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Effect of mouse *Sim2* gene on the cell cycle of PC12 cells

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Abstract

Sim2 gene plays an important role in the pathogenesis of Down syndrome (DS). To observe the effect of mouse Sim2 (mSim2) on the cell cycle of PC12 cells in vitro and explore the role of Sim2 in the pathogenesis of DS, we cloned the full open reading frame of *mSim2* into the pcDNA3 vector and transfected it into PC12 cells, before analysing the effect of mSim2 on the cell cycle. A eukaryotic expression vector of mSim2 (pcDNA3-mSim2) was successfully constructed. There was notable expression of *mSim2* mRNA in the cells transfected with pcDNA3-Sim2. Flow cytometry showed that there were more cells in G_0/G_1 phase in the Sim2-transfected cells than that in the controls ($P < 0.01$), and significantly fewer in G_2/M phase ($P < 0.01$). The mRNA and protein expressions of cyclin E decreased in the Sim2-transfected cells, while p27 expression increased significantly ($P < 0.01$). It is concluded that Sim2 may play an important role in the pathogenesis of DS by inhibiting the cell cycle, which is related to the decreased expression of cyclin E and increased expression of p27.

Keywords: *Sim2*; Down syndrome; Cell cycle; Cyclin E; p27

1. Introduction

Down syndrome (DS) is the most common autosomal aneuploidy disease occurring in 1.03–1.30 of 1000 live births (Epstein, 1995). Although the genetic basis of DS has not been fully elucidated, many genes have been found in a region associated with DS on human chromosome 21. A minimum region of HC21, named Down syndrome chromosomal region (DSCR), is associated with many phenotypic characteristics of DS (Delabar et al., 1993). As a step towards understanding the pathogenesis of DS, many investigators have been making enormous efforts on isolation of all the genes located on this small chromosome.

mSim2 is a murine homolog of *Drosophila* single-minded gene, the product of which plays a pivotal role in the central nervous system (CNS) midline development of *Drosophila* (Reeves et al., 2001). It is located on the C3.3–C4 band of mouse chromosome 16, which is homologous with human chromosome 21q22 (Gardiner et al., 2003). *mSim2* gene is expressed in brain including central nervous system and ventral diencephalon from embryo to adult, and the mutations in this single gene cause a complete loss of CNS midline cells in mouse (Dahmane et al., 1995; Vialard et al., 2000). Interestingly, *Sim2* mRNA is also expressed in branchial arches, ribs and limbs in developing rodent and human embryos, which are apparently associated with the sites of symptoms of DS. Recently, two papers revealed that the Sim2-overexpressing transgenic mice have mild impairment of learning and memory, abnormal behavior and sensitivity to pain (Ema et al., 1999; Chrast et al., 2000). Although all these data have led to the hypothesis that *Sim2* gene may play a crucial role in the mental retardation of DS, the mechanism how Sim2 exerts its influence on mental retardation is still unclarified.

The PC12 cell line, derived from a rat adrenal medullary pheochromocytoma tumor, is widely used in vitro model for studying the neural differentiation and survival (Greene and Tischler, 1976). In the present study exploring the role

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of Sim2 in the pathogenesis of DS, mSim2 was transiently transfected into PC12 cells, and the effect of Sim2 on the cell cycle and cycle-related proteins was detected by flow cytometry, reverse transcription-polymerase chain reaction (RT-PCR) and immunocytochemistry methods.

2. Materials and methods

2.1. Cell culture

PC12 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 5% v/v heat-inactivated horse serum, 10% v/v heat-inactivated fetal bovine serum (Invitrogen, USA), 2 μmol/L glutamine, 50 U/ml penicillin, and 100 mg/L streptomycin. The cells were routinely grown on 60-mm diameter uncoated polystyrene dishes at 37 °C in a humidified atmosphere with 5% CO_2.

2.2. Construction of the mSim2 expression vectors

Total RNA was obtained from a fetal male Kunming mouse after decapitation by means of total RNA Isolation kit (Invitrogen, USA). First-strand complementary DNA was generated with oligo$(dT)_{18}$ (MBI, Lithuania). The *mSim2* coding sequence was amplified by using two nucleotide primers: 5′-CTGGATCCATGCGCGCGGTCTTCCCG-3′, which had a *BamHI* site at the 5-end and an initiating codon, and 5′-CTGAATTCTCACAGATTGACACC TG-3′, which had an *EcoRI* site and a terminating codon. A polymerase chain reaction was performed with DNA polymerase Pfu for 35 cycles by denaturing at 94 °C for 45 s and annealing at 55 °C for 45 s, with extension at 72 °C for 90 s. The vector pcDNA3 containing a *BamH I–EcoR I* digested *mSim2* fragment pcDNA3-mSim2 was constructed. The sequence of the inserted *mSim2* fragment was confirmed by DNA sequencing. Plasmid DNA used for the transfection experiments was prepared using a Plasmid Maxi kit (Qiagen, Chatsworth, CA).

2.3. Transient transfection of expression vectors

PC12 cells were seeded on 35-mm polystyrene dishes at a density of 1×10^6. The constructed vector, pcDNA3-mSim2 and mock plasmid, pcDNA3, were transfected into PC12 cells by Lipofectamine™ reagent (Invitrogen, USA) according to the manufacturer's instruction. Then the cells were incubated for 48 h in the standard culture medium.

2.4. Semi-quantitative RT-PCR

The mRNA expression of mSim2, cyclin E and p27 was detected by RT-PCR method. Briefly, total RNA was extracted from PC12 cells with TRIzol reagent (Invitrogen, USA) according to the instructions of the manufacturer. RNA samples were quantified by measurement of optic absorbance (A) at 260 nm in a spectrophotometer, with the A_{260}/A_{280} ratio ranging from 1.8 to 2.0, which indicated a high purity of the extracted RNA. The concentrations of RNA were calculated at A_{260}. Aliquots of total RNA (2.0 μg) from each sample were reverse-transcribed into cDNA according to the instructions of the First-strand cDNA Synthesis kit manufacturer (MBI, Lithuania). Equal amounts of the product of the reverse transcription reaction were subjected to PCR amplification. We coamplified the housekeeping gene *β-actin* to allow for a semi-quantifiable comparison of the PCR products. Amplification consisted of 5 min of denaturation at 94 °C followed by 30 cycles (for *β-actin*, 27 cycles) of 45 s at 94 °C, 45 s at 55 °C, and 60 s at 72 °C. The final extension was set for 10 min at 72 °C. After amplification, 5.0 μl of each PCR reaction product was electrophoresed on a 1.5% (w/v) agarose gel containing ethidium bromide (0.50 μg/ml). The product sequences were determined using Big-Dye Primer Cycle Sequencing and Big-Dye Terminator Cycle Sequencing kits (Perkin–Elmer) with the ABI3700 automated sequencer. The mRNA levels of mSim2, cyclin E and p27 were normalized with *β-actin* mRNA levels. The mean mRNA level of the untreated cells was defined as 1.0 arbitrary unit. At least three independent PCR procedures were performed to allow for statistical analysis.

All PCR primers were synthesized by Sangon Biotech, China. The sequences and the amplified lengths are shown in Table 1. The DNA marker (DL2000) was obtained from Takara, China.

2.5. Flow cytometry

For cell cycle analysis, the cells were seeded at a density of 10^6 cells/ml. After harvested by centrifuging, the pellet was washed once with phosphate-buffered saline (PBS), resuspended in 1 ml of cold 70% ethanol, and stored overnight at −20 °C prior to flow cytometric analysis. The cells were washed twice and then stained with 50 μg/ml propidium iodide in 0.1% sodium citrate plus 0.1% Triton X-100 for 30 min. The stained cells were analyzed by a fluorescence cell sorter (Becton and Dickinson, USA) and the data were analyzed.

2.6. Immunocytochemistry

After a brief rinse with PBS, the cultured cells were fixed in freshly prepared ice-cold 4% paraformaldehyde for 1 h, and were then treated with 0.05% Triton X-100 in PBS for 10 min for the enhancement of the penetration of immunoreagents. The fixed cells were incubated in the following solutions: 1 h in 3% BSA–PBS; 18 h in 3% BSA–PBS containing mouse anti-rat cyclin E or p27 antibody (Santa Cruz, USA). Then the immunocytochemistry process was performed according to the instructions of the SP Immunocytochemical kits (Zhongshan Biotech, China). The slides covered with cells were observed under microscope. The number of PC12 cells stained with cyclin E or p27 antibody was determined by counting the number of positive-stained cells in a total of at least 200 cells under magnification of 200×.

2.7. Statistical analysis

All data were presented as mean ± SE. One-way analysis of variance followed by Q test using SPSS 11.0 Software was performed to determine statistical significance. A P value < 0.05 was considered statistically significant.

3. Results

3.1. Cloning the mSim2 coding sequence

The amplified fragment of *mSim2* was about 2.5 kb as expected. The DNA sequencing result also suggested a successful plasmid construction (Fig. 1).

3.2. Expression of mSim2 in PC12 cells

As shown in Fig. 2, *mSim2* mRNA was almost undetectable in both normally cultured and mock-transfected PC12 cells.

Table 1
The primers used for PCR and the length of the product

Templates	Primer sequences	Product length (bp)
mSim2	Sense: 5′-ACTGGAGAACTAGTC-3′ Antisense: 5′-CACATACAGCGGGCTC-3′	622
Cyclin E	Sense: 5′-CTGGCTGAATGTTTATGTCC-3′ Antisense: 5′-GCTTGGGCTTTGTCCAGC-3′	380
p27	Sense: 5′-AAC CTC TTC GGC CCG GTC AAT C-3′ Antisense: 5′-TGC TCC ACA GTG CCA GCA TTC G-3′	467
β-Actin	Sense: 5′-CCTTCCTGGGCATGGAGTCCTG-3′ Antisense: 5′-GGAGCAATGATCTTGATCTTC-3′	208