Construction of R2C cell line overexpressing DLD

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Abstract

Dihydrolipoamide dehydrogenase (DLD) is one of the components of the pyruvate dehydrogenase complex, the alpha-ketoglutarate dehydrogenase complex, the branched chain amino acid dehydrogenase complex and the glycine cleavage system. DLD plays an important role in cAMP and ROS metabolism, whereas cAMP can affect the synthesis of steroid hormones. Therefore, DLD is involved in regulating the synthesis of steroid hormones. R2C cell line overexpressing DLD was constructed by lentiviral vector in this study. It showed that the ratio of 8 μg/mL Polybrene and 10 μL of the virus was the best concentration ratio for infection of DLD-overexpressing lentiviruses. The results of fluorescence microscopy and Western blotting showed a successful construction of R2C cell line overexpressing DLD.

Keywords

Dihydrolipoamide dehydrogenase; R2C cells; Lentiviral vector.

1. Introduction

Dihydrolipoamide dehydrogenase (DLD) belongs to the family of pyridine nucleoside disulfide oxidoreductase[1]. DLD is one of the components of the pyruvate dehydrogenase complex, the alpha-ketoglutarate dehydrogenase complex, the branched chain amino acid dehydrogenase complex and the glycine cleavage system[2, 3].DLD is usually present in the form of homodimer, and each subunit carries an active disulfide bond and a non-covalently bound NAD^+ or NADH molecule[4, 5].Inhibition of DLD will also significantly inhibit the production of ROS and cAMP levels, which reveales that ROS, cAMP as a downstream signal molecules regulated by DLD[6].It is important to note that, cAMP as an important intracellular second messenger can activate PKA, through the PKA-ERK1/2 pathway to regulate the synthesis of steroid hormone in Leydig cells. It means that DLD can be an upstream protein of cAMP to regulate the steroid hormone synthesis via PKA-ERK1/2 pathway. Therefore, R2C cell line overexpressing DLD was constructed for further study on the regulation of DLD in steroid hormone synthesisin this study.

2. Experimental detail

2.1 Materials

F12 medium, fetal bovine serum and horse serum were purchased from Life Technologies. pLVX-EF1a-IRES-ZsGreen1, Lenti-PacFIV Expression Packaging Kitwere purchased from GeneCopeia. Dihydrolipoamide dehydrogenaserabbit antibody was purchased from abcam. 293T cells were purchased from Clontech. R2C cells were purchased from ATCC.

2.2 Reagent formulation

LB medium

Chemicals	Amount
Yeast Extract	5.0 g
Typton	10.0 g
NaCl	5.0 g
Ultra-pure water volume to	1 L

If the solid medium is added agar powder 15 g, 121 ℃autoclave 20min, stored at 4 ℃.

Electrophoresis buffer

Chemicals	Amount
Tris-base	3.03 g
SDS	1.0 g
Gly	4.4 g
Ultra-pure water volume to	1 L

Transfer membrane buffer

Chemicals	Amount
Tris-base	5.8 g
SDS	0.37 g
Gly	2.9 g
Methanol	200 mL
Ultra-pure water volume to	1 L

2.3 Experimental Procedure

A: Recombinant overexpression vector

Total RNA was extracted from the rat's liver and then reverse transcribed to cDNA by TaKaRa (6210A).The product was used for PCR reaction to obtain the target sequence. The primer sequence and PCR reaction procedure as follow:

Primer sequence:

Sense:5'-CGACTAGTATGCAGAGCTGGAGTCGT-3' (Tm=66.2°C)

Anti-sense:5'-GCGGATCCTTAAAAGTTGATTGGTTTGCC-3' (Tm=66.2°C)

Reaction system:

Reaction component	Amount
Primer STAR (Premix)	25 μL
Primer F	2 μL(10 μM)
Primer R	2 μL(10 μM)
Template cDNA	2 μL
RNase-free Water	19 μL
Total Volume	50 μL

Reaction procedure:

98 ℃, 30s;
55 ℃, 30s; } 30 cycles
72 ℃, 30s;
72 ℃, 7min;

The target sequence and the pLVX-EF1a-IRES-Zs Green1 vector were both digested and then ligated. The product was transformed into DH5α cultured overnight for 12-16 hrs.

Colonies were randomly picked and inoculated in 3 mL of ampicillin-resistant LB liquid medium shaken at 37 °C for 12-16 hrs.

Bacteria liquid (2 μL) was used as a template for PCR reaction, and positive clones were sequenced.

B: Recombinant lentiviral vector was transfected into 293T cells

Extraction of endotoxin-free recombinant plasmids.

Two days before transfection, 293T cells were cultured in a 10-cm dish(1.5×10^6 cells) in 10 mLof DMEM medium supplemented with 10% heat-inactivated fetal bovine serum, and incubated at 37 ℃ with 5% CO_2.

Diluted 2.5 μg of recombinant lentiviral plasmid and 5.0 μL(0.5 μg/μL) of Lenti-Pac HIV mix into 200 μl of Opti-MEM® I (Invitrogen), marked as tube 1. Diluted 15 μL of EndoFectin Lenti into 200 μL of Opti-MEM I,marked as tube2. Mixed gently and put solution of tube 2 into tube 1.

Incubated the cells at 37 ℃ with 5% CO_2 overnight (12 hours). Replaced fresh DMEM medium supplemented with 5% heat-inactivated FBS and added 1/500 volume of the TiterBoost reagent to the culture medium.

Aftter 72 hrs，collected the pseudovirus-containing culture medium by centrifuge at 500 ×g for 10 min to get rid of cell debris. Filtered the supernatant through 0.45 μm polyethersulfone (PES) low protein-binding filters.

The supernatant stocks should be aliquoted and stored at -80 ℃.

C: R2C cells were infected with DLD-overexpressing lentiviruses

R2C cells were cultured in a 24-well plate(6×10^4 cells/well) in 0.5mLof F12 medium supplemented with 15% HS and 2.5% FBS, and incubated at 37 ℃ with 5% CO_2.

For each well, virus suspension was diluted in complete medium with Polybrene at a final concentration of 4, 8, 12 μg/mL.

R2C cells was infectedwith0.5 mL of diluted viral supernatant. Cells were incubated in a 37 ℃ incubator with 5% CO_2 overnight.

Fluorescence intensity was observed by fluorescence microscopy, and the protein expression of DLD was identified by Western blotting

2.4 Statistical analysis

All data were presented as mean±SD and statistically significant was determined by one-way ANOVA. *P* value less than 0.05 was considered statistically significant.

3. Results and discussion

3.1 Construction of recombinant lentiviral expression vector

Total RNA was extracted and reverse transcribed into cDNA, amplified by PCR and identified by 1% agarose gel electrophoresis. The target band was found at the 1500 bp position. The target gene and lentiviral vector were digested and ligated, and then transformed into DH5α. Randomly picked colonies and identified by PCR, as shown in Figure 1.

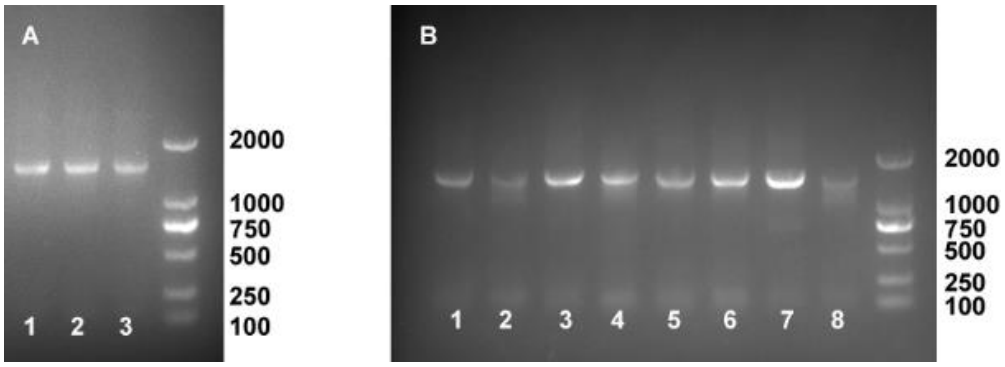

Fig 1 Construction of recombinant lentivirus vector. (A) 1, 2, 3: DLD gene amplification; (B) 1, 2, 3, 4, 5, 6, 7, 8: PCR identification for different colonies of DH5α transformed with DLD- lentivirus vector . The 7th sample was sent to detect the complete sequence.

3.2 The optimal proportion of lentiviruses and Polybrene of infection

Virus suspension (1.25, 2.5, 5, 10μL) was diluted in 0.5 mL of complete medium with Polybrene at a final concentration of 4, 8, 12μg/ml respectively. After infection, Western blotting was conducted to detect the expression of DLD inR2C cells. The results showed that the protein expression of DLD in the concentrations of 4 ng/mL, 8 ng/mL, 12ng/mL of Polybrene and10μLof the virus was significantly higher than that of the control group (Fig. 2) (P<0.05, P<0.001). The ratio of 8 μg/mL Polybrene and10 μL of the virus was the best concentration ratio. So the proportion of the concentration was selected as the follow-up infection.

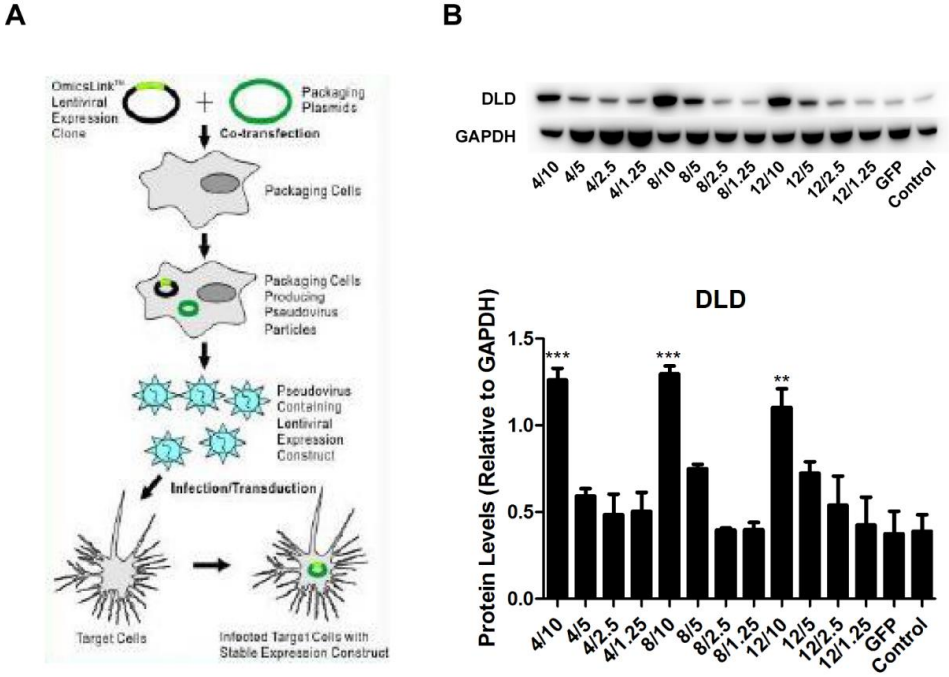

Fig.2 Schematics of lentivirus production and infection of target cells (A), expression of DLD protein in R2C cells infected with different concentrations of Polybrene and virus (B).The ratio of 8 μg/mL Polybrene and10 μL of the virus was the best concentration ratio for infection of DLD-overexpressing lentiviruses. Data represent as mean ± SD, n=3. **P< 0.01, ***P<0.001 compared with the control.

3.3 Identification of DLD overexpression inR2C cells

Lenti-pLVX-EF1α-IRES-ZsGreen had green fluorescent label protein. When the lentivirus successfully infected cells, cells would express green fluorescence, indicating the successful introduction of DLD gene. As results shown in Figure 3, the intensity of green fluorescence could represent the expression of the target gene. The intensity of fluorescence is stronger, the expression of DLD is higher. At the same time, the protein expression of DLD was validated by Western blotting.

Compared with that in Control cells, the expression of DLD protein in R2C cells infected with recombinant lentiviral vector was significantly increased (P<0.01), indicating that DLD R2C cell line was successfully constructed.

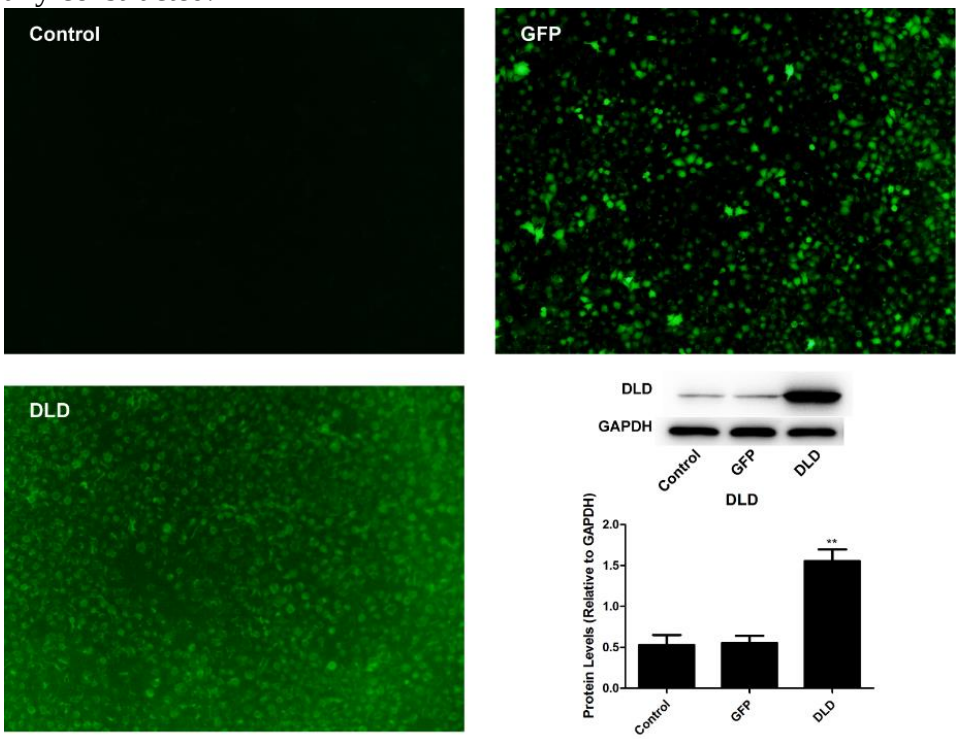

Fig.3 Identification of DLD overexpression in R2Ccells by Immunofluorescence and Western blotting. Compared with that in Control cells, the expression of DLD protein in R2C cells infected with recombinant lentiviral vector was significantly increased. Data represent as mean ± SD, n=3. **P< 0.01 compared with the control.

4. Conclusion

In this study, we found that the ratio of 8 μg/mL of Polybrene and 10 μL of the virus was the best concentration ratio for infection of DLD-overexpressing lentiviruses. The results of fluorescence microscopy and Western blotting showed a successful construction of R2C cell line overexpressing DLD.

Acknowledgements

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