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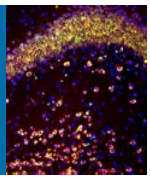

Original aritical

Establishment and detection of Dpysl2 in vitro

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Abstract

Background/aim: To construct recombinant vector of over expression and siRNA of Homo sapiens dihydropyrimidine 2 (Dpysl2) gene. **Methods:** Fragment containing Dpysl2 ORF gene or Dpysl2-siRNA was inserted into recombinant plasmid HIV, then the recombinant of over expression vector was identified by digestion with Xhol and EcoR1, and sequencing of ORF cloning. Pseudovirion containing the recombinants were produced by virus packaging with 293Tα cell, and then the pseudovirion was transfected into HT1299 cell to detect the virus titer. PC12 were divided into two groups: transfected pseudo virus particles and the control. RT-PCR and WB analysis were performed to observe the changes of Dpysl2 expression after transfection. **Results:** Successful recombinant vector of over expression and siRNA were identified by digestion with Xhol and EcoR1 and sequencing results. At 48h post-transfection, both of the mRNA and protein level of PC12 were up-regulated in transfected group compared to control group, $P<0.05$ and $P<0.01$, respectively. **Conclusion:** Recombinant vectors of Dpysl2 could effectively regulate the expression of Dpysl2.

Key words: *Dpysl2; Lentiviral vector; Xhol; EcoR1*

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Introduction

Homo sapiens dihydropyrimidine 2 (Dpysl2) belongs to dihydroorotase (DHOase) family and hydantoinase/dihydropyrinidinase enzyme subfamily. Each subunit contains a zinc ions, which can catalyze the reaction of 5, 6-dihydrouracil + H2O = 3-ureidopropanoate and also take part in catabolic processes in pyrimidine bases biological program (Mirel DB, et al., 1998). Dpysl2 also known as collapsin response mediator protein (CRMP). CRMP of mammals is mainly expressed in the early differentiation process of neurons, which means that they played a certain role in the growth of neurons (Crooks DR, et al., 2007; Gruer MJ, et al., 1997; Lall MM, et al., 2008; Yan X, et al., 2009). In the central nervous system, Dpysl2 pays a certain role in the process of structure and adjustment i.e. neural differentiation, release of neurotransmitters and microtubule stability (Silva PN, et al., 2013). In the penumbra of cerebral cortex and striatum with stroke, the expression of Dpysl2 and alpha-spectrin 2 (spna2) was up-regulated in mast cells. So, it suggested these genes in axon growth and plasticity pay an important role (Saraswathy S, et al., 2011). Additionally, Dpysl2 also pays an important role in the process of axonal formation, its dysfunction may result in abnormal neural development (Zhao X, et al., 2006). Nakata and his colleagues found that 3' end gene polymorphism of Dpysl2 was associated with schizophrenia in Japanese mouths, especially related to paranoid delusions. Although there is no evidence to demonstrate Dpysl2 itself being a susceptibility gene of schizophrenia, study showed that it may interact with other candidate genes of schizophrenia (Zhao X, et al. 2006). However, this experiment provides a basis for the development of therapeutic drugs in neural damage and mental illness through cloning vector construction of Dpysl2 expression.

Methods

Construction of lentiviral recombinant of Dpysl2 shRNA and gene overexpression

DNA transduction of lentiviral plasmid vector with Lv122 HIV skeleton

Recombinant lentiviral vector (10ng) from Guangzhou Funeng Gene Limited Company was added to Stbl3TM competent cell in ice for 30min, then the mixture was immediately transferred to pre-set water bath pot to heat in the 42°C for 60s, continued ice bath for 2 min, added 400μL SOC medium without antibiotics, placed in a 200rmp/min shaker to shake for 1h, then dipped into the bacterial liquid in 37°C incubator overnight. In next day, monoclonal bacterial plaques were collected and picked it into liquid LB (5ml) with antibiotics, placed on a 220rmp/min shaker to shake for 12h. The bacteria were harvested and prepared for the next step to extract plasmid vector DNA.

Extraction of plasmid vector DNA through alkaline lysis with E.Z.N.A.®Plasmid Miniprep Kit (100)

The bacteria were collected into EP tube (1.5ml), then centrifuged in the speed of 12000rmp/min for 1min. After collected supernatant, the tube was again centrifuged in the speed of 12000rmp/min for 1min, and the supernatant was also sucked with pipette. Then, we added Solution (250μl) with Rnase (100μg/ml) , and let them well mixture in the vortex, followed by adding Solution (350μl) with gently inversing. At last, we added Solution (300μl), inversed and well mixed them, and immediately placed in -20°C refrigerator for 10min. After centrifugation (12000rmp/min, 10min), the supernatant was transferred to silica gel column, and then centrifuged (12000rmp/min, 1min). Followed removing the subnatant, we added HB Buffer (500μl) and centrifuged again. After drained subnatant, we joined DNA Wash Buffer (750μl) and centrifuged (12000rmp/min 1min). Finally, the silica gel column was transferred to a new tube, added TE Buffer (40μl), stood for 5min, and then centrifuged (10000rmp/min, 1min). The extracted DNA samples were collected into 1.5ml EP tube, placed in -20°C refrigerator. At the same time, a small samples were taken to measure the concentration by spectrophotometer. Meanwhile electrophoresis was used to detect the extractive quality of plasmid.

Digestion of lentiviral plasmid vector DNA by restriction endonuclease and gel recycle after electrophoresis

Plasmid DNA samples extracted by alkaline lysis were successively digested with Xho1 and EcoR1. According to the enzyme activity units, we designed the system of digestion reaction. After digested by Xho1 and EcoR1 , samples were placed in PCR instrument at 37°C for 4h. When it finished, electrophoresis experiment was executed at 90v voltage for 30min. Finally, UV transmission reflectometer was used to cut gel and TaKaRa Agarose Gel DNA rification Kit Ver.2.0 was used to recycle the gel.

Extraction of RNA in brain tissue

Rats was anesthetized with peritoneal injection of 3.6% chloral hydrate. Scalp was opened with scissor along the median sagittal, then skull was exposed and cut with scissor along the median sagittal, exposing the whole brain. The cerebral cortex (150mg) of both sides were taken, placed in glass homogenizer with 1.5ml Trizol liquid and completely homogenized. Then the brain tissue homogenate was centrifuged (12000rmp/min, 10min), supernatant was extracted, chloroform (300μl) was added, mixture was vigorously shaken for 15s. After standing for 15min, it was centrifuged again, , the upper layer was extracted followed by adding isopropanol (750μl) and shaking for 5-10 times, then the mixture was centrifuged (12000rmp/min, 8min) after standing for 10min, the supernatant was discarded, 1 ml 75% ethanol was added and gently shaken several times. The mixture was centrifuged (7500rmp/min, 5min), the supernatant was discarded, water droplets was dried, then the rest was dissolved with DEPC (20μl) and saved at -80°C. Finally, agarose gel with 1.5% formaldehyde denaturing was performed to detect the integrity of RNA.

Amplification of Dpysl2 and gel recovery experiment

According to sequences of Dpysl2 gene in rats, primers were designed as follows: forward primer: 5'-ATCCACGCTGTTTTGACC-3', reverse primer: 5'-CCGGACACGCTGAACTTGT-3'. The primers, containing Xho1 and EcoR1 restriction sites, purchased from Dalian Takara Company. The cDNA was synthesized by reverse transcribed in PCR instrument. After synthesized, it was performed amplification. Finally, the PCR products of DNA (Dpysl2 gene) were purified by electrophoresis, implemented digestion and gel recovery.

Extraction of recombinant DNA

Connection of Dpysl2 gene and plasmid vector DNA

A total of 25μl reaction system was established including plasmid vector DNA (4μl), Dpysl2 gene with enzymic sticky ends (12μl), T4-DNA ligase (1μl), 10×T4-DNA ligase Buffer (2.5μl) and no-nuclease-free water (5.5μl). The above system was placed in a 16°C water bath for 10h and DNA samples were taken to join competent cells, the mixture was concentrated in ice for 30min. After finished, it was immediately placed in 42°C hot water bath for 90s, and then placed in the ice bath for 1min. Followed by addition LB broth (1ml), the mixture was placed in 30°C thermostatic shaker and shaken at 200rmp/min for 1h. Then it was placed into LB solid medium with ampicillin, and gently shaken. The plate was placed overnight in 37°C incubator. The single colony was picked at next day, rinsed in LB liquid medium and cultivated in 37°C constant temperature shaker overnight.

Extraction of recombinant DNA Recombinant DNA was extracted with E.Z.N.A ®Plasmid Miniprep Kit (100). This operation was same as extraction of plasmid vector DNA.

Digestion and electrophoresis of recombinant

Digestion reaction system (30μl) consisted of Dpysl2 recombinant DNA (4μl), Xhol (1μl), EcoR1 (1μl), 10×K Buffer (3μl) and no-nuclease-free water (21μl). The above system was placed into PCR instrument at 37°C for 4h. Meanwhile, the method and procedure of electrophoresis were same as described above.

Sequencing experiment of recombinant

The recombinant samples were sent to Shanghai Biological Engineering Limited Company for sequencing. These samples were sequenced using DNA automatic sequencer. After sequencing, they were implemented virus packaging.

Dpysl2-ORF-HIV virus packaging

Transduction of Dpysl2-HIV lentiviral recombinant DNA

Methods is same as before.

Preparation of plasmid with no-endotoxin

The remaining bacterial suspension (1ml) was added to LB medium with Amp (+) liquid (100ml). Then the mixture was shaken at 37°C constant temperature shaker for 14h. The

Table 1 Information of the four shRNA fragments.

Clone Name	Symbol	Location	Length	Target Sequence
MSH028938-1-HIVU6(OS211077)	Dpysl2	274	19	cgatcgtcttctgatcaaa
MSH028938-2-HIVU6(OS299794)	Dpysl2	939	19	tgaaccggtccatcactat
MSH028938-3-HIVU6(OS299795)	Dpysl2	62	19	cagaggattgcatctgttt
MSH028938-4-HIVU6(OS299796)	Dpysl2	2532	19	ggaaaggttccatgtgtct

bacteria were collected (each tube 100ml) and centrifuged (3200rmp/min, 20min). After discarding the supernatant, 10ml Solotion with Rnase (100ug/ml) was added into the tube, and well mixed on the vortex device, then Solution (10ml) was joined and gently mixed by inversion. The mixture was placed in -20°C for 10min after mixing with 10ml Buffer N3, then centrifuged at12000rmp/min for 10min. The supernatant was transferred to a new 50ml filtration column and filtered, the subnatant was collected. An equal volume of ETR Binding Buffer was joined, after reversed and mixed, the mixture was added to silica gel column, which were centrifuged (12000rmp/min, 2min). Each tube was successively added with 3ml ETR Wash Buffer, 3ml Buffer EHB and 3ml DNA Wash Buffer, all of which were followed by centrifuging (12000rmp/min, 2min). Finally, the silica gel column was transferred to a new EP tube, and dissolved by Endonxin-Free Elution Buffer (40μl). After standing for 5min, it was centrifuged (12000rmp/min, 2min). The concentration was measured and reserved at -20°C surroundings.

Viral packaging and determination of titer

Postthawed 293Ta cell lines and H1299 cell lines were culated into six-well plates. Then passage of 293Ta cell lines and H1299 cell lines were conducted at the cell concentration of 1×105 /ml. Cell growth was observed. If the density of cell growth reached 80% of the entire plate, medium was changed and viral packaging was started. To prepare the inoculation system, the diluted system of transfection reagent was prepared, consisting of 3ul reagent and 30ul opti-MEM. Lentivirus plasmid was diluted in the same way. Then, diluted reagent was added to dilute DNA and transfection complex formed at RT for 25minutes.

After that, the mixture was added to six-well plate, gently mixed and placed in an incubator with 5% CO2 at 37°C for 12h. The original serum free medium was replaced with medium (1ml) containing 5% fetal calf serum, antibiotics and 2.0μl virus titer enhancer. The plate was cultured in incubator with 5% co2 in 37°C for 48h. Then the cells were observed in an inversion fluorescence microscope. When all cells emitted labeled fluorescent, virus were collected in biological safety cabinet. Total cells were blown up using a pipette, and then collected to centrifuged (500g/min, 10min). The supernatant was filtered through a 0.45um filter.

When the H1299 cells covered 80% of six-well plates, medium was replaced with medium (2ml) containing polybrene (5ug/ml), 5% fetal calf serum and double antibiotics medium. Virus was orderly added in five-hole: 0.1μl, 0.5μl, 2μl, 10μl, 50μl. The sixth well was used as the control. After adding virus they were mixed and six-well plates were placed at 4°C refrigerator for 2h, then placed to incubator with 5% CO2 in 37°C for 12h and then replaced with 10% fetal calf serum and double antibiotics medium. They were placed to incubator with 5% CO2 in 37°C for 48h. The images were taken using two-dimension heavy phase of bright-field and fluorescence and calculating the ratio of fluorescent cells (R). Cells were digested and counted. Total number of cells per hole were calculated (M): T=RM*1ml/0.1μl=10^4RM Titer/ml.

Establishment of Dpysl2-shRNA-HIV Transduction of HIVU6 skeleton lentivirus vector plasmid

The methods are same like with ORF clones.

Digestion and connection

Information of HIVU6 plasmid showed that the enzyme site in 5' cloning was BamHI, 3'cloning site was EcoRI, and Hairpin sequence was TCAAGAG. Four ShRNA fragments were designed as sthowed in table 1.

Screening of effective interference fragment

Firstly, PC12 cell was resurrected , cultivated 2 days and prepared to passage. After 24h, cell growth was observed. When the cells bedding area was more than 60%-70%, they were transfected. Seven experimental groups were designed i.e. control, transfection reagent, vector control, shRNA-1-Dpysl2, shRNA-2-Dpysl2, shRNA-3-Dpysl2, and shRNA-4-Dpysl2. The medium with serum was discarded and D-hanks without serum were used to wash. Then, 1.5ml opti-MEM (GIBICO) medium without serum was added. For each transfection sample, the mixture was prepared following these steps. For one hole in six-well plates, transfection reagent was diluted by the opti-MEM medium without serum and incubated at room temperature for 5min. Dpysl2-shRNA (1.5ug) was diluted by the 250μl opti-MEM medium without serum, incubated for 5min and they were added into the diluted transfection reagent. They were gently shaken and incubated at room temperature for 20min to forme a shRNA-Dpysl2-lipo2000 complex. After that, the mixture was transferred to the prepared well and well mixed. All groups were placed to incubator with 5% CO2 in 37°C for 6h, and then the medium was replaced with the high glucose DMEM culture medium containing 10% calf serum, and cultured for 48h. Then, lysis of cells was performed to collect total RNA using the method described above. The concentration of 3μl RNA sample was measured by ND-1000 spectrophotometer. The first cDNA chain was synthesized following the instructions of RevertAidTM First Strand cDNA Synthesis Kit. These reactants were added to form PCR system in ice bath including 5×reaction Buffer 4μl, RibolockTM Ribonuclease Inhibitor (20μg/μl) 1μl and 10mm dNTP mix 2μl. Reactants were gently shaken, centrifuged for 3-5s and incubated in 37°C for 5min. The

1μl RevertAidTM M- MuLV Reverse Transciptase was added to react with these reactants and the mixture was incubated at 42°C for 60min. After heated 70°C for 10min, the reaction was terminated, and then ice-cooled. In PCR tube was orderly added the following reagents i.e. 2×PCR Master Mix 12.5μl, upstream primer 0.5μl, downstream primer 0.5μl, cDNA temple 1μl and PCR water nuclear-free 10.5μl. The temperature of annealing was 52°C. PCR results were implemented the electrophoresis. The optical density was measured by Gene Tool software. The data were presented as mean± SD and analyzed by SPSS11.5 software. The statistical analyses were performed using one way analysis. The value of $p<0.05$ was considered significant (*).

Dpysl2-shRNA recombinant packaging and titering

The methods was same as Dpysl2-HIV virus packaging.

Transfection of Dpysl2-ORF-HIV and Dpysl2-shRNA-HIV virus particles

PC12 cells were cultured. Cell growth was observed after 24h. When the cells bedding area were more than 60%-70%, virus transfection was performed. The method used was the same as described above. Four types of virus (10μl) were orderly and independently added to shRNA negative control, Dpysl2-shRNA-HIV, Dpysl2-ORF-HIV negative control and Dpysl2-HIV. The control group was added with basal medium. After addition virus, they were placed into 4°Crefrigerator for 2h, and then transferred to 37°C incubator with 5% Co2. After 12h, medium was replaced with the medium with 10% fetal calf serum and double antibiotics. Then cells were placed at 37°C incubator with 5% Co2 for 48h. Fluorescence image were collected. After image acquisition, harvested cells were used for RT-PCR and WB detection.

Detection of Dpysl2 using RT-PCR and WB

Method used to detect the Dpysl2 using RT-PCR have already been described above.

For WB analysis, cells was washed 3 times using 0.01m PBS and then added with RIPA buffer. Cells were whipped by scraper, suspension was collected, and then placed in ice-water bath and treated by ultrasonic cleaning machine for 3-5min. When the chain of DNA were broken, it was centrifuged at 4°C (12000g/min, 15min). The supernatants were collected, placed into 0.5ml centrifuge tube and stored at -20°C.

BCA reagent was used to detect protein concentration. Then, SDS-PAGE electrophoresis was performed. A total of 50ug protein was added for each group. Samples were stacked firstly using the force of 100V constant voltage, and then was separated in 80V constant voltage for 3h. Then, the protein was transferred to the PVDF member using the semi-dry transferred method. The PVDF membrane was blocked in 5% skim milk at room temperature for 1h and then incubated with primary antibody (rabbit anti-dihydropyrimidine, 1:500, Abcam, ab205039) overnight at 4°C. Membranes was wash 3 times by TBST, enzyme-labelled secondary antibody was added and incubated at room temperature for 2h. The membrane was washed three times and ECL was used to form the images in gel imager.

Results

Information of Dpysl2 carrier

Product code was named as MSH028938-HIVU6. Carrier size was 9762bp, length of HIV-ORF expression vector was 1719bp and shRNA cloning vector length was 9047bp. Pronucleus screening was labelled as Ampicillin and eukaryotic screening maker was Puromycin. Reporter gene was Egfp, 5' cloning sites was BamHI and 3' cloning sites was EcoRI. Hairpin sequence was TCAAGAG. Specific structure was showed as figure 1.

Vector construction

Authentication results of ORF cloning

After Dpysl2-HIV recombinant being digested by BamHI and EcoRI, we obtained the expected results i.e. ORF length of coding genes located in the makers of 1500bp-2370bp through electrophoresis as showed in lane 2 (fig 2).

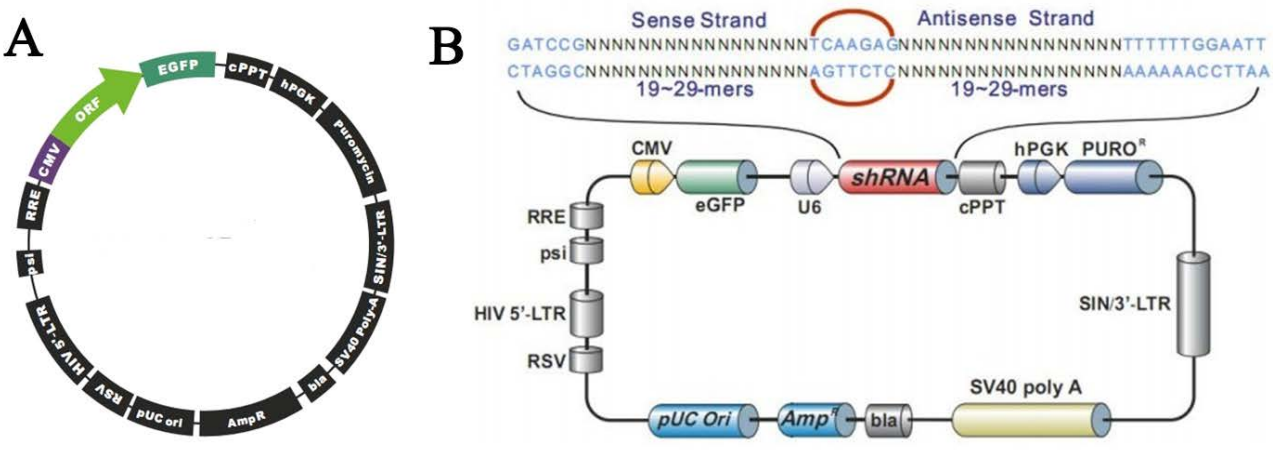

Figure 1 Dpysl2 carrier information. *A Vector information for HIV-Dpysl2-ORF cloning. B Vector information for HIV- Dpysl2-SH cloning.*

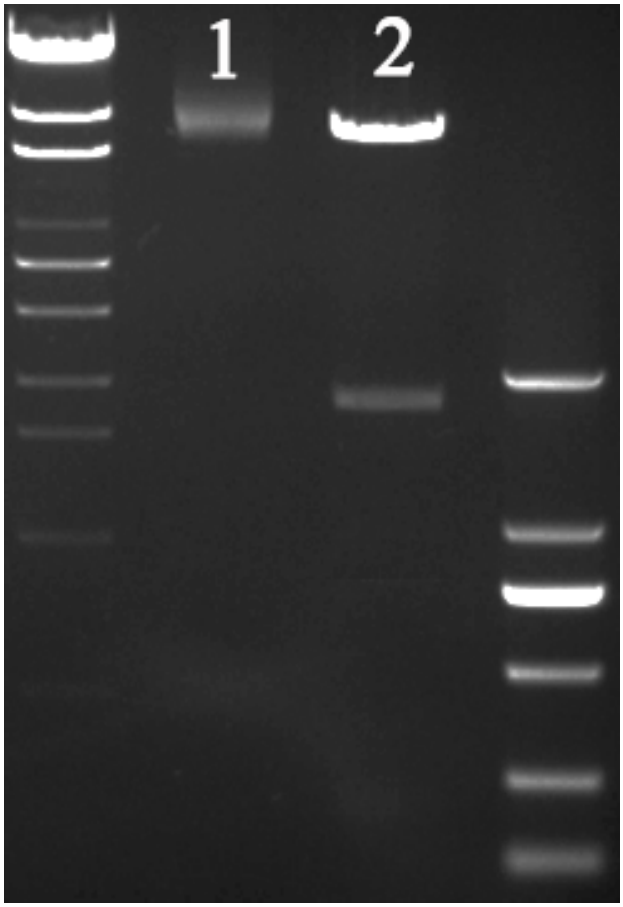

Figure 2 Authentication of Dpysl2-HIV recombinant by electrophoresis. *Enzyme digestion result of Dpysl2-HIV (unlabled lane from left to right. DNA Ladder 6000,10000). Lane1: Dpysl2-HIV plasmid. Lane2: plasmid digested by BamHI and EcoRI. There are two expected bands (~8043/2000bp).*

Sequencing of ORF cloning

Sequencing of ORF cloning was performed by TaKaRa Bio. Product code was EX-Mm01932-Lv122 and register numberwas NM-009955. Compared with data in CNBI, coding sequence was exactly consistent (fig 3).

shRNA cloning sequencing

SH cloning shown that results were consistent with interference fragment sequence.

Virus packaging and titering

When virus were packaged for 48h, we began collecting pseudo-viral particles. But before this process, we collected the fluorescence images through inverted fluorescence microscope (fig 4).

Viruses were collected, concentrated and purified. After viruses transfected in HT1299 cells, the viral titer was measured, R was worked out through fluorescence cell count and bright-field cell count (fig 5).

R Dpysl2-HIV=0.87, R sh-Dpysl2-HIV=0.96, titer: T Dpysl2-HIV =7.2×108pfu/ml, Tsh-Dpysl2-HIV =1.74×108pfu/ml

Transfection PC12 cells

Virus were transfected with PC12 cells. After 48h, images was collected, the results were showed as follows (fig 6).

RT-PCR test

Results showed that Dpysl2 mRNA expression was increased after ORF cloning transfection, but Dpysl2 mRNA expression was decreased after shRNA cloning transfection (fig 7).

Western Blot test

Dpysl2 protein expression was increased after ORF cloning transfection, but Dpysl2 protein expression was decreased after shRNA cloning transfection (fig 8).

Discussion

This experiment successfully constructed shRNA and ORF expression cloning vector of Dpysl2. Successful transfection was verified by PCR and WB analysis. Recombinant could up-regulate or down-regulate Dpysl2 that had biological activity. Thereby, Dpysl2 gene vector plays an important role in the regulation of neural damage and mental illness.

In cultured neurons, we found that Dpysl2 was associated with neuronic polarity and axonal extension. In the developmental process of nervous system, Dpysl2 proteins were expressed in different parts, but its esoteric function

Figure 3 Authentication of Dpysl2ORF -HIV recombinant by sequencing. *Sequence and vector information for EX-Mm01932-Lv122 were showed.*

was still not clear (Lu GT, et al., 2009). Studies have shown that phosphorylation of Dpysl2 locates in hippocampus during embryonic development (Applequist SE, et al., 1995). Nakata and his colleagues found that 3' end gene polymorphism of Dpysl2 was associated with schizophrenia in Japanese mouths, especially paranoid delusions. However, other study showed that Dpysl2 did not play a major role in schizophrenia (Ganapathy-Kanniappan S, et al., 2012). RNA interference refers to a phenomenon with highly conserved, double-stranded RNA-induced and homologous mRNA efficient specific degradation during evolution. We can specifically eliminate and close the expression of specific

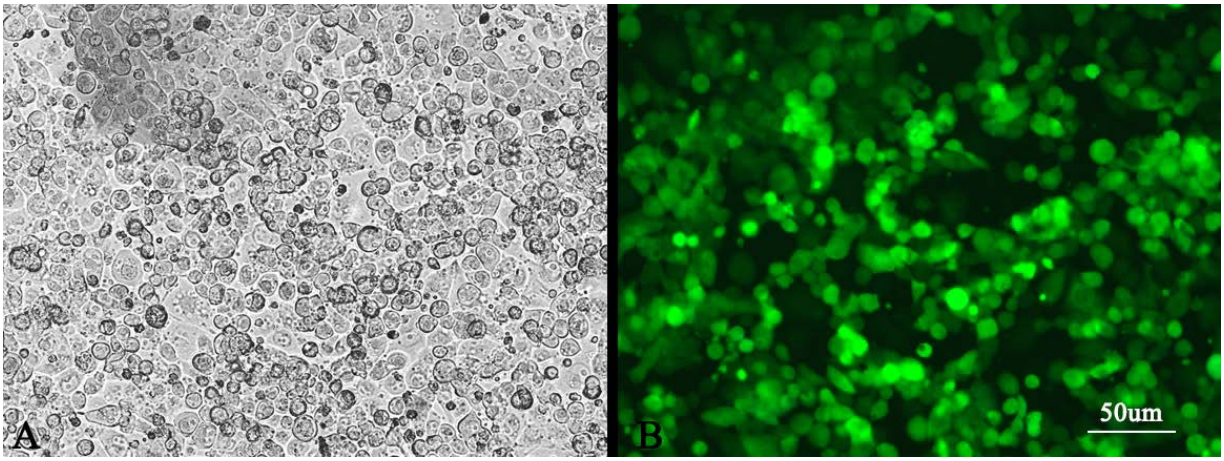

Figure 4 Bright-field and fluorescence image of 293Ta used for viral packaging for 48h. *A Bright-field image of transfected 293Ta. B Fluorescence photo of transfected 293Ta showed that many cells had fluorescence.*

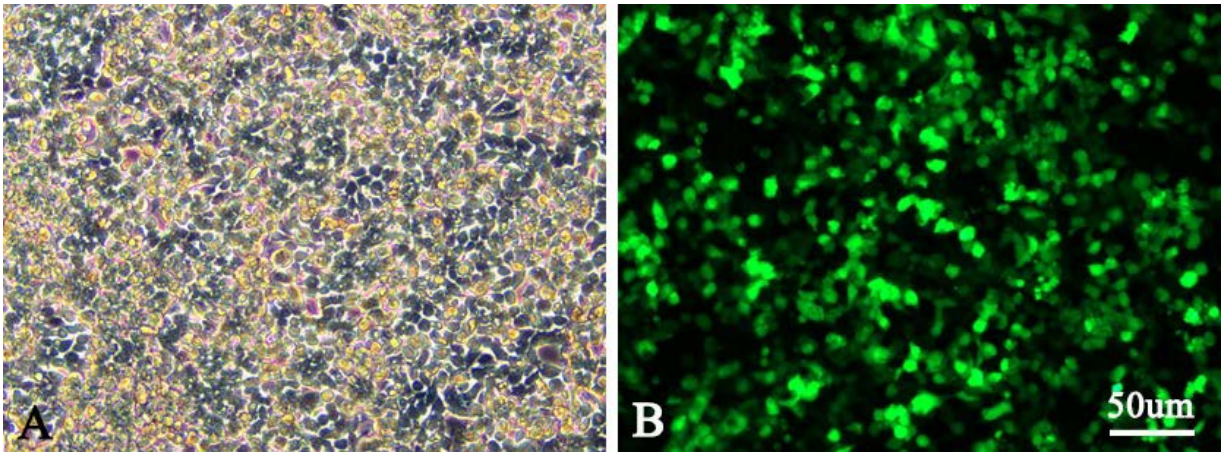

Figure 5 Bright field and fluorescence photos of transfected HT 1299 cells. *A Bright-field image of transfected HT1299 cells. B Fuorescence photo of HT1299 cells transfected with 10ul lentivirus.*

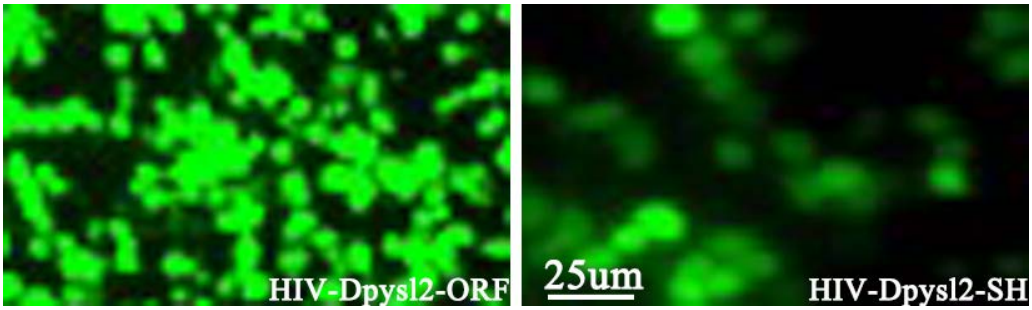

Figure 6 Fluorescence photo of PC12 transfected the virus. *Stable expression of HIV pseudovirion particles in PC12 cells after transfection 48h.*

genes using RNAi technology. Therefore, this technology has been widely used to explore genes function, infectious diseases and cancer. The advantages of siRNA expression vector mainly came from that it can be proceeded for long-term studies due to the vector with antibody maker can inhibit the expression of target gene in cell. Viral vectors can also be used for siRNA expression with the benefit of that it can directly and efficiently transfect cells to study gene silencing and avoid the low efficiency of plasmid transfection. Meanwhile, establishment and identification of vectors containing interference fragment and ORF sequence, respectively, which illustrated that our investigative targets play a role to control the gene expression in-vivo. In summary, we have successfully constructed HIV lentiviral vectors containing shRNA or ORF cloning to down-regulate or up-regulate the expression of Dpysl2. After PC12 cells transfected with the two kinds of lentivirus, expression of Dpysl2 was increased after ORF cloning transfection, but

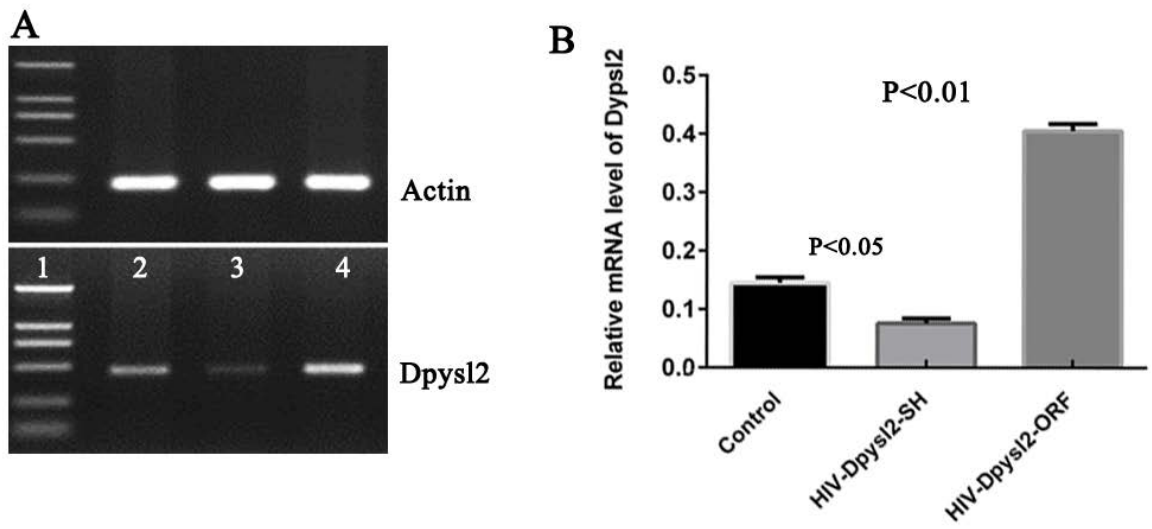

Figure 7 Dpysl2 mRNA expression after ORF and shRNA transfection. *A PCR produces were detected by agarose gel electrophoresis for each group. B Histogram of Dpysl2 mRNA expression showed that Dpysl2 carriers could significantly regulate the expression of Dpysl2. β-actin, a house-keeping gene was used as an internal control. Lane1: DNA marker; Lane 2: Control; Lane 3: Dpysl2-shRNA-HIV; Lane 4: Dpysl2-ORF-HIV. Data were represented as the mean ± SD.*

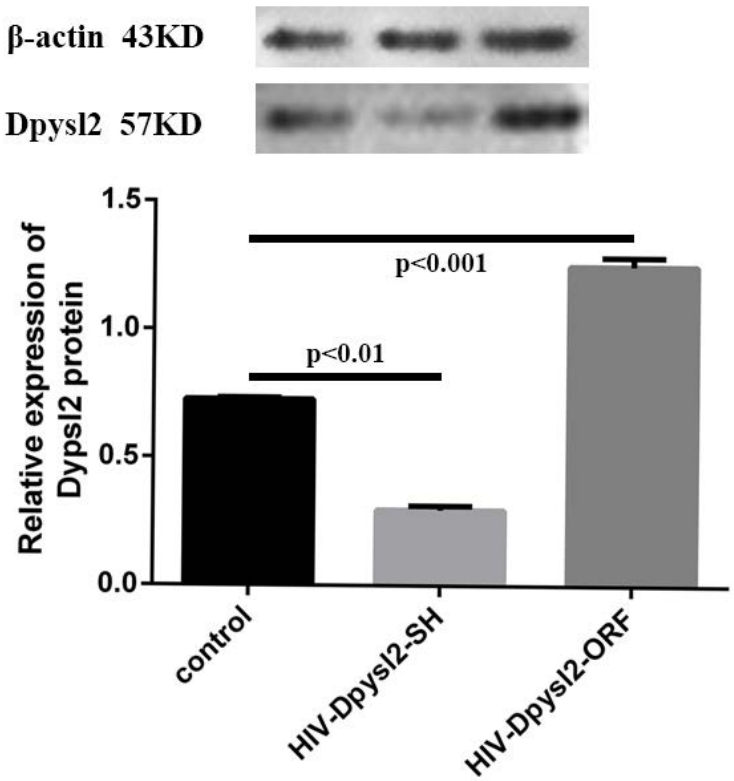

Figure 8 Dpysl2 protein expression after ORF and shRNA transfection. *WB bands of each group and Histogram of Dpysl2 protein showed that Dpysl2 carriers could significantly regulate the translation of Dpysl2. β -actin, a house-keeping gene was used as an internal control. Lane1: DNA marker; Lane 2: Control; Lane 3: Dpysl2-shRNA-HIV; Lane 4: Dpysl2-ORF-HIV. Data were represented as the mean ± SD.*

decreased after shRNA cloning transfection.

In this experiment, the constructed vectors of Dpysl2 shRNA and ORF expression cloning can be used for studies in vitro. In addition, it also can be applied in-vivo and its effects of neurobiology laid the foundation for future investigation. Therefore, the construction of these vectors have great wide application foreground.

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Conflicts of interest

There is no significant conflict interest in this study.

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