Optimization of the method for the differentiation of mesenchymal stem cells into steroidogenic cells

Haifeng Hong [1, 2, a], Haiqing Cui [1, 2, b], Xing Wei [1, 2, c,*]

[1]College of Life Science and Technology, Jinan University, Guangzhou 510632, China

[2]Institute of Biomedicine, Jinan University, Guangzhou 510632, China

*Corresponding Author: Xing Wei; Phone: +86-20-85222759

[a]726776107@qq.com, [b]921004549@qq.com, [c]2433461420@qq.com

Abstract

Mesenchymal stem cells (MSCs), which can self-renew and differentiate into distinct multiple cell lineages, are defined as multipotent cells and have the ability to differentiate into different cells. As people get old, the serum testosterone falls down to abnormal levels resulting in hypogonadism. Stem cell transplantation has become a promising alternative to testosterone replacement. Steroidogenic factor 1 (SF-1), also known as Ad4BP or NR5A1, is a transcriptional factor belonging to the nuclear receptor superfamily and plays a key role in pituitary development, steroid production process and adrenal carcinogenesis. Previous studies have successfully differentiated umbilical cord mesenchymal stem cells (UC-MSCS) into steroidogenic cells after infection with adenovirus containing SF-1 and treatment with dibutyryl-cAMP (dbcAMP). In this study, we constructed a recombinant lentiviral vector containing SF-1, and optimized the method for the differentiation of mesenchymal stem cells intosteroidogenic cells, including the use of the different multiplicity of infection (MOI) of infection and the different concentrations of dbcAMP, this study provided a valuable method for differentiation of mesenchymal stem cells into steroidogenic cells.

Keywords

SF-1; testosterone; multiplicity of infection; steroidogenic cells。

1. Introduction

Steroidogenic factor-1 (SF-1), also known as Ad4BP or NR5A1, is a member of the orphan nuclear receptor family and plays a key role in steroidogenesis, regulation of brain and pituitary hormones and adrenal tumors, it's an important regulator of adrenal and gland development and function [1-3]. SF-1 knockout show adrenal and gonadal hypoplasia, and also show decreased levels of pituitary ghrelin, such as LH and FSH, so that SF-1 is thought to be an important regulatory factor of pituitary-adrenal or pituitary-gonadal axis differentiation [4-5]. To confirm the conserved role of the SF-1 gene, Tomoko transferred SF-1 to human BMSCs and found that the infected cells secrete a variety of hormones, including hormones secreted by the adrenal and gonads [6]. Besides, other important regulatory factors such as WT1, DAX-1, PBX-1, CITED2 and WNT4 during adrenal and gonadal development were introduced into human bone marrow mesenchymal stem cells and found that only SF-1 can regulate the secretion of steroid hormone by stem cells, indicating that SF- 1 is the most important regulator of gonadal and adrenal development. Previous studies have shown that adenovirus or lentivirus mediated forced expression of SF-1 could transform human BMCs and embryonic stem (ES) cells into steroidogenic cells [6, 7].

In our previous study, we have differentiated umbilical cord mesenchymal stem cells into steroidogenic cells after being infected with adenovirus containing SF-1 and treated with dibutyryl-cAMP (dbcAMP) [8]. In this study, we constructed a recombinant lentiviral vector containing SF-1, and optimized the method for the differentiation of mesenchymal stem cells intosteroidogenic cells,

including the use of the different multiplicity of infection (MOI) of infection and the different concentrations of dbcAMP.

2. Materials and methods

2.1 Cell culture

HEK 293T cells (ATCC) and human UC-MSCs were cultured in DMEM and DMEM-F12 respectively, supplemented with 10% fetal bovine serum (Gibco, USA), and were maintained in a 37 °C incubator, saturated humidity and 5% CO_2. The human UC-MSCs were isolated and identified by our laboratory as previously described [8]. At approximately 90% confluence, cells were harvested using 0.25% trypsin (Gibco) and passaged for expansion.

2.2 Plasmid construction

The human SF-1 cDNA (NM_004959.4) was synthesized by Genewiz and cloned into the plasmid pUC57-SF-1. The following primer sequences were used for amplification of SF-1 containing restriction sites. The forward and reverse primers were 5'-AATGCGGCCGCTAAATGG ACTATTCG TACGAC GAGG-3'and 5'-CGGG ATCCCGTCA AGTCTGCT TGGCT TA-3', respectively. The PCR product and the lentiviral vector pLVX-EF1α-MCS-IRES-EGFP (Clonetech) were both digested and then ligated. The product was transformed into DH5 cultured overnight for 16-20 hrs. Colonies were randomly picked and used as a template for PCR reaction, and positive clones were sequenced.

2.3 Lentiviral preparation

When 293T cells reached 80% confluency, transfected using PEI with lentiviral vector pLVX-EF1α-SF-1-IRES-EGFP and packaging vectors pMD2G and psPAX2. Lentiviral supernatant was collected 48 hours after transfection, and then concentrated using Amicon Ultra Centrifugal Filter Devices (Millipore). Filtered the supernatant through 0.45 μm polyethersulfone (PES) low protein-binding filters. The virus stocks should be aliquoted and stored at -80°C.

2.4 Optimization of lentiviral infection and MSCs differentiation

Human UC-MSCs were inoculated with above lentiviral particles at different multiplicity of infection (MOI =0, 10, 50, 100, and 200) in the presence of polybrene (10 μg/ml final concentration). The mRNA expression levels of SF-1 was identified by quantitative RT-PCR.

After determined the proportion of MOI, the infected MSCs were cultured in s DMEM-F12 supplemented with 10% (v/v) FBS, 100U/ml penicillin and 100g/ml streptomycin. Meanwhile, in order to differentiation mesenchymal stem cells into steroidogenic cells, the medium was supplemented with dbcAMP (SIGMA-ALDRICH) at different concentrations of 0 μM, 100 μM, 250 μM, 500 μM, 1000 μM. Medium was changed every 2 days for 7 days. On 7th day, the cells culture media of all groups were collected for measurement of testosterone and cortisol concentrations by ELISA. ELISA kits were usd according to manufacturer's instruction (CUSABIO, Wuhan, China). The OD value at 450 nm was detected using a Microplate Reader (BD)

2.5 Quantitative real-time PCR

We performed quantitative analysis of the mRNA expression levels of SF-1. Total RNA of human UC-MSCs after differentiation was isolated using of total RNA Kit I(Omega bio-tek) and then reverse transcribed into cDNA using the StarScript II First-strand cDNA Synthesis Kit (Genstar, Beijing, China) following the manufacturer's instructions. RealStar Green Fast Mixture (Genstar, Beijing, China) was used for Quantitative Reverse Transcription–Polymerase Chain Reaction(RT-PCR) analysis. The SF-1 qRT-PCR primers were as follows: Forward primer: 5′-TACCACTACGGACTGCTCACG-3′, Reverse primer: 5′-CTGCGTCTTGTCGATCTTGC-3′. Quantitative RT-PCR was conducted as previously described. The relative mRNA expression level were calculated using the $2^{-\Delta\Delta Ct}$ method.

2.6 Statistical analysis

All data were expressed as means ± SEM. Statistical evaluation were performed using an unpaired Student's t-test or one-way ANOVA for more than two groups. *P-values<0.05 were considered statistically significant.

3. Result and discussion

3.1 Recombinant lentiviral vector construction

The agarose gel electrophoresis of the PCR amplification showed that thecorrect size of target gene fragments (SF-1) (Figure 1A). The SF-1 and lentiviral vector were digested and ligated, and then transformed into DH5α. Randomly picked colonies and identified by PCR (Figure 1B). The result revealed that the SF-1 gene fragments were correctly subcloned into the lentiviral vector.

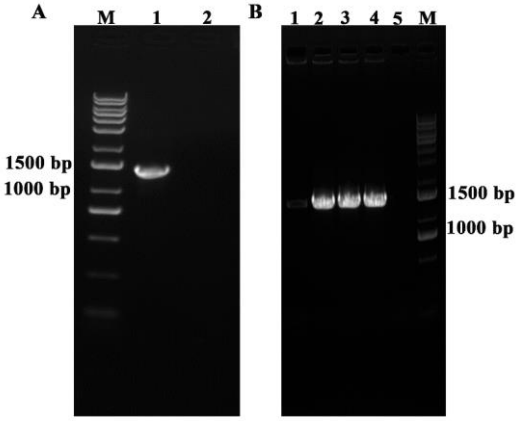

Figure 1. PCR of the SF-1 gene fragment and cloning into lentiviral vector. (A) Lane M: DNA marker; lane 1: PCR products of SF-1 gene fragment; lane 2: negative control (no DNA template). (B) Lane M: DNA marker; lane 1, 2, 3, 4: PCR products of different colonies of DH5α transformed with SF-1 lentiviral vector.

3.2 Lentiviral preparation

Lentiviral particles were collected from the supernatant of 293T cells at 24, 48, and 72 h following transfection. After 48 h transfection, EGFP expression was observed under a fluorescence microscope (Figure 2), and the result showed that the lentiviral vectors were successfully transferred into 293T cells.

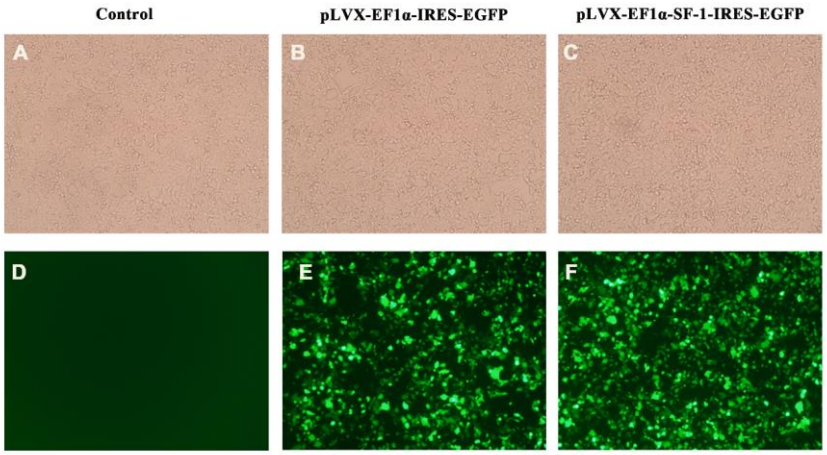

Figure 2. The EGFP fluorescent protein expression in 293T cells. Control: no vector control; pLVX-EF1α-IRES-EGFP: transfection with pLVX-EF1α--IRES-EGFP vector; pLVX-EF1α-SF-1-IRES-EGFP: transfection with pLVX-EF1α-SF-1-IRES-EGFP vector; all groups were observed at 48 h following transfection; A, B and C: white-light; D, E and F: fluorescence.

3.3 Testing different multiplicity of infection (MOI)

Human UC-MSCs were inoculated with different multiplicity of infection (MOI) of lentiviral particles (MOI=0, 10, 50, 100, and 200) in the presence of polybrene (10μg/ml final concentration). After 72 hours, we detected the mRNA expression levels of SF-1(Figure 3). The results showed that the mRNA expression levels of SF-1 reached the maximum when the MOI=100, so this MOI was selected as the follow-up infection.

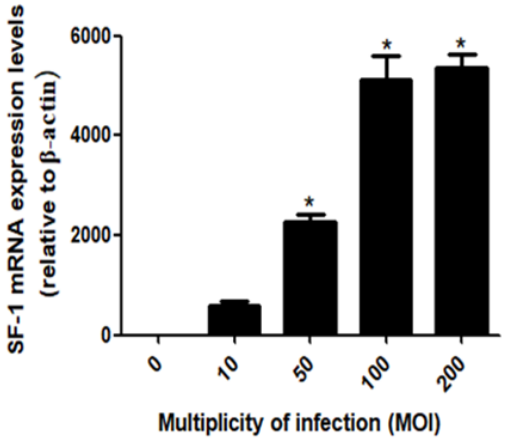

Figure 3. Quantitative RT-PCR detected the SF-1 mRNA expression levels. The 0, 10, 50, 100, 200μM: Human UC-MSCs were infected with lentiviral particles at different multiplicity of infection (MOI =0, 10, 50, 100, and 200). Relative mRNA expression levels were calibrated to β-actin. Values are means + SD (n=3). *p<0.01 compared to control.

3.4 Testing different concentrations of dbcAMP

In order to determine the optimal concentration of dbcAMP, we measured the testosterone (Figure 4A) and cortisol (Figure 4B) secreted after induction. The result showed that the differentiation of UC-MSCs treated with dbcAMP were significantly higher than that of the control group. The secretion of steroid hormones reached the maximum in the 500μM dbcAMP group.

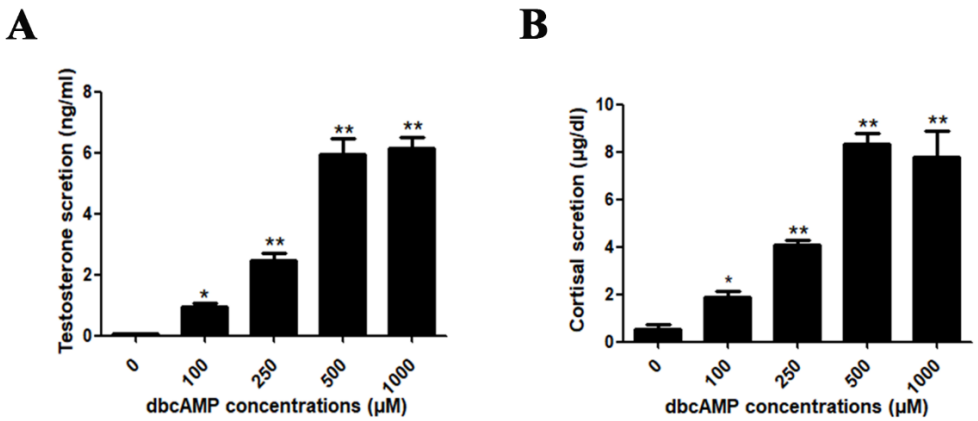

Figure 4. Measurement of secreted steroid hormones including testosterone (A) and cortisol (B). The 0 μM, 100 μM, 250 μM, 500 μM and 1000 μM: Differentiation of UC-MSCs was induced with the concentrations of 100 μM, 250 μM, 500 μM and 1000 μM dbcAMP respectively. The medium was collected from all groups and was measured by ELISA kits. The OD value at 450 nm was detected using an ELISA reader. Values are means + SD (n = 3). *p<0.05, **p<0.01 compared to control group (0 μM).

4. Conclusion

In this study, we successfully constructed a recombinant lentiviral vector containing SF-1, we found that multiplicity of infection (MOI) of 100 was good proportion of infection UC-MSCs with lentiviruse containing SF-1. After infection and differentiation, the results showed 500μM dbcAMP was good concentration for differentiation of mesenchymal stem cells into steroidogenic cells. This study provided a valuable method for differentiation of mesenchymal stem cells into steroidogenic cells.

Acknowledgments

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