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Astrocytes induce proliferation of oligodendrocyte progenitor cells via connexin 47-mediated activation of Chi3l1 expression

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Abstract. – OBJECTIVE: Demyelinating neurodegenerative diseases are some of the most important neurological diseases that threaten the health of the elderly. Astrocytes (ASTs) play an important role in the regulation of the growth and development of oligodendrocytes (OLs) and oligodendrocyte progenitor cells (OPCs), which participate in remyelination. This study investigated the mechanism by which ASTs promote the proliferation of OPCs via connexin 47 (Cx47) in OPCs.

MATERIALS AND METHODS: Under direct-contact co-culture conditions, we performed Cx47 siRNA interference in ASTs and OPCs and tested the cell proliferation ability by flow cytometry and with 5-ethynyl-20-deoxyuridine (EdU). We then detected Chi3l1 expression by Western blotting and immunofluorescence. Next, after the addition of exogenous Chi3l1 protein to OPCs under monoculture conditions, we tested the cell proliferation ability by flow cytometry and EdU.

RESULTS: After siRNA interference with Cx47, the expression of Chi3l1 decreased from 1.10±0.91 to 0.30±0.08, and the proportion of new OPCs decreased from 48.7±3.8% to 28.4±6.6%. Moreover, upon addition of exogenous Chi3l1 protein under OPCs mono-culture conditions, the expression of cyclin D1 increased from 0.68±0.09 to 1.16±0.14, leading to an increased number of OPCs in the S phase, from 7.37±1.38% to 13.55±1.60%.

CONCLUSIONS: Cx47/Chi3l1 plays an important role in the promotion of OPCs proliferation by ASTs. ASTs can promote the expression of Chi3l1 via Cx47 in OPCs, and then activate the expression of cyclin D1 and regulate the cell cycle of OPCs, thereby promoting cell proliferation. This study provides a new target for the treatment of neurodegenerative diseases.

Key Words
Astrocytes, Oligodendrocyte progenitor cells, Cx47, Chi3l1.

Introduction

Many neuropsychiatric diseases, such as Alzheimer's disease (AD), multiple sclerosis (MS), and epilepsy, are associated with myelin sheath damage[1-3]. Promotion of the regeneration and repair of myelin is crucial for the treatment of diseases caused by myelin damage. However, oligodendrocytes (OLs), as the only myelin-forming cells in the central nervous system (CNS), do not exhibit cell division, and the proliferation of these cells is dependent on the proliferation and differentiation of oligodendrocyte progenitor cells (OPCs). Astrocytes (ASTs) are the largest, most abundant, and most widely distributed glial cells in the CNS. These cells can transmit biological information via secretory cytokines and intercellular gap junction (GJ) channels and support and nourish the growth and development of other nerve cells[4-7], as well as provide the lipids necessary for myelination. ASTs play an important role in regulating the growth and development of OLs and can promote the proliferation of OPCs[8]. GJs are direct channels for material and information exchange between adjacent cells. GJs are composed of two half-channel connections, and each half channel is composed of 6 connexins joined by non-covalent bonds, allowing the passage of some substances with molecular weights <1 kD[9]. To date, the expression of 21 connexins has been detected in humans, and of these connexins, OLs mainly express connexin 47 (Cx47), Cx32 and Cx29, and ASTs mainly express Cx43, Cx30 and Cx26[10-13]. In the CNS, stable expression of Cx47 in OLs requires the presence of ASTs, and OPCs can be coupled to ASTs via Cx47[14]. Downregulation of the expression of Cx47 leads to myelin

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sheath damage and can cause severe demyelination-associated diseases, such as MS[15]. Chitinase 3-like protein 1 (Chi3l1) is a tumour-associated cytokine that promotes the growth of tumour cells[16,17], but the promotion of OPC proliferation by Chi3l1 has not been reported in the literature.

Materials and Methods

Experimental Materials

The following experimental materials were used in this study: Neonatal (P0-P3) Sprague-Dawley (SD) rats (provided by the Experimental Animal Center of Chongqing Medical University, the conditions of animal housing fully complied with the national standards for the management and protection of laboratory animals, all investigations have been approved by the Ethics Committee), neuroblastoma cell line B104 (kindly provided by the Army Medical University, Chongqing, China), DMEM/F12 medium (SH30023.01B, HyClone, South-Logan City, UT, USA), 0.25% trypsin (40127ES60, Gibco, Grand Island, NY, USA), N-2 supplement (A1370701, Gibco, Grand Island, NY, USA), cyan-streptomycin double-antibody solution (C0222, Beyotime Biotechnology, Shanghai, China), fetal bovine serum (FBS, 10099-141, Gibco, Grand Island, NY, USA), ethylenediaminetetraacetic acid (EDTA, Beyotime Biotechnology, Shanghai, China), Cx47 antibody (BS7447, Bioworld Technology, Shanghai, China), Chi3l1 antibody (sc-393484, Santa Cruz Biotechnology, CA, USA), PDGFαR antibody (sc-338, Santa Cruz, CA, USA), Cyclin D1 antibody (bs-20596R, Bioss Technology, Beijing, China), GAPDH antibody (bs-2188R, Bioss Technology, Beijing, China), FITC-labeled goat anti-rabbit IgG (H+L) (A0562, Beyotime Biotechnology, Shanghai, China), Cy3-labelled goat anti-mouse IgG (H+L) (A0521, Beyotime Biotechnology, Shanghai, China), DAPI dihydrochloride (C1002, Beyotime Biotechnology, Shanghai, China), recombinant Chi3l1 (RPB463Ra01, CloudClone Corp, Wuhan, China), cDNA Synthesis Kit (RR047A, TaKaRa, Dalian, China), EdU Kit (C10310-1, C10310-3, RiboBio, Guangzhou, China), siRNA Kit (RiboBio, Guangzhou, China).

Collection of B104 Supernatant

Frozen B104 cells were thawed in a water bath at 37°C. After centrifugation at 1000 rpm for 5 min, the cells were resuspended in a medium containing 12% serum. Then, the cells suspension was used to inoculate a culture flask, and the medium was replaced every other day. After reaching 70-80%, the cells were washed with PBS, and medium containing 1% N-2 supplement was added. The cells were incubated at 37°C for 4 days, and then, the supernatant of the cells was centrifuged, filtered, and stored at -80°C.

Primary Culture of OPCs

The neck skin of newborn (P0-P3) SD rats was sterilized with 75% alcohol. Next, each rat was decapitated, and the head was placed in pre-chilled PBS. The scalp and skull were cut along the middle of the skull, and the brain tissue was removed. The telencephalon was separated from the rest of the brain. Then, the brain tissue was slashed with a scalpel, and medium containing 12% FBS was added. The cell suspension was plated in a poly-D lysine (PDL)-pre-coated Petri dish, and the medium was replaced every other day. After 4 days of culture, medium containing 1% N-2 supplement and 20% B104 supernatant was added to the cells, and the medium was replaced every other day. The cells were cultured for 7-9 days and then digested using 0.1% EDTA for 10 min with an appropriate amount of pipetting. The cell suspension was plated in a PDL-pre-coated Petri dish and the medium was replaced every other day. After culturing the cells for 3-4 days, the cells were treated as needed for experiments.

Primary Culture of ASTs

Newborn SD rats were treated as described in "primary culture of OPCs". The brain tissue was trypsinized for 1 min and centrifuged at 1000 rpm for 5 min. The cells suspension were plated in a Petri dish, and medium containing 12% FBS was added. After incubating the cells for 20 min at 37°C, the supernatant was plated in a petri dish pretreated with PDL. The medium was replaced every other day. After reaching 80-90% confluency, the cells were trypsinized, centrifuged at 1000 rpm for 5 min, resuspended in 12% serum-containing medium, and plated in a PDL-pre-coated dish, and the medium was replaced every other day. After 3-4 days of cell culture, the cells were treated as needed for experiments.

Co-cultivation of ASTs and OPCs

Mono-cultured OPCs and ASTs were used to inoculate the same culture dish, adding medium containing 20% B104 supernatant and 1% N-2 supplement, and the medium was replaced every other day. After 3-4 days of

culture, the cells were treated as needed for experiments.

Flow Cytometry for Cell Cycle Detection

The cells were digested and collected, washed 3 times with PBS, and centrifuged at 1000 rpm for 5 min, and then, the supernatant was discarded, and the cells were fixed with 75% alcohol at 4°C overnight. The cell cycle was then detected by flow cytometry.

RT-PCR

Total RNA was extracted from the OPCs according to the manufacturer's instructions for the RNAiso Plus total RNA extraction reagent (TaKaRa, Otsu, Shiga, Japan). The extracted RNA was reverse transcribed to cDNA by following the instruction for the M-MLV RTase cDNA Kit. The upstream primer sequence Cx47 was GAGGATGAGGACGAGGAACCA, and the downstream primer sequence was CTCCTACTCCTGCTCCTTGGT; the CFX 96 RT-PCR system was used for detection. The reaction conditions were as follows: pre-denaturation at 95°C for 30 s, 40 cycles of denaturation at 95°C for 5 s and annealing at 60°C for 30 s and extension at 72°C for 10 min.

Immunofluorescence

Cells were fixed in 4% paraformaldehyde for 30 min, washed with PBS, treated with 0.5% Triton X-100 for 10 min, washed with PBS again, blocked with goat serum for 1 h, treated with primary antibody, and incubated at 4°C overnight. After that, the cells were washed with PBS, treated with fluorescent secondary antibody, protected by incubation at 37°C for 1 h, washed with PBS, incubated with DAPI for 5 min, and washed with PBS again. The cells were then sealed with anti-fluorescence quencher, and the fluorescence was detected by confocal laser scanning microscopy. The fluorescence intensity was analyzed by ImageJ. The dilution ratio of Chi3l1 was 1:50; the dilution ratio of PDGFαR was 1:100; the dilution ratio of Cx47 was 1:50; the dilution ratio of FITC-labeled goat anti-rabbit IgG (H+L) was 1:500 and the dilution ratio of cy3-labeled goat anti-mouse IgG (H+L) was 1:500.

EdU-Based Detection of Cell Proliferation

The OPCs were inoculated into a 24-well plate and incubated with 5-ethynyl-20-deoxyuridine (EdU). Fluorescent staining was performed according to the manufacturer's instructions for the EdU Kit, and the fluorescence was detected by a fluorescence microscope.

Western Blot Analysis of Protein Expression

After extracting total cellular protein, the BCA assay detected the protein concentration. Loading buffer (5X) was added to the protein, and the sample was boiled for 10 min. When loading the protein, we ensured that the amount of protein loaded in each group was consistent. The protein was then electrophoretically transferred to a membrane that was blocked with 5% skim milk for 2 hours. After incubating the primary antibody overnight at 4°C, the membrane containing the proteins was incubated with the secondary antibody. Next, the blot was visualized, and ImageJ software was used for image analysis. GAPDH was used as an internal standard to analyze the expression of each protein. The dilution ratios were 1:200 for Chi3l1, 1:500 for Cx47, 1:500 for Cyclin D1 and 1:2000 for GAPDH.

Statistical Analysis

In this study, we used one-way analysis of variance, and a difference with $p<0.05$ was considered statistically significant. All the data were processed using SPSS17 (Chicago, IL, USA).

Experimental Groups

Group A: OPCs were collected under OPCs and ASTs direct-contact co-culture conditions.

Group O: OPCs were collected under OPCs mono-culture conditions.

Group Cx47si: OPCs were collected after siRNA interference with Cx47 under ASTs and OPCs co-culture conditions. The specific siRNA interference sequence for Cx47 is CCGAGAAGACTGTCTTCTT.

Group NCsi: OPCs were collected under blank interference with Cx47 under ASTs and OPCs co-culture conditions.

Group 5 ng/ml: OPCs were collected after addition of 5 ng/ml Chi3l1 under OPCs mono-culture conditions.

Group 10 ng/ml: OPCs were collected after addition of 10 ng/ml Chi3l1 under OPCs mono-culture conditions.

Results

The Expression of Cx47 Increased in OPC when AST-OPC Direct-Contact Co-Culture

Examined the expression levels of Cx47 in OPCs under OPC-mono-culture conditions (group O) and AST-OPC co-culture conditions

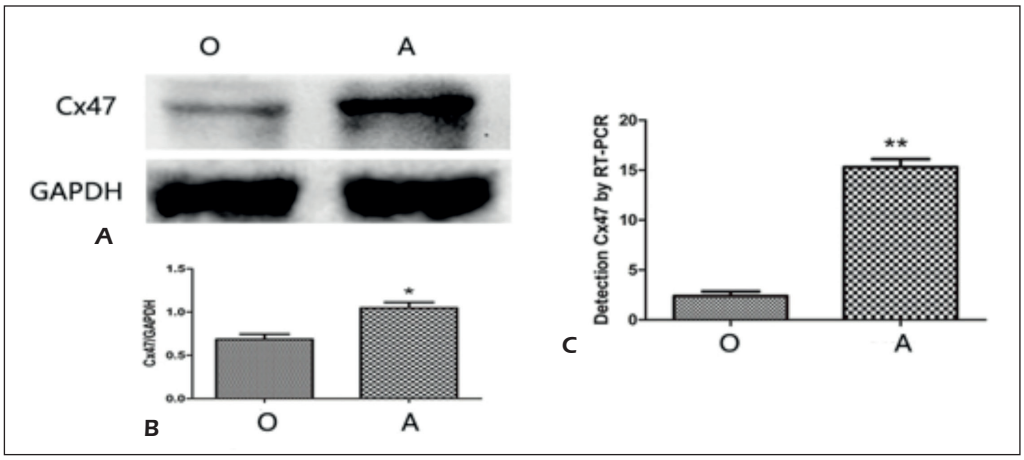

Figure 1. Expression of Cx47 in group O and group A. **A**, Western blot detection of Cx47 protein expression in group O and group A, (**B**) Statistical analysis of the expression of the Cx47 protein in group O and group A detected by Western blot; *p<0.05, group O vs. group A, n=3, (**C**) RT-PCR detection of Cx47 gene expression in group O and group A; **p<0.01, group O *vs.* group A, n=5.

(group A). Western blot analysis showed that the band intensity of Cx47 increased from 0.68±0.08 in group O to 1.05±0.09 in group A (Figure 1A and B), RT-PCR results showed that the expression of Cx47 increased from 2.2±0.58 in group O to 15.3±2.40 in group A (Figure 1C). The experiments showed that AST can promote the expression of Cx47 in OPCs.

Reduced Proliferation of OPCs after siRNA Interference with Cx47

After the addition of Cx47 siRNA under AST and OPC co-culture conditions, Western blot analysis showed that the band intensity of Cx47 decreased from 1.042±0.121 in group A and 1.046±0.163 in group NCsi to 0.456±0.207 in group Cx47si (Figure 2A-B). Immunofluorescence experiments showed that the fluorescence intensity of Cx47 decreased from 1116.20±252.74 in group A and 881.35±263.97 in group NCsi to 254.1.2±53.93 in group Cx47si (Figure 2C). The experiments showed that siRNA interference with Cx47 was successful. Under the above conditions, the results of flow cytometry showed that the proportion of the S phase was 17.35±2.03% in group A and 14.85±1.92% in group NCsi, decreasing to 7.44±1.10% in group Cx47si (Figure 2D,F). The results of EdU showed that the proportion of green fluorescently labeled neonatal cells decreased from 48.7 ± 3.8% in group A and 47.8 ± 5.1% in group NCsi to 28.4 ± 6.6% in group Cx47si (Figure 2E,G). The results suggest that cell proliferation was reduced after specific siRNA interference with Cx47.

Reduction of Chi3l1 Expression in OPCs after siRNA Interference with Cx47

After specific siRNA interference with Cx47 under the ASTs and OPCs co-culture conditions, the expression of Chi3l1 in OPCs was detected by Western blot and immunofluorescence. Western blot analysis showed that the band intensity of Chi3l1 decreased from 1.10±0.91 in group A and 1.03±0.27 in group NCsi to 0.30±0.08 in group Cx47si (Figure 3A-B). The immunofluorescence results showed that the red fluorescence intensity of the specific marker Chi3l1 decreased from 1081.54±327.25 in group A and 1282.33±381.64 in group NCsi to 407.33±106.84 in group Cx47si (Figure 3C). The experimental results indicated that the expression of Chi3l1 in OPCs decreased after siRNA interference with Cx47 under the ASTs and OPCs co-culture conditions.

Under Mono-Culture OPCs Conditions, the Exogenous Chi3l1 Protein Increased the Proliferative Ability of OPCs and the Expression of Cyclin D1

After adding 5 ng/ml and 10 ng/ml Chi3l1 under OPCs mono-culture conditions, the proliferative ability of the OPCs was detected by flow cytometry and with EdU. The flow cytometry results showed that the proportion of cells entering the S phase increased from 7.37±1.38% in group O to 10.63±2.33% in group 5 ng/ml and 13.55±1.60% in group 10 ng/ml (Figure 4A,C). The EdU results showed that the relative proportion of green fluorescently labeled neonatal cells increased from 22.92 ± 2.90% in group O

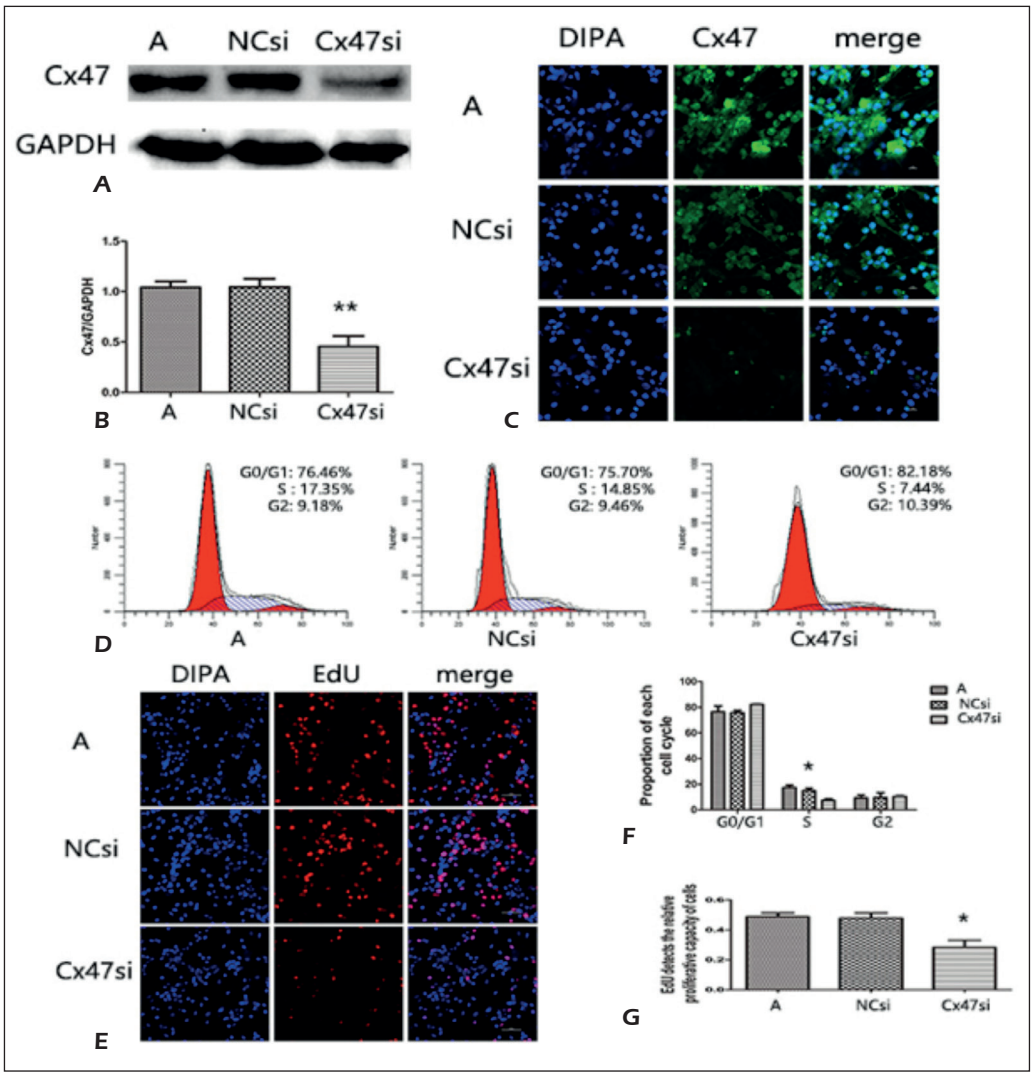

Figure 2. Proliferation ability of OPCs after siRNA interference with Cx47. **A**, Detection of the expression of Cx47 in group A, group NCsi and group Cx47si by Western blot. **B**, Statistical analysis of the expression of Cx47 in the three groups after siRNA interference with Cx47; ***p*<0.01, Cx47si *vs* NCsi, n=3. **C**, Measurement of the immunofluorescence intensity of Cx47 in group A, group NCsi and group Cx47si. Blue fluorescence indicates the nucleus and green fluorescence indicates Cx47, n=5. **D**, Detection of changes in the cell cycle after siRNA interference with Cx47 by flow cytometry. **E**, EdU-based detection of cell proliferation of after siRNA interference with Cx47. Red fluorescence indicates EdU-labeled neonatal cells. **F**, Statistical analysis after siRNA interference with Cx47. Changes in cell cycle, compared with group A and group NCsi. The proportion of cells in the S phase of the Cx47si group decreased; **p*<0.05, Cx47si *vs*. NCsi, n=3. **G**, Statistical analysis of changes in relative cell proliferative capacity after interference with Cx47; **p*<0.05, Cx47si *vs.* NCsi, n=5.

to 31.08 ± 3.44% in group 5 ng/ml and 45.32 ± 1.74% in group 10 ng/ml (Figure 4B,D). Western blot detection of the cyclin D1 protein in group O and group 10 ng/ml showed that the expression of cyclin D1 increased from 0.68±0.09 in group O to 1.16±0.14 in group 10ng/ml (Figure 4E-F). The experiments show that the proliferation of OPCs was enhanced, and the expression of cyclin D1 increased after the addition of exogenous Chi3l1 protein.

Discussion

The incidence of age-related neurodegenerative diseases is increasing annually. A growing number of studies have indicated different degrees of myelin damage and demyelination in neurodegenerative diseases such as AD, MS, and multiple system atrophy (MSA). Myelin damage is also an important cause of disease[18-23]. Myelin is formed by mature OLs surrounding axons, and

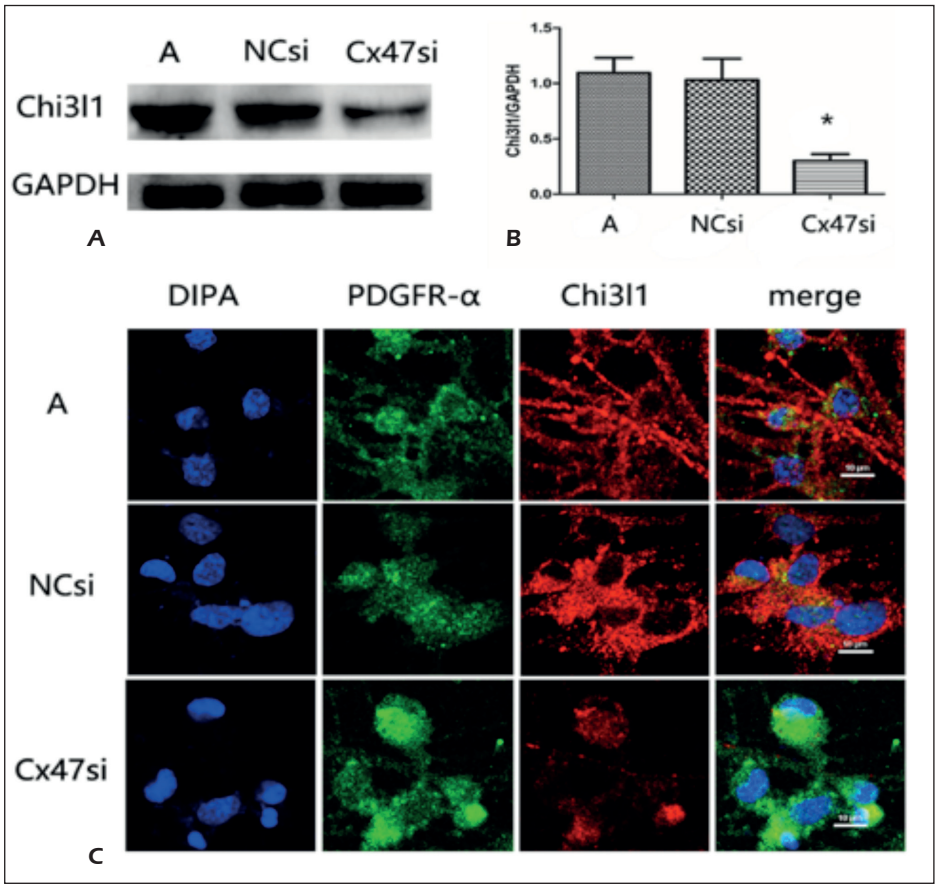

Figure 3. Expression of Chi3l1 after siRNA interference with Cx47. **A**, Western blot analysis of Chi3l1 express in the A, NCsi and Cx47si groups. **B**, Statistical analysis of Chi3l1 expression in the A, NCsi and Cx47si groups; *p<0.05, Cx47si vs. NCsi, n=3. **C**, After siRNA interference with Cx47, the expression of Chi3l1 was observed by immunofluorescence. The blue color indicates DIPA-labeled nuclei; the green indicates was PDGFR-α-labeled OPCs; and the red color indicates specifically labeled Chi3l1 (n=5).

the proliferation of OLs relies on the proliferation, differentiation and maturation of OPCs. Because OLs have the unique ability to form myelin in the CNS, development of methods to promote OPC proliferation is crucial for treating neurodegenerative diseases. ASTs are the most abundant cells in the CNS and play important roles in all stages of proliferation, migration and maturation of OPCs. Additionally, ASTs can secrete some cytokines, such as C-X-C motif chemokine 12 (CXCL-12), basic fibroblast growth factor (FGF-2) and Insulin-like growth factor 1 (IGF-1), to promote OPC proliferation; however, ASTs can transfer biological information to OPCs via GJ channels to promote OPC proliferation[7,24-26]. GJs play an important role in OPCs proliferation via ASTs, but the mechanism has not yet been elucidated. Four types of gap connection channels exist between ASTs and OPCs, namely, the Cx47/Cx43 type, Cx47/30 type, Cx32/Cx43 type and Cx32/C30 type[10,11,15,27,28]; Cx47/Cx43 type-channels are the most prevalent[28]. The existence of ASTs is of great significance for the stable expression of Cx47 by OPCs. In the present work, we examined the expression levels of Cx47 in OPCs under two different culture conditions, "AST-OPC co-culture" and "OPC mono-culture". The expression level of Cx47 was decreased in the OPC mono-culture group. This finding is consistent with previous research. GJs are channels used for the exchange of cellular information and material between adjacent cells and have notable effects on cell proliferation. We interfered with Cx47 expression in co-cultured OPCs and found that the proliferative capacity of OPCs was significantly reduced. The results showed that ASTs can promote the proliferation of OPCs by enhancing the expression of Cx47 in OPCs. Most of the current research has not explored the signaling pathway downstream of Cx47. In the present study, we performed Cx47 siRNA

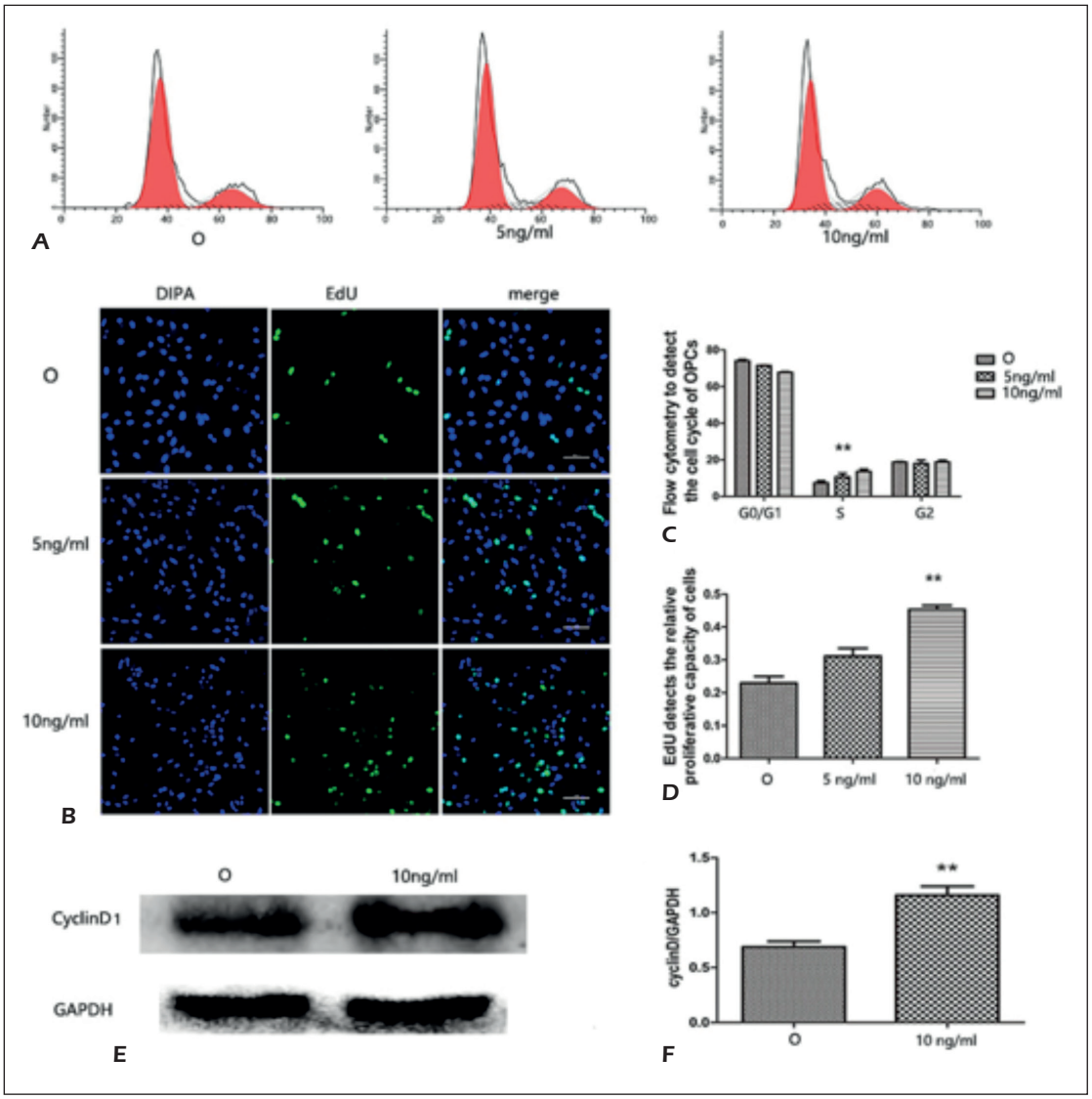

Figure 4. Changes in the proliferative capacity of OPCs after treatment with exogenous Chi3l1 protein. **A**, Detection of the OPCs cell cycle after the addition of 5 ng/ml and 10 ng/ml exogenous Chi3l1 protein by flow cytometry. **B**, EdU-based-detection of the cell proliferative ability after addition of exogenous Chi3l1 protein. Green fluorescence indicates EdU-labeled neonatal cells. **C**, Statistical analysis of changes in the cell cycle after the addition of exogenous Chi3l1 at different concentrations; ** $p<0.01$, group O *vs.* 5 ng/ml vs. 10 ng/ml, n=3. **D**, Statistical analysis of the proportion of new cells after adding different concentrations of exogenous Chi3l1; ** $p<0.01$, group O *vs.* 5 ng/ml vs. 10 ng/ml, n=5. **E**, Western blot analysis of the expression levels of Cyclin D1 after the addition of 10 ng/ml Chi3l1. **F**, Statistical analysis of cyclin D1 expression in group O and group 10 ng/ml; **$p<0.01$, group O *vs.* 10 ng/ml, n=3.

interference on co-cultured OPCs and found that the expression of Chi3l1 was decreased after interference with Cx47. These experimental results showed that Chi3l1 is the downstream signal of Cx47 that transmits information to OPCs. Chi3l1, also known as YKL-40 and GP-39, is a member of the glycoside hydrolase 18 family. Due to its lack of enzymatic chitin hydrolysis activity, this protein is not classified as a chitinase[29]. Chi3l1 can be secreted by a variety of cells, such as fibroblasts, macrophages, endothelial cells, vascular smooth muscle cells, and chondrocytes[30-33]. In many inflammation-related diseases, such as bacterial infections, rheumatoid arthritis, chronic nephritis and asthma, the expression of Chi3l1 is elevated. Chi3l1 is also highly expressed in various tumour cells, such as lung cancer, glioblastoma, colon cancer, stomach cancer and breast cancer cells. As

a factor that promotes tumor growth, Chi3l1 plays an insulin-like growth factor (IGFI)-like role in tumor cell proliferation, promoting tumor growth, invasion and metastasis[34,35]. In some malignant tumors, Chi3l1 is considered to be a tumor marker, and higher expression of Chi3l1 indicates a greater degree of malignancy[34-36]. This result shows that Chi3l1 can promote cell proliferation. Chi3l1 is also expressed in the CNS, but the promotion of OPC proliferation by Chi3l1 has not been confirmed. In this study, we added exogenous Chi3l1 protein to mono-cultured OPCs and found that the cell proliferation ability and cyclin D1 expression were increased. The results indicate that Chi3l1 can promote OPC proliferation in the CNS.

Conclusions

We demonstrated that Cx47 plays an important role in promotion of the proliferation of OPCs by ASTs. ASTs can promote the expression of Cx47 in OPCs, in turn promoting the expression of Chi3l1 in OPCs, and can then activate the expression of cyclin D1 to regulate the cell cycle of OPCs, thereby promoting cell proliferation. This study provides a new target for the treatment of neurodegenerative diseases.

Author Contributions

This work was financially supported by the Yuzhong District Science and Technology Plan Project in Chongqing China (No. 20160111) and the Natural Science Foundation of China (NSFC 81671259). Lu Jiang is mainly responsible for the experimental design and experimental research in this study. Wenjin Zhang assisted in the development of cell culture and cell experiments. Dan Xu guided the experimental design and data analysis. Yan Peng and Yong Tang were the project leaders and guided the preparation of the manuscript. All the authors have read and approved the final text.

Conflict of Interests

The authors declare no conflict of interest

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