresponding secondary antibody at room temperature for 2 h. β-Tubulin and GAPDH were used as control. The primary antibodies used for western blot analysis were rabbit anti-Nkx2.1 antibody (1:1000; Abcam, Cambridge, USA), rabbit anti-Cyclin D1 antibody (1:1000; Abcam), mouse anti-Shh antibody (1:1000; Abcam), mouse anti-Smo antibody (1:500; Santa Cruz Biotechnology), rabbit anti-Ptch1 antibody (1:1000; Cell Signaling Technology, Boston, USA)

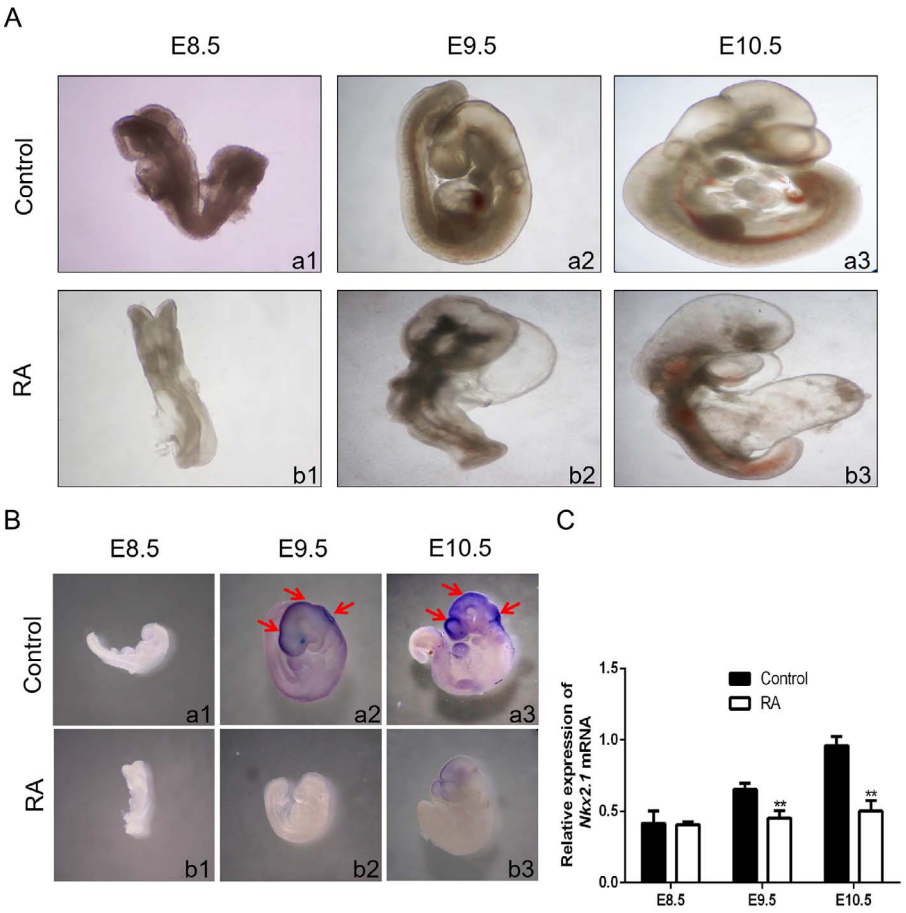

Figure 1. Nkx2.1 expression is decreased in RA-treated mouse embryos (A) Obvious abnormality was observed in the brain of embryos of RA-treated mice. a1, a2, and a3 indicate embryos of control mice at E8.5, E9.5, and E10.5, respectively; and b1, b2, and b3 indicate embryos of RA-treated mice at E8.5, E9.5, and E10.5, respectively. a1, b1: 100×, a2, b2, b3: 50×, a3: 25×. (B) Whole mount *in situ* hybridization was performed to detect Nkx2.1 in mouse embryos at E8.5–E10.5. Blue stain (red arrowheads) represents Nkx2.1 expression. (C) qRT-PCR was used to detect mRNA level of *Nkx2.1* in normal and malformed brain tissue at E8.5–E10.5. $^{**}P < 0.01$ vs control group.

and mouse anti-GAPDH antibody (1:1000; Abcam). The secondary antibodies used were HRP-conjugated goat anti-rabbit IgG (1:5000; ZSGB-BIO, Beijing, China) and HRP-conjugated goat anti-mouse IgG (1:5000; ZSGB-BIO).

Cell apoptosis assay

GreenNuc^{TM} Caspase-3 Assay Kit (Beyotime, Shanghai, China) was used to detect cell apoptosis according to manufacturer's instructions. In brief, N2a cells were seeded in 24-well plates (3×10^4 cells/well), and 48 h after siRNA-Nkx2.1 transfection, cells were washed twice with PBS and treated with 5 μM GreenNuc^{TM} Caspase-3 substrate for 30 min. After washing twice with PBS, DAPI (Solarbio) was added and incubated for 8 min. The cells were visualized with a fluorescence microscope (Nikon, Tokyo, Japan) and apoptotic cells were counted in 10 randomly selected microscopic fields (magnification, ×200). The cells were analyzed using Image J software. Moreover, Annexin V-FITC/PI apoptosis detection kit (KeyGEN, Suzhou, China) was also used for the apoptosis assay. Forty eight hours after siRNA-Nkx2.1 transfection, cells were trypsinized with 0.25% trypsin without ethylenediaminetetraacetic acid (EDTA) and centrifuged at 85 *g* for 5 min. The cells were resuspended in 500 μl binding buffer and stained with 5 μl Annexin V-FITC and 5 μl PI for 15 min at room temperature. The apoptosis rate was measured with a BD FACSCanto II flow cytometer (BD Biosciences, San Jose, USA). All experiments were performed in triplicates.

Cell cycle and proliferation detection

The cell cycle detection kit (KeyGEN) was used for cell cycle analysis. Following fixation of N2a cells in 70% ethanol at 4°C overnight, cells were washed twice with PBS and treated with RNase A and propidium iodide (PI) at 37°C for 1 h. The cell cycle in each group was detected by flow cytometry. Cell-Light Edu Apollo DNA *in vitro* Kit Cell (RiboBio, Guangzhou, China), a 5-Ethynyl-2′-deoxyuridine (EDU)-based DNA synthesis assay [26], was used to detect the cell proliferation. EDU-positive cells were visualized and imaged with a fluorescence microscope (Nikon). Proliferative cells were counted in 10 randomly selected microscopic fields (magnification, ×100) and were analyzed using Image J software.

Statistical analysis

Data are presented as the mean ± standard deviation. SPSS17.0 was used for data analysis. Statistical analysis was performed using

Table 1. Embryonic phenotypes of mice treated by RA

RA (mg/kg)	Pregnant mice N	Embryos N	Normal N (%)	Resorption N (%)	Growth retardation N (%)	NTDs N (%)	Other malformation N (%)
28	14	118	13 (11.1)	6 (5.1)	13 (11.1)	75 (63.5)	6[a],5[b] (9.2)

N: number. NTDs: neural tube defects.
[a]Craniofacial malformation.
[b]Polydactyly.

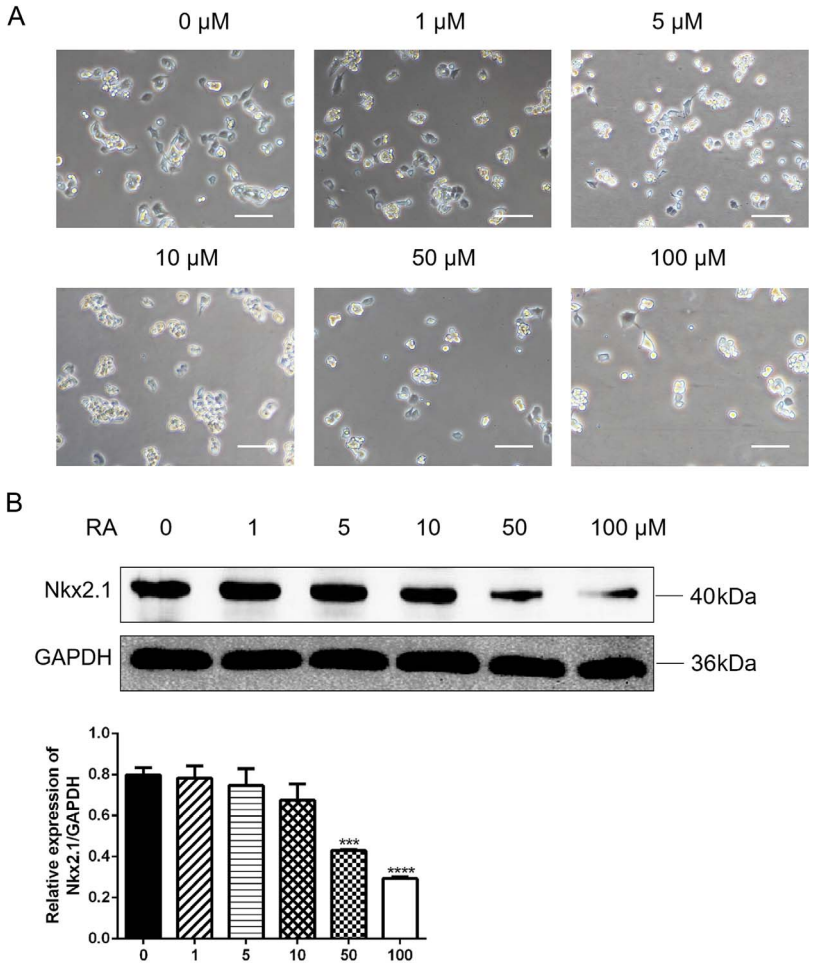

Figure 2. RA regulates Nkx2.1 expression *in vitro* (A) Morphology of N2a cells induced by 0, 1, 5, 10, 50, and 100 μM RA. Scale bar: 500 μm. (B) Nkx2.1 protein expression in N2a cells was analyzed by western blot analysis. GAPDH was used as the loading control. ***$P < 0.001$, ****$P < 0.0001$ vs control group.

Student's *t*-test. Differences were considered statistically significant when $P < 0.05$.

Results

Effect of Nkx2.1 on abnormal brain tissue induced by excessive RA

In order to identify protein factors involved in brain development, we analyzed previous RNA-seq results [13] and chose *Nkx2.1* as a candidate to investigate its function. Compared to normal mouse embryos at E8.5, E9.5 and E10.5, mouse embryos treated with 28 mg/kg RA showed obvious overall growth retardation along with a small and hypoplastic brain vesicle (Fig. 1A). In addition, statistical analysis was performed to grade the severity of phenotype, which showed that the rate of NTDs embryos was 63.5% with 5.1% embryonic resorption rate (Table 1). *In situ* hybridization results indicated that *Nkx2.1* is mainly expressed in the brain of normal embryos especially at E9.5 and E10.5, and an obvious decrease was found in RA-induced embryos (Fig. 1B). The same results were obtained by quantitative RT-PCR analysis (Fig. 1C). Together, tissue specific location of *Nkx2.1* and reduced expression of Nkx2.1 in RA-induced NTDs model suggested that *Nkx2.1* plays an important role in embryo development, especially in brain.

RA regulates Nkx2.1 expression *in vitro*

To examine whether the expression of *Nkx2.1* is regulated by RA, we first treated the N2a cells with 0, 1, 5, 10, 50 and 100 μM RA for 48 h, and investigated the effect of RA on N2a cell viability. As shown

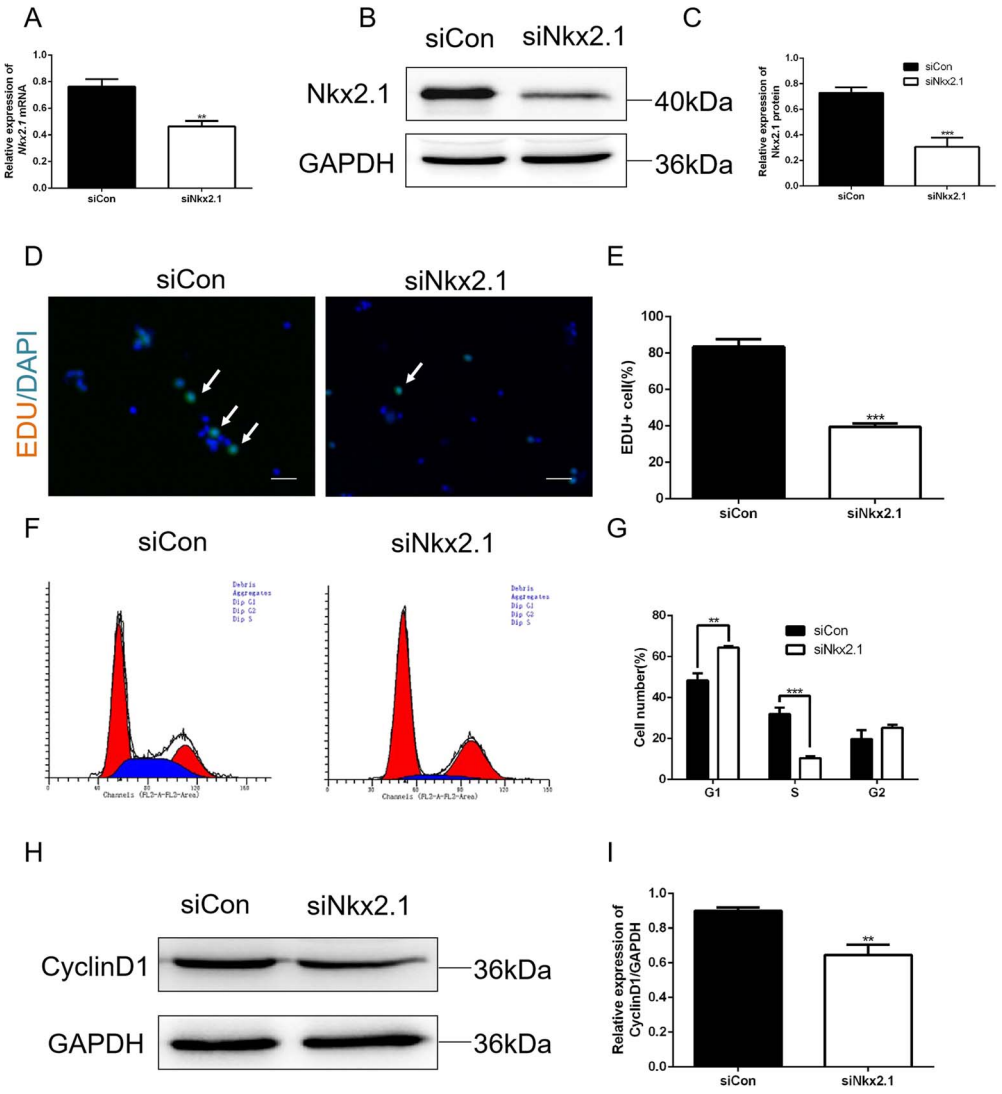

Figure 3. Knockdown of Nkx2.1 affects N2a cell proliferation (A) mRNA level of *Nkx2.1* gene was analyzed by qRT-PCR in the siNkx2.1 and siCon groups. The *β-actin* gene was used as a control. (B,C) The Nkx2.1 protein level in the siNkx2.1 and siCon groups was evaluated by western blot analysis, and the expression of GAPDH was used as the loading control. (D,E) The effect of Nkx2.1 knockdown on cell proliferation was detected using Cell-Light Edu Apollo DNA *in vitro* Kit and was quantified. Images of immunofluorescence cell staining against the EDU (shown in green), and the nuclei were counterstained using DAPI (shown in blue). Scale bar: 200 μm. (F,G) The cell cycle distribution in the siNkx2.1 and siCon groups was analyzed by flow cytometry. (H,I) Cyclin D1 protein levels in the siNkx2.1 and siCon groups were evaluated by western blot analysis (n = 3). GAPDH was used as the loading control. $^{**}P < 0.01$, $^{***}P < 0.001$ vs siCon group.

in Fig 2A, with increased concentration of RA, the cell viability decreased rapidly at 50 and 100 μM RA. These results indicated that RA inhibited N2a cell proliferation. Furthermore, the Nkx2.1 expression under different concentrations of RA was detected by western blot analysis, and the results showed that RA treatment downregulated *Nkx2.1* in N2a cell (Fig. 2B).

Knockdown of *Nkx2.1* represses cell proliferation and increases cell apoptosis

The abundance of *Nkx2.1* mRNA was significantly decreased in Nkx2.1 siRNA-transfected cells (siNkx2.1 group) compared with that in Nkx2.1 cells transfected with control siRNA (siCon group) (Fig. 3A). The expression of *Nkx2.1* was consistent both at mRNA and protein level (Fig. 3A-C), confirming that *Nkx2.1* was successfully knocked down in N2a cells. Experiments with three replicates were performed with three samples in each group. The average transfection rate was 75%. The transfection rate of siCon group was 81%, and the transfection rate of siNkx2.1 group was 73%.

Figure 3D and E showed that the ratio of EDU-positive cells was significantly decreased in siNkx2.1 group compared with siCon group, indicating that Nkx2.1 down-regulation inhibited the proliferation of N2a cells. Cell cycle results showed that cell quantity in S and G1 phase was reduced and increased, respectively in the siNkx2.1 group compared with the siCon group (Fig. 3F,G). In addition, CyclinD1 protein level was significantly decreased in the siNkx2.1 group as revealed by western blot analysis (Fig. 3H,I). These results showed that knockdown of Nkx2.1 blocked the process of cell cycle from G1 to S phase, resulting in cell cycle arrest at G1

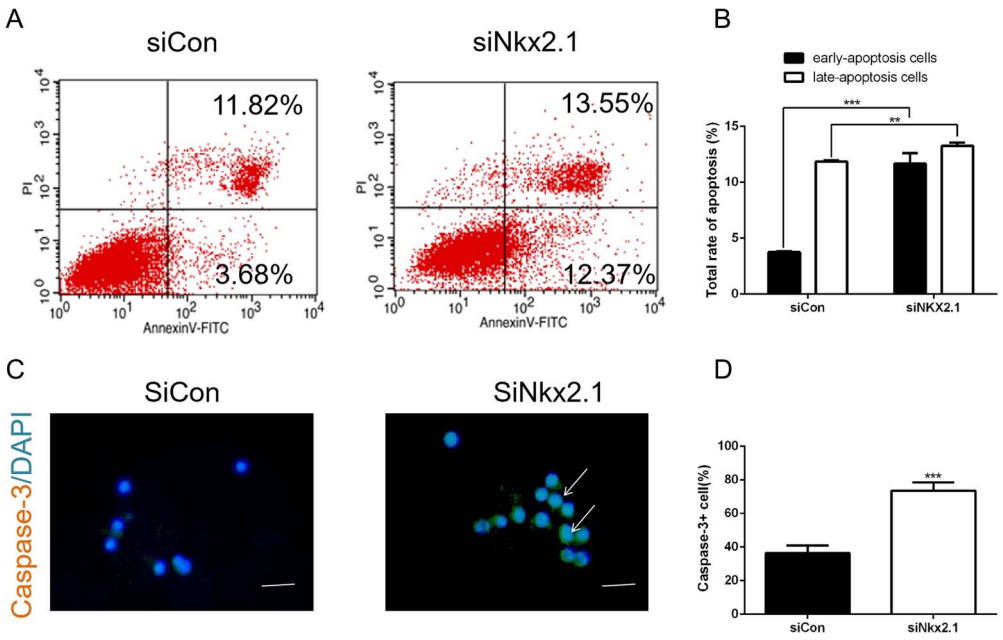

Figure 4. Knockdown of Nkx2.1 causes N2a cell apoptosis (A,B) The cell apoptosis in the siNkx2.1 and siCon groups was analyzed by flow cytometry. (C,D) The cell apoptosis in the siNkx2.1 and siCon groups was detected using the GreenNuc^{TM} Caspase-3 Assay Kit. Images of caspase-3 are shown in green, and the nuclei were counterstained using DAPI, shown in blue. Scale bar: 100 μm. $^{**}P<0.01$, $^{***}P<0.001$ vs siCon group.

phase by reducing CyclinD1, indicating that *Nkx2.1* play a role in N2a cell proliferation.

It was also found that the Nkx2.1 siRNA-transfected N2a cells showed high rate of early and late apoptosis compared with cells in the siCon group (Fig. 4A,B). The GreenNuc^{TM} Caspase-3 assay results showed higher ratio of caspase-3-positive cells in the siNkx2.1 group than the siCon group, indicating that knockdown of *Nkx2.1* increased N2a cell apoptosis (Fig. 4C,D).

Knockdown of *Nkx2.1* gene represses the Shh signaling pathway

Sonic hedgehog (Shh), Ptch1, and Smo are the main components of the Shh signaling pathway. Protein levels of Shh, Ptch1 and Smo were significantly decreased in RA-induced abnormal brain compared with those in the normal brain (Fig. 5A,B). Similarly, the expressions of Shh, Ptch1 and Smo were also impaired in the siNkx2.1 group compared with those in the siCon group (Fig. 5C,D), suggesting that *Nkx2.1* is an important regulator for the Shh signaling pathway. Higher rate of early and late apoptosis was found in the cyclopamine-treated N2a cells (Fig. 5E,F), indicating that repression of the Shh signaling pathway accelerated cell apoptosis. Taking together, *Nkx2.1* is a possible regulator for brain development by activating the Shh signaling pathway.

Discussion

In this study, through *in situ* hybridization we first found that *Nkx2.1* was mainly localized in mouse brain and its expression was significantly lower in RA-induced abnormal brain than in normal group, which is consistent with previous high-throughput transcriptome sequencing results [13]. All these data suggest that *Nkx2.1* plays a potential role in regulating mouse embryonic brain development.

NTC relies on the precise synergy of multiple mechanisms: the convergent extension of neural plates, the migration of neural crest cells, the proliferation and apoptosis of neuroepithelial cells, and apical cytoskeleton microfilament contraction. Under the combined effects of genetic and environmental factors, the destruction of any mechanism may cause the neural tube not to be completely closed, which will cause different types and degrees of NTDs. In fact, there are many cell lines involved in NTC, and choosing any one of the cell lines can not summarize all of them. The most common cells in the current research on neural tube development are primary embryonic stem cells and neural stem cells. N2a cell is a mouse neuroblastoma cell line that is easy to passage, culture and transfect, and N2a cells have been extensively used as a well-characterized model for the study of neuronal differentiation and axonal growth. Previously, it has been shown that neuronal NLRC5 regulates MHC class I expression in N2a cells and during hippocampal development [27,28]. The overall objective of the current study was to understand the importance of *Nkx2.1* in N2a cells, which might lay a foundation for resolving the pathogenesis of RA-induced abnormal brain. So, we first evaluated *Nkx2.1* expression by whole mount *in situ* hybridization during key period of brain development (E8.5–E10.5). The expression of *Nkx2.1* was decreased in RA-induced embryos compared with that in the normal control. A proper balance between cell proliferation and apoptosis is essential during embryonic development [29]. Cell cycle regulation is very important for brain development [30]. The cell cycle is divided into different phases: quiescent and gap 1 (G0/G1), synthesis (S), gap 2 (G2) and mitosis (M) [31]. We constructed *Nkx2.1*-targeting shRNA (siRNA-Nkx2.1) plasmid to silence its expression in N2a cells, and investigated the effects of *Nkx2.1* on cell proliferation and apoptosis. It was confirmed that knockdown of *Nkx2.1* blocked cell progression from G1 to S phase via reducing CyclinD1 activity, which caused an obvious decrease in EDU-positive cells. Moreover, the number of caspase-3-positive cells was significantly increased in *Nkx2.1*-knockdown cells. These results

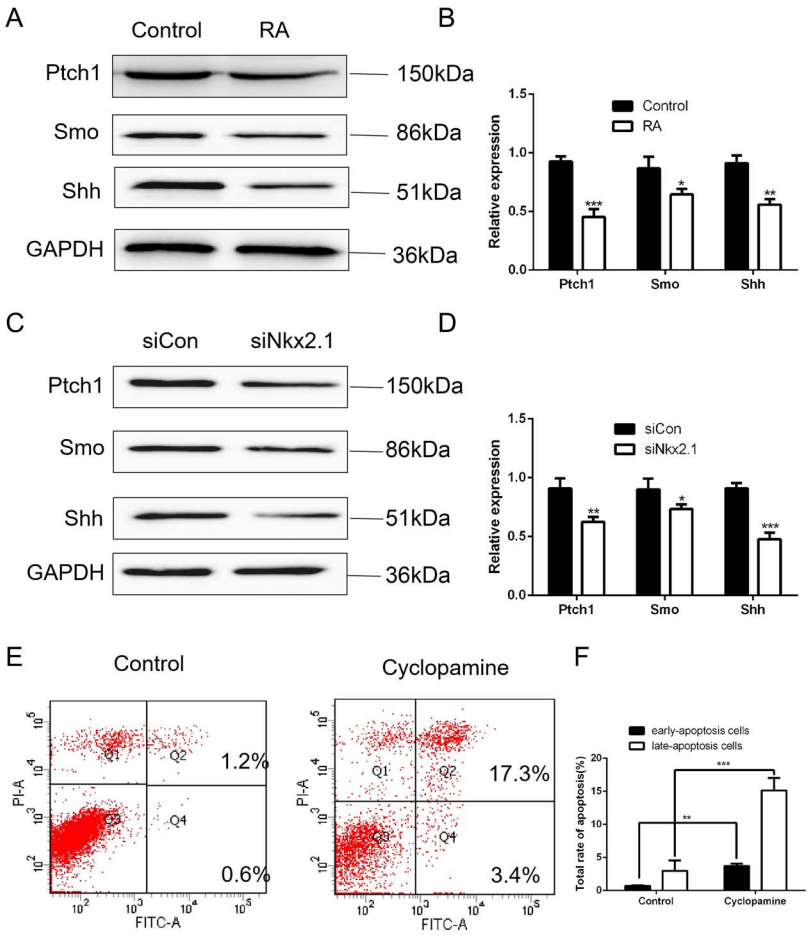

Figure 5. Knockdown of Nkx2.1 affects the Shh signaling pathway (A–D) The Shh, Ptch1, Smo protein levels were evaluated in indicated groups by western blot analysis, and the expression of GAPDH was used as the loading control. (E,F) The cell apoptosis in the control and cyclopamine-treated cells were analyzed by flow cytometry. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs control group or siCon group.

indicated that Nkx2.1 is an important factor for brain development by regulating the balance between cell proliferation and apoptosis, suggesting that Nkx2.1 could be an important pathogenesis factor in RA-induced CNS abnormalities.

In the early stage of CNS development, Shh protein is abundantly expressed in the spinal cord [32]. A functional loss of Shh causes an imbalance between cell proliferation and apoptosis in brain development and cardiovascular diseases [33,34]. In order to reveal the potential role of Nkx2.1 in brain development by regulating Shh signaling pathway, we detected the main components (Shh, Ptch1, and Smo) of this pathway at cellular and embryonic level. The results indicated that Shh signaling pathway was repressed when *Nkx2.1* expression was downregulated. Moreover, cyclopamine is an effective inhibitor to repress Shh signaling pathway by acting on Smo [35]. In this study, 30 μM cyclopamine was used to treat N2a cells for investigating the effect caused by the repression of Shh signaling pathway [36]. We found that cyclopamine increased cell apoptosis when compared with the control cells. We further proved that the effect of *Nkx2.1* gene knockdown and cyclopamine were consistent, confirming that *Nkx2.1* not only activates the shh signaling pathway but also inhibits cell apoptosis. We concluded that the Shh signaling pathway is an important participant in *Nkx2.1*-regulated brain development.

In summary, Nkx2.1 plays an important role in activating the Shh signaling pathway, which ensures the balance between cell proliferation and apoptosis for normal brain development. Our results provide a new understanding of Nkx2.1 function in brain development and will have far reaching implications for diseases and neurobiology. Due to different pathogenesis of brain development, it will be of great interest to determine whether Nkx2.1 also has pro-differentiation and other effects on normal brain development.

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