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Expression of rat oligodendrocyte transcription factor 2 in COS-7 cells following its eukaryotic expression vector construction

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Abstract. – OBJECTIVES: The basic HLH transcription factor Olig is a key regulator for differentiating the oligodendrocyte lineage cells during development. Oligodendrocyte transcription factor 2 (Olig2) plays a crucial role in differentiating the oligodendrocytes in the spinal cord. We aimed to construct and investigate the eukaryotic expression recombinant plasmid in the rat Olig2.

DESIGN, TIME AND SETTING: The experiment was performed at the Laboratory of Neurobiology, Xuzhou Medical College from October 2011 to March 2012.

MATERIALS AND METHODS: The pEGFP-N1 vector was purchased from Invitrogen. JM101 competent cells and COS-7 cells were preserved at the Laboratory of Neurobiology, Xuzhou Medical College, China. The Olig2 cDNA fragment was cloned by RT-PCR with the total RNA from the neonatal rat spinal cord, and subsequently cloned into pGEM-T vector. The confirmed Olig2 fragment was then cloned into the pEGFP-N1 vector. The right recombinant was transfected into COS-7 cells by lipofectamine 2000. The expression of the Olig2 in COS-7 cells was detected by RT-PCR and immunoblot analysis. Enzyme digestion and sequencing of the recombinant plasmid; and expression of the Olig2 were analyzed by fluorescence microscope and western blot.

RESULTS: The correct pEGFP-N1-Olig2 cloning was verified by restriction endonuclease digestion and sequencing. The western blot analysis indicated that the Olig2-GFP fusion protein was expressed in the COS-7/pEGFP-N1-Olig2 cells at 72 h.

CONCLUSIONS: The pEGFP-N1-Olig2 vector was constructed successfully. The Olig2-GFP fusion protein was expressed in the COS-7/pEGFP-N1-Olig2 cells. This study lays the foundation for further research in gene therapy for central nervous system demyelinating diseases.

Keywords:
Oligodendrocyte transcription factor 2, Eukaryotic expression vector, Demyelinating diseases.

Introduction

The oligodendrocyte transcription factor 2 (Olig2) is a basic helix-loop-helix (BHLH) that plays an important role in regulating the development of the nerve cells. The Olig 2 is widely expressed in the central nervous system (CNS), and is related to the oligodendrocyte. It is a key transcription factor used to differentiate the neural stem cells (NSCs) from the oligodendrocytes (OLs) or motor neuron (MN)[1-4]. In traumatic spinal cord injury, multiple sclerosis or other demyelinating diseases, the Olig2 plays a crucial role in myelin repair mediated by the oligodendrocytes[5-9]. In this study, we clone the full-length sequence of the rat Olig2 gene coding and construct its eukaryotic expression vector, then transfer it into the COS-7 cells (kidney fibroblast cells of African green monkey) for expression. This lays the foundation for further application in gene therapy for CNS demyelinating diseases.

Materials and Methods

Design

The gene cloning was constructed, and a single sample was observed.

Date and place

The research was done in the Neurobiology Laboratory of Xuzhou Medical College from October 2011 to March 2012.

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Materials

Cells, plasmids, strains and major reagents	Sources of materials
pEGFP-N1	Invitrogen Inc., Carlsbad, CA, USA
Lipofectamine 2000	Invitrogen Inc., USA
ReverTra Ac	TOYOBO Inc., Osaka, Japan
pGEM-T Vector kit	Promega Inc., Madison, WI, USA
NBT/BCIP kit	Promega Inc., USA
DNA purification kit	Promega Inc., USA
Plasmid extraction kits	Promega Inc., USA
Gel extraction kit	Nanjing Tiangen Biology Inc., Nanjing, China
Restriction endonucleases Hind II I Kpan I	NEB Inc., Hitchin, UK
T4 DNA Ligase	NEB Inc., UK
DMEM cultured, newborn calf serum	GIBCO Inc., Carlsbad, CA, USA
E. coli JM101, COS-7 cells	Neurobiology Laboratory of Xuzhou Medical College, China
Primary antibody	Invitrogen Inc., USA
Rabbit anti-mouse Olig2 polyclonal antibody	

Methods

Primer design

The Olig2 cDNA sequences (NM 001100557) of the Sprague Dawley (SD) rats logged on the Genbank, HindIII and Kpan I restriction sites, were designed in the forward and reverse primer. The termination codon which followed Kpan I was removed and the two bases (AA) were added. The primer sequences were as follows:

- forward primer:
  5'-GGAAGCTTATGGACTCGGACGCC-3'
- reverse primer:
  5'-GCGGTACCGTCTTGGCGTCGGAGG-3'

Total RNA extraction and Olig2 cDNA amplification by RT-PCR

Spinal cord tissues of neonatal SD rats were homogenized in a denaturing solution. Total RNA was extracted from the tissue using the TRIzol method. Using the extracted total RNA as a template, ReverTra Ace and random primers were used for reverse transcription (RT-PCR). The reaction parameters were as follows: 94°C pre-denaturation for 2 min; 94°C pre-denaturation for 30 s, 55°C annealing for 40 s, 72°C refolding for 50 s, and were amplified for 30 cycles. We used 1% agarose gel electrophoresis to analyze the fragment size of the PCR products.

Construction and identification of the pGEM-T-Olig2 vector

Target fragments were recovered and purified using 1% low melting agarose gel electrophoresis and DNA purification kit. We combined the purified gene fragments and pGEM-T vector as per kit instructions, and transformed them into JM101 competent cells (prepared by calcium chloride method). White plump single colony was randomly picked and inoculated into LB liquid medium (Amp +), 37°C and shaken for 12 h. After plasmid extraction, the products were determined by restriction enzyme digestion using Hind III and KpanI. The positive plasmids of the double digested results were taken and sent to the Shanghai Invitrogen Company for forward and reverse sequencing. The corrected plasmid was determined by sequencing results and named pGEM-T-Olig2.

Construction and identification of the pEGFP-N1-Olig2 vector

The pGEM-T-Olig2 recombinants were digested using Hind III and Kpan I. Digested target genes were recovered and purified using a 1% low melting agarose gel electrophoresis and DNA purification kit. The purified target genes were connected with the pEGFP-N1 vector dual-

digested by the same restriction endonucleases, and transformed into JM101 competent cells. Plasmids were extracted and identified by Hind III and Kpan I double digestion and sequencing. The correct plasmids identified by sequencing were extracted, purified and named pEGFP-N1-Olig2.

COS-7 cells culture and plasmid transfection

COS-7 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) medium with 10% newborn calf serum. When the cells reached a density of 90% to 95%, the recombined pEGFP-N1-Olig2 plasmids and pEGFP-N1 plasmids were transfected into COS-7 cells using Lipofectamine TM 2000. The non-transfected COS-7 cells were observed under fluorescence microscopy as the control group. After 72 h-culture, cells in the three groups were collected and amplified using RT-PCR according to the conditions above and the Olig2 and β-actin (550bp) primers were added for PCR. After 72 h-culture, cells in the three groups were collected and their protein concentrations measured by Lowry method.

Western Blot

In order to separate the total protein, samples were placed in 10% SDS-PAGE for gel electrophoresis and electroblotted in nitrocellulose membrane (NC membrane). The NC membrane were sealed in 5% nonfat dry milk for 3 h, and a rabbit anti-mouse Olig2 polyclonal antibody 1:2500 was added and, shocked in a shaker for 1h at room temperature, then, incubated overnight at 4°C. We, then, added a goat anti-rabbit antibody labeled with alkaline phosphatase, shocked it in the shaker for 2h at room temperature, stained it with NBT/BCIP, and scanned it using gel electrophoresis imaging system.

Outcome

The PCR amplified productions were identified using electrophoresis. We identified the recombinant plasmids using restriction enzyme digestion. The results of the recombinant gene sequencing and the expressions of the Olig2 in COS-7 cells were observed under fluorescent microscope and identified by western blot.

Designer, operator and evaluator

The trial design and procedure were conducted by the first and corresponding author. All investigators had received training. Blind evaluation was not used in the experiment.

Results

Amplification of Olig2 cDNA

Using the total RNA of the rat hippocampal tissue as a template, we amplified the Olig2 cDNA. The electrophoresis showed that the amplified cDNA matched the target gene fragments (990bp) (Figure 1).

Construction and identification of pGEM-T-Olig2 vector

We combined the Olig2 cDNA fragment with the pGEM-T vector. The electrophoresis results showed that there were two DNA fragments displayed as selected positive recombinants double digested with Hind III/Kpan I. The fragments matched the vector pGMT-T (3.0kb) and Olig2 cDNA (990bp) (Figure 2). The sequencing results showed no mutation, which indicated that the Olig2 gene was cloned into pGEM-T vector.

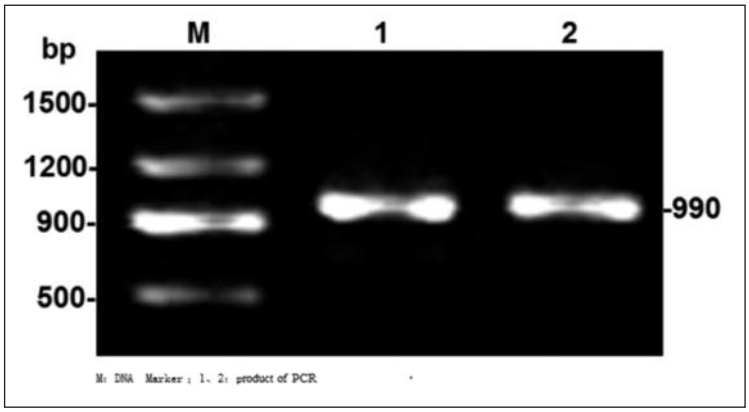

Figure 1. Electrophoresis analysis of Olig2 cDNA amplified by PCR.

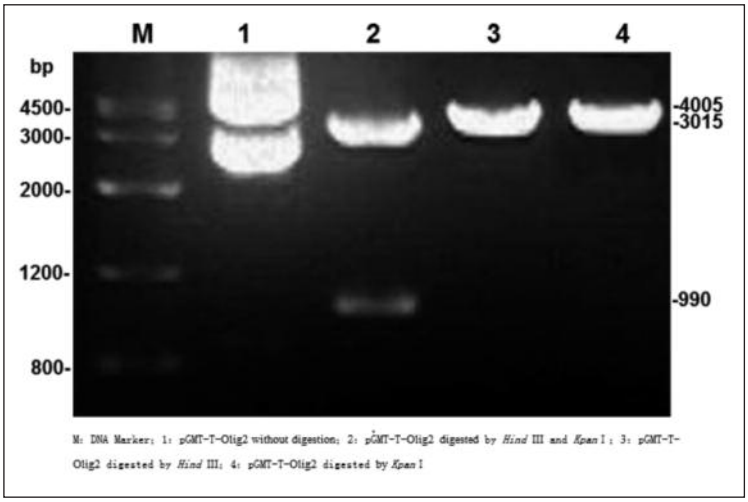

Figure 2. Electrophoresis analysis of enzymatic result of pGMT-T-Olig2.

Construction and identification of the pGEM-T-Olig2 vector

The pGMT-T-Olig2 identified by sequencing were digested by the double enzymes, and the target fragments were recovered and combined with the vector pEGFP-N1. The electrophoresis results showed that there were two DNA fragments displayed as selected positive recombinants double digested using HindIII/Kpan I. The molecular mass of the fragments matched the pEGFP-N1 vector (4.7 kb) and Olig2 cDNA (990bp) (Figure 3). The sequencing results revealed that the Olig2 gene was successfully cloned into the pEGFP-N1 vector.

pEGFP-N1-Olig2 expressed in COS-7 cells

The pEGFP-N1-Olig2 were transfected into COS-7 cells using liposome-mediated method and labelled as the experimental group (COS-7/pEGFP-N1-Olig2). The pEGFP-N1 were transfected into COS-7 cells, and labelled as the negative control group (COS-7/pEGFP-N1). The COS-7 cells without transfection were labelled as the blank control group. Under a fluorescence microscope, the pEGFP-N1-Olig2 began to show green fluorescence after 48 h. The GFP expression increased with time and peaked at 96 h (Figure 4). The RT-PCR results showed that the COS-7/pEGFP-N1-Olig2 could amplify the specific gene fragments of around 990bp, and the control group did not spread out of this fragment (Figure 5). The three groups cells collected at 72 h were analysed by western blot using anti-Olig2 antibody (Figure 6), and the COS-7/pEGFP-N1-Olig2 was displayed by an electrophoretic band at about 60kD. However, there was no band displayed in COS-7 or COS-7/pEGFP-N1.

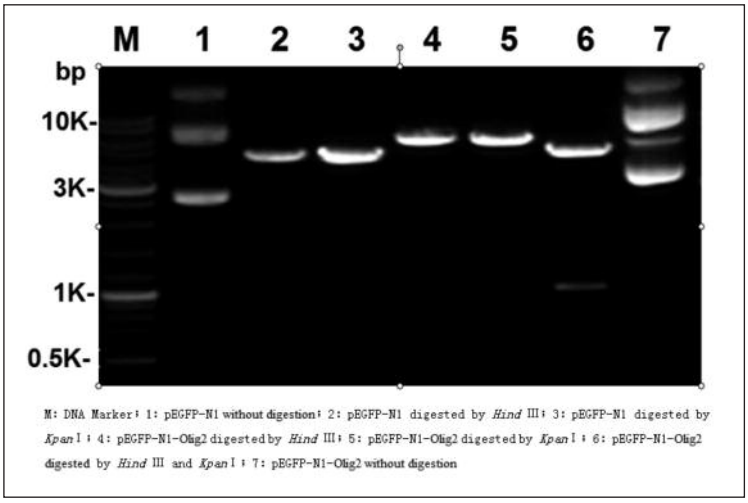

Figure 3. Electrophoresis analysis of enzymatic result of pEGFP-N1-Olig2.

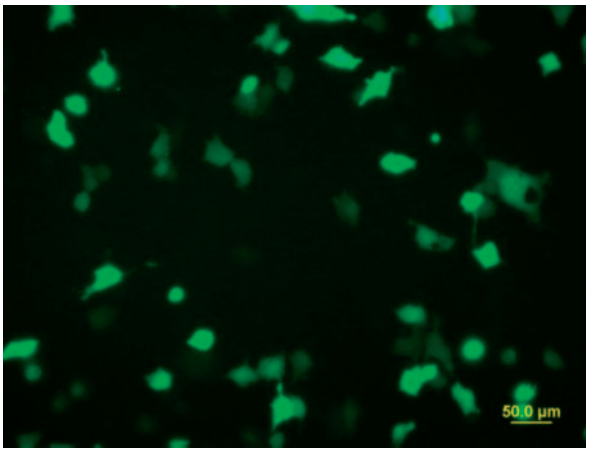

Figure 4. COS-7 cells transfected with pEGFP-N1-Olig2 were observed by fluorescence microscope at 96h×200.

Discussion

The oligodendrocyte transcription factor family includes Olig1, Olig2 and Olig3 that are members of the bHLH family, and were discovered by Takebayashi et al in 2000[10]. Olig1 and Olig2 are two of the earliest oligodendrocyte transcription factors and named for their decisive roles in maturation of OLs. During the embryonic phase, the Olig1 and Olig2 mainly exist in the neuroepithelium cells of the neural tube in the ventral side, which is in the motor neuron progenitor (pMN) region.

In recent years, studies have shown that Olig1 and Olig2 play a key role in oligodendrocytes and myelin formation during the brain and spinal cord development, but have different divisions[11-14]. Single or simultaneously knockout of the Olig1 and Olig2 genes will affect the development of OLs. A single knockout of the Olig2 will severely affect the growth of motor neurons (MN) in the ventral spinal cord. The MN which originates in the pMN region are replaced by the V2 intermediate neuron[1]. Further studies have shown that the Olig2 expression during development of the activated Ngn1 and Ngn2 genes, causes MN differentiation in pMN region of the spinal cord. The Olig2 and Nkx2.2 are activated together, causing the MN in the pMN region of the spinal cord to grow the oligodendrocyte precursor cells (OPCs)[2,15]. Olig1 promotes the survival and maturation of OPCs during the development of the spinal cord.

In the treatment of traumatic spinal cord injury, multiple sclerosis and other central demyelinating diseases, studies focus on remyelination of the endogenous and exogenous OLs, or OPCs. Recent studies have found that the Olig2 has a positive effect on repairing the central demyelinating after injury. Studies by Copray et al[16] have shown that NSCs could differentiate into OLs by inducing over expression of the Olig2 *in vitro*. Fancy et al[9] detected that the OPCs expression of the Olig2 and Nkx2.2 increased in the damaged areas of the cuprizone induced rats demyelination model. They further differentiated into OLs to facilitate remyelination[17].

Artificial interventional expression of the Olig families could bring some favorable factors for the demyelination. Kim et al[5] transferred the virus carrying Olig2 gene into the lesion site of the spinal cord injury, and discovered that the mature oligodendrocytes had increased in the lesion site and that Sox10 was unregulated and the rats' movement function had improved. Hwang et al[7] studies showed that the Olig2 gene overexpression in human neural stem cells promoted differentiation from human immortal neural stem cells into oligodendrocyte. The rat's movement

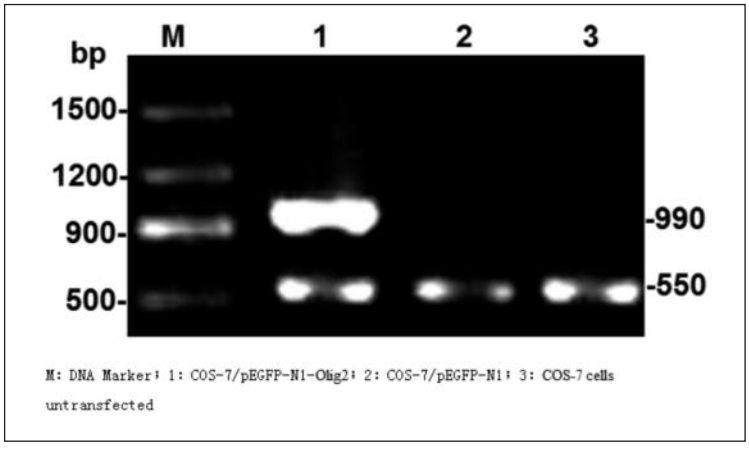

Figure 5. The expression of Olig2 mRNA in cultured COS-7 cells detected by RT-PCR.

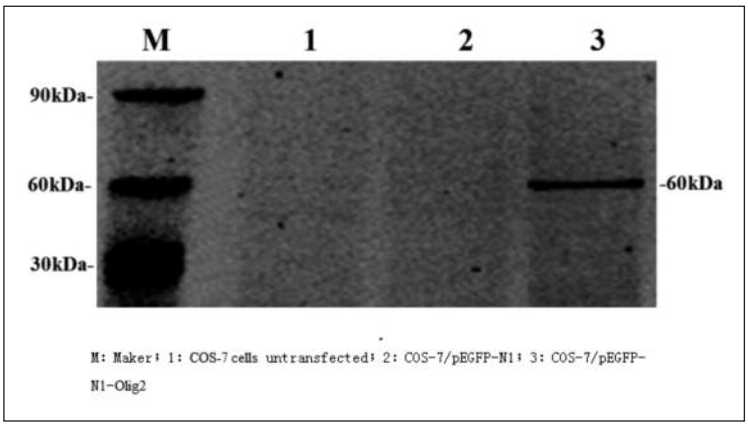

Figure 6. The expression of Olig2 in the cultured COS-7 cells transfected after 72 h by western blotting.

function recovery was enhanced by transplanting the Olig2 genes into the rat's spinal cord. It proved that the Olig2 was a favorable factor for repairing demyelinating injury. Termination codon TAG of target gene Olig2 before EGFP was removed from the pEGFP-N1-Olig2 vector, so that the Olig2 would continue translating. The termination codon was removed and the two bases (AA) were added, to ensure that the sequence did not shift and the EGFP protein was correctly translated. With green fluorescence, we confirmed the expression of the Olig2 target protein. In this study, full-length sequence of the Olig2 gene coding region was cloned from the spinal cord tissue of SD neonatal rats, which ensured that the plasmid was transfected and expressed correctly. The molecular weight of the Olig2 protein was 32kD, and the GFP protein was 27 kD; thus, the Olig2-GFP fusion protein (including fusion peptide chain) was around 60 kD.

Conclusions

This study constructed the pEGFP-N1-Olig2 eukaryotic expression vector, which was transfected into eukaryotic COS-7 cells using liposome-mediated method. The results of RT-PCR and the green fluorescence confirmed that the Olig2 expressed in COS-7 cells. The western blot results confirmed the expression of the fusion protein with the activity of Olig2 antigen. This study lays the foundation for further gene therapy research for central demyelinating diseases.

Conflict of interest

The Authors declare that they have no conflict of interests.

References

1) Zhou Q, Anderson DJ. The bHLH transcription factors OLIG2 and OLIG1 couple neuronal and glial subtype specification. Cell 2002; 109: 61-73.
2) Takebayashi H, Nabeshima Y, Yoshida S, Chisaka O, Ikenaka K, Nabeshima Y. The basic helix-loop-helix factor olig2 is essential for the development of motoneuron and oligodendrocyte lineages. Curr Biol 2002; 12: 1157-1163.
3) Cheng X, Wang Y, He Q, Qiu M, Whittemore SR, Cao Q. Bone morphogenetic protein signaling and olig1/2 interact to regulate the differentiation and maturation of adult oligodendrocyte precursor cells. Stem Cells 2007; 25: 3204-3214.
4) Ligon KL, Fancy SP, Franklin RJ, Rowitch DH. Olig gene function in CNS development and disease. Glia 2006; 54: 1-10.
5) Kim HM, Hwang DH, Choi JY, Park CH, Suh-Kim H, Kim SU, Kim BG. Differential and cooperative actions of Olig1 and Olig2 transcription factors on immature proliferating cells after contusive spinal cord injury. Glia 2011; 59: 1094-1106.
6) Labombarda F, Gonzalez SL, Lima A, Roig P, Guennoun R, Schumacher M, de Nicola AF. Effects of progesterone on oligodendrocyte progenitors, oligodendrocyte transcription factors, and myelin proteins following spinal cord injury. Glia 2009; 57: 884-897.
7) Hwang DH, Kim BG, Kim EJ, Lee SI, Joo IS, Suh-Kim H, Sohn S, Kim SU. Transplantation of human neural stem cells transduced with Olig2 transcription factor improves locomotor recovery and enhances myelination in the white matter of rat spinal cord following contusive injury. BMC Neurosci 2009; 10: 117.
8) Hu JG, Shen L, Wang R, Wang QY, Zhang C, Xi J, Ma SF, Zhou JS, Lü HZ. Effects of olig2-overexpressing neural stem cells and myelin basic protein-activated T cells on recovery from spinal cord injury. Neurotherapeutics 2012; 9: 422-445.
9) Fancy SP, Zhao C, Franklin RJ. Increased expression of Nkx2.2 and Olig2 identifies reactive oligodendrocyte progenitor cells responding to demyelination in the adult CNS. Mol Cell Neurosci 2004; 27: 247-254.

10. Takebayashi H, Yoshida S, Sugimori M, Kosako H, Kominami R, Nakafuku M, Nabeshima Y. Dynamic expression of basic helix-loop-helix Olig family members: implication of Olig2 in neuron and oligodendrocyte differentiation and identification of a new member, Olig3. Mech Dev 2000; 99: 143-148.
11. Grinspan J. Cells and signaling in oligodendrocyte development. J Neuropathol Exp Neurol 2002; 61: 297-306.
12. Miller RH. Regulation of oligodendrocyte development in the vertebrate CNS. Prog Neurobiol 2002; 67: 451-467.
13. Zhou Q, Wang S, Anderson DJ. Identification of a novel family of oligodendrocyte lineage-specific basic helix-loop-helix transcription factors. Neuron 2000; 25: 331-343.
14. Zhou Q, Choi G, Anderson DJ. The bHLH transcription factor Olig2 promotes oligodendrocyte differentiation in collaboration with Nkx2.2. Neuron 2001; 31: 791-807.
15. Richardson WD, Smith HK, Sun T, Pringle NP, Hall A, Woodruff R. Oligodendrocyte lineage and the motor neuron connection. Glia 2000; 29: 136-142.
16. Copray S, Balasubramaniyan V, Levenga J, de Bruijn J, Liem R, Boddeke E. Olig2 overexpression induces the in vitro differentiation of neural stem cells into mature oligodendrocytes. Stem Cells 2006; 24: 1001-1010.
17. Islam MS, Tatsumi K, Okuda H, Shiosaka S, Wanaka A. Olig2-expressing progenitor cells preferentially differentiate into oligodendrocytes in cuprizone-induced demyelinated lesions. Neurochem Int 2009; 54: 192-198.