Insight of a novel functional gene related to differentiation of neural stem cells

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Abstract A novel functional gene P12 was isolated from neural stem cells cultured in differentiation medium. The fully length cDNA of P12 gene was cloned and sequenced. Result showed that it contains an open reading frame encoding a protein of 291 amino acids. Further, this gene was transferred into neural stem cell. Functional analysis suggests that the expression of p12 protein is closely correlated with differentiation of neural dendrite configuration. In addition, to obtain encoding protein, P12 sequence was also expressed in Pichia pastoris yeast.

Key words neural stem cells, differentiation, protein expression, Pichia pastoris.

1 Introduction

Neural stem cells (NSCs) are a subtype of progenitor cells in the nervous system that can differentiate into neurons and glia\(^1\rightarrow3\). Due to their feature of self-renewal, NSCs have expectations for treatment of nervous system diseases such as Parkinson’s disease, Huntington’s disease, spinal cord injury and so on. They are under active consideration as a source of donor tissues for neuronal cell therapy\(^4\rightarrow5\). However, the mechanisms of how NSC differentiate into different kinds of cells remain unknown. From the perspective of gene expression, it is still not very clear how many genes are involved in their differentiation. It is likely that the differentiation is regulated by intrinsic signals as well as the external environment\(^6\).

Our previous research demonstrated SHD11 gene was highly expressed during the differentiation of neural stem cells. Analysis of protein sequence indicated that it has a 98 % homology with dendritic cell factor\(^7\). In view of these results, we hope to clone this gene. To our surprise, we found that a novel fragment, named P12, was always expressed simultaneously with SHD11, so we cloned and insight this functional gene.

2 Materials and methods

2.1 Preparation of total RNA and synthesis of cDNA

Neural stem cell line C17.2 was cultured in feeding medium (83 % DMEM, 10 % FBS, 5 % HS, 1 % L-Glu, 1 % P/S/F) and pelleted by centrifugation at low speed. Cells were washed in 1 x PBS balanced salt solution twice and digested for 3 min with 0.05 % trypsin-EDTA-D-Hanks, 0.5 g/L trypsin, 0.2 g/L EDTA-Na, D-Hanks Balanced salt solution without CaCl\(_2\), MgCl\(_2\). 6H\(_2\)O.

2.2 Detection of P12 gene specific expression in tissues

Three tissues (liver, spleen and cerebral cortex) were excised from a 1 month old mouse, and total RNA was isolated with total RNA isolation kit (Sangon). RT-PCR was performed with the primers specific to the P12 transcript: primer Pxy1(5’-CATATG GATCC CATG GCGG CGCC AAGG GGAA-3’) and Pxy2 (5’-ACTAGGATCCGA TTTCTGAGTGAGCAATGC-3’). The products were semi-quantified by β-actin.

2.3 Cloning of P12cDNA

To amplify P12 gene, 50 – 100 ng cDNA was used as template in a total volume of 100 μL containing 10 mM Tris/HCl, 50 mM KCl, 2.5 mM MgCl\(_2\), 200 μM dNTP, 1 μM of each primer and 2.5 U Taq DNA polymerase\(^8\). PCR was carried out by using primers Pxy5 (5’-
C TATG GTACCATGGCGGCGCCAAAGGGGAA-3’) and Pxy6 (5’-ACTAGGTACCGATGC-3’), at which Kpnl site was introduced. The reaction mixtures were denatured for 3 min at 94 °C followed by 30 cycles: denaturation for 1 min at 94 °C, annealing of primers for 1 min at 50 °C, and extension for 1 min at 72 °C. The final elongation step was extended to 10 min at 72 °C.

The PCR products were purified with the NUIQ-10 column PCR product purification kit (Sangon) and cloned into the plasmid vector PMD8-T vector purchased from Takara. Sequencing was carried out in Company (Sangon).

2.4 Expression of P12 gene in C17.2 cell

The recombinant plasmid pEGFP-C1 containing P12 was transfected into cell line C17.2 by Sofast™ Transfection Reagent™. To obtain optimal transfection efficiency with Sofast™, the cell density should be 60% – 80% confluent. The cells were seeded in 24-well plates for 4 h before gene transfection. 0.6 µg plasmid DNA was diluted in 30 µL serum-free and antibiotic-free DMEM containing 1 – 2 µL Sofast™, mixed gently, and incubated for 15 – 20 min at room temperature. 60 µL Sofast™/DNA complexes was added into each well while gently swirling the plate and incubated cell at 37 °C in a CO2 incubator for 24 – 48 h. Gene cloned into the MCS will be expressed as fusion protein with the C-terminus of EGFP.

2.5 Expression of P12 gene in yeast Pichia pastoris

The yeast Pichia pastoris was chosen as the heterologous host to express P12 gene. A yeast colony was picked up and grow at 30 °C at 200 r/min in a shaking incubator until culture reach an OD600 = 1.0. Cells were centrifuged for 5 min at 4 000 r/min at 4 °C. The cell pellet was resuspended with 40 mL of ice-cold sterile water by swirling the tube and centrifuged again.

Of the 80 µL cells were mixed with 10 µg of pPICZαA-P12 that linearized by SacI and transferred to an ice-cold (0 °C) 0.2 cm electroporation cuvette and incubated on ice for 5 min. A pulse length of 5 ms (7 500 V/cm) was used to electroporate. 1 mL of ice-cold 1 M sorbitol was immediately added to the cuvette and transferred the cuvette contents to a sterile tube. The tube was incubated for 1.5 h at 30 °C and YPDS plates containing 100 µg/mL Zeocin were spreaded with 10, 25, 50, 100 and 200 µL of cell mixture respectively, and incubated for 2 to 3 d at 30 °C until colonies formed.

Positive clones were selected and used to identify the protein expression. A clone was inoculated in 25 mL of BMGY and grow at 30 °C in a shaking incubator to OD600 5.0. Cells were harvested by centrifuging for 5 min at 15 – 3 000 xg at room temperature and cultured in BMMY with the beginning OD600 = 1.0. In order to induce expression, methanol was added to adjust a final concentration of 0.5% (V/V). The supernatants were collected every 24 h and the protein expressions were analyzed by Coomassie-stained SDS-PAGE.

3 Results and discussions

3.1 Cloning and sequencing of P12 gene

The PCR product of P12 gene was cloned to plasmid PMD18-T and sequenced in company (Sangon, Shanghai). The sequence of P12 is shown in Fig. 1. In this sequence, an ORF can be found between position 1 and 876 which encodes a protein of 291 amino acids. Analysis of genomic DNA by Mouse database indicated that it located in the 14th chromosome.

3.2 Expression of P12 gene in C17.2

Plasmid pEGFP-C1 was chosen to construct expression vector pEGFP-C1-P12, which contained an 876 bp fragment of P12 showed in Fig. 2 (b). And Fig. 2 (a) showed the model of the construction. The pEGFP-C1-P12 was transferred with the Sofast™ Transfection Reagent into C17.2. The green fluorescence could be detected by using fluorescence microscope when the pEGFP-C1-P12 was translated with pEGFP-C1-P12, the shape turned round and without neuritis (see Fig. 3 (a)), and it was different from the control NSCs (see Fig. 3 (b)) which have already differentiated with neuritis configuration. P-value analysis of neuritis indicates a significant difference (P < 0.001) (see Fig. 3 (c)).

3.3 Tissues specific expression of P12

In order to better understand the tissues expression pattern, P12 gene expression in liver, cerebral cortex and spleen tissues from 1 month old mouse were evaluated by semi-quantified RT-PCR analysis (see Fig. 4). RT-PCR result showed a down regulation of P12 in...