Use of a new expression vector for the induction of bone marrow mesenchymal stem cells into steroidogenic cells

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Abstract

Male serum testosterone levels decreased with age, showing a drop in physical decline, autonomic dysfunction and sexual dysfunction and a series of corresponding clinical symptoms. SF-1, a member of the nuclear receptor superfamily, is a key transcriptional regulator of genes involved in the hypothalamic-pituitary-steroidogenic axis. With the development of cell therapy, people try to increase androgen secretion by transplanting testicular stromal cells and have achieved some clinical effects. In this study, we constructed a recombinant lentiviral plasmid and successfully transferred the lentiviral plasmid containing SF-1 gene into BM-MSCs. Finally, we tested the expression level of steroidogenic genes CYP17A1 and CYP11A1 and the secretion of steroid hormones. The results showed that the mRNA expression levels of CYP17A1, CYP11A1 and secretion of steroid hormones were significantly higher in the steroidogenic differentiation group than control group. Our study lays the foundation for using BM-MSCs to treat partial androgen deficiency of the aging male (PADAM).

Keywords

SF-1, stem cell, testosterone.

1. Introduction

Steroid generation factor-1 (officially known as NR5A1, but hereafter called SF-1) was originally discovered by Keith Parker and KenIchirou Morohashi in the early 1990s following the search for a master-regulator of steroidogenesis [1]. SF-1, a member of the nuclear receptor superfamily, is a key transcriptional regulator of genes involved in the hypothalamic-pituitary-steroidogenic axis [2,3]. In mammalian testis determination and differentiation, SF-1 is a positive regulator of SOX9 (Sry-box 9) and Anti-Müllerian Hormone (AMH) [4,5]. And It is also involved in the regulation of the expression of steroidogenic genes such as CYP11A1 [6], CYP17A1 [7], HSD3B2 [8] and CYP19A1 [9]. SF-1is expressed in the adrenal gland and reproductive axis during fetal development and in adult life, and variations in genes encoding these factors are associated with an ever expanding range of adrenal and reproductive conditions [10].

Male serum testosterone levels decreased with age, showing a drop in physical decline, autonomic dysfunction and sexual dysfunction and a series of corresponding clinical symptoms and signs, known as partial androgen deficiency of the aging male (PADAM) [11]. At present, testosterone replacement therapy is mainly used for the lack of androgen secretion, however, this method has a great side effect on the prostate. With the development of cell therapy, people try to treat androgen secretion by transplanting testicular stromal cells and have achieved some clinical effects. Stem cells are a group of self-renewing pluripotent cells that can differentiate into a variety of functional cells [12] and it may be used in the treatment of PADAM.

In this study, BM-MSCs were successfully induced and differentiated into steroidogenic cells by constructing recombinant lentiviral plasmid carrying SF-1 gene and transferring the lentivirus into

BM-MSCs. The results showed that differentiated BM-MSC could secrete more testosterone than the control group, which is of great significance for the treatment of PADAM in the future.

2. Methods

2.1 Experimental materials

Human SF-1 gene was isolated from plasmid pUC57-SF-1; BstB I and BamH I enzymes were purchased from Thermofisher; The lentiviral plasmid pLVX-EF1α-AcGFP1-N1was purchased from Takara Biology; Lentiviral packaging plasmids psPax2 and pMD2G were purchased from Addgene; Transfection Reagents Polyetherimide (PEI) was purchased from Polyscience.

2.2 The construction of recombinant lentiviral vector

The SF-1 gene with two restriction enzyme (BstB I/BamH I) recognition sequences was obtained from plasmid pUC57-SF-1 by PCR. The forward and reverse primers were 5'-CGCTTCGAATTA TGG ACTATTCGTACGACG A-3' and 5'-TAAGGATCCAGAGTCTGCTTGGCTTGCAGCA-3', respectively. To prepare the recombinant plasmid DNA for cell transfection, the plasmid pLVX-EF1α-AcGFP1-N1was double digested with BstB I and BamH I at 37 °C for 1 hour. Then, the PCR-amplified fragment SF-1was ligated to the plasmid after digestion using DNA ligase. Recombinant lentiviral plasmid was verified by restriction enzyme digestion.

2.3 Cells culture and transfection

HEK 293T cells and human BM-MSCs were cultured in DMEM and DMEM-F12 respectively, supplemented with 10% FCS (Gibco, USA). Cells were maintained under standard cell culture conditions (humidified air-5 % CO_2, 37 °C). At about 90% confluence, cells were harvested using 0.25% trypsin (Invitrogen) and passaged for amplification. Cells in logarithmic growth phase were used for all experiments.

The lentivirus containing SF-1 was packaged and expanded with the aid of PEI by co-transferring the pLVX-EF1α-SF-1-AcGFP1-N1 vector and two plasmids psPAX2 and pMD2G into 293T cells. Lentiviral particles were collected from 293T cells supernatant at 24, 48, 72 and 96 h after transfection, respectively. Then, concentrated using Amicon Ultra Centrifugal Filter Devices (Millipore), the virus is stored at -80 °C for later use. At the same time, plasmid pLVX-EF1α-AcGFP1-N1 group and normal culture group were set as control.

Human BM-MSCs were cultured in 6-well plates at a density of 5×10^5 cells / well overnight. The next day, lentivirus pLVX-EF1α-SF-1-AcGFP1-N1 and pLVX-EF1α-AcGFP1-N1 were each added to BM-MSCs medium at an MOI of 100, respectively. Then, the cells were cultured in steroidogenic differentiation media containing DMEM-F12 supplemented with 10% (v / v) FBS, 500μM dibutyryl-cAMP (dbcAMP), 100 U /ml penicillin and 100 μg / ml streptomycin. The medium was changed every 2 days for a total of 7 days. Meanwhile, a normal culture MSCs group as a negative control was set. Fluorescent microscopy was used to observe fluorescent expression on day 4 after lentiviral transfection.

2.4 Real-time RT-PCR analysis

The mRNA expression levels of CYP17A1 and CY11A1 were detected by Real-time RT-PCR. The HiPure Total RNA Micro Kit (Magen, Guangzhou, China) was used to extract total RNA and the RNA was reverse transcribed into positive cDNA using the StarScript II First-strand cDNA Synthesis Kit (Genstar, Beijing, China). RealStar Green Fast Mixture (Genstar, Beijing, China) was used for quantitative (real-time) PCR analysis. The PCR amplified mRNA was quantified and the results were normalized against β-actin expression. The $2^{-\Delta\Delta}$Ct method was used to calculate the relative mRNA expression level.

Primers used were as follow:

	Forward (5′-3′)	Reverse (5′-3′)
CYP17A1	CATGCTGGACACACTGATGCAA	CGTAGAGCTTCTTCTTCACCTGA
CYP11A1	GCGGGCTCCGGAAATTACTC	CTGGTAGATGGCATCAATGAATCG
β-actin	CGTGGACATCCGCAAAG	AAGGTGGACAGCGAGGC

2.5 Measurements of steroid hormones

7 days after transfection, the stem cell-differentiated group and the undifferentiated cell-conditioned medium were collected. Then, test the content of testosterone and cortisol in the two groups of cell culture medium by ELISA kit, according to manufacturer's instruction (Diagnostics Biochem Canada Inc, London, Ontario, Canada). The OD value at 450 nm was measured using a microplate reader.

2.6 Statistical analysis

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

3. Results and discussion

3.1 The construction of recombinant lentiviral vector

First, we cloned the SF-1 gene containing the double enzyme sites (BstB I / BamH I) from plasmid pUC57-SF-1. The result of agarose gel electrophoresis showed that the target gene(SF-1) band after PCR amplification was correct (Figure 1A). Next, we ligated the gene to the vector pLVX-EF1α-AcGFP1-N1. Then, we verified the construction of the recombinant plasmid vector by double digestion (Figure. 1B), the results of agarose gel electrophoresis showed that we successfully ligated the gene SF-1 to the lentiviral plasmid.

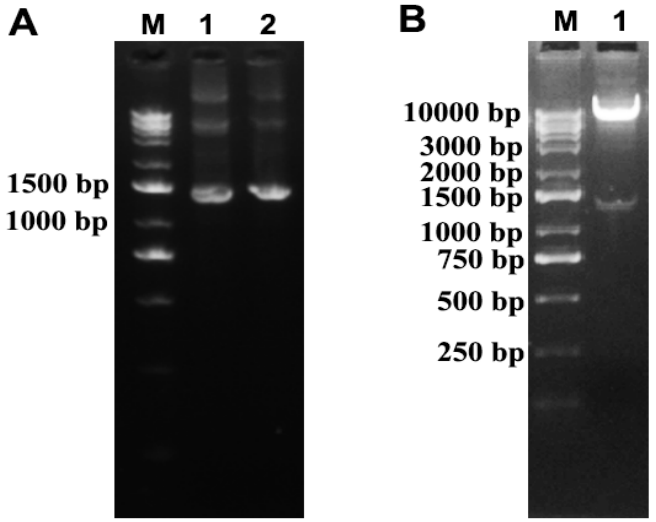

Figure 1. PCR amplification of SF-1 gene and construction of a recombinant lentiviral vector. (A) Line M: DNA marker; line 1 and 2: two parallel experiments of PCR products of the SF-1 gene. (B) Line M: DNA marker; line 1: digested DNA products of recombination lentiviral vector pLVX-EF1α-SF-1-AcGFP1-N1 by enzymes BstB I and BamH I.

3.2 Lentivirus preparation

A total of three groups were set, (1) control group: normal cultured 293T cells group; (2) pLVX-EF1α-AcGFP1-N1 group: add pLVX-EF1α-AcGFP1-N1 vector and two plasmids psPAX2 and pMD2G into 293T cells group; (3) pLVX-EF1α-SF-1-AcGFP1-N1 group: add pLVX-EF1α-SF-1-AcGFP1-N1 vector and two plasmids psPAX2 and pMD2G into 293T cells group. Lentiviral particles were collected from 293T cells supernatant at 24, 48, 72 and 96 h after transfection, respectively.

During this time, fluorescence detection was performed 48 h after transfection to verify the plasmid transferred 293T cells (Figure 2). Both pLVX-AcGFP1 group and pLVX-SF-1-AcGFP1 group were detected the expression of the fluorescent protein AcGFP1, the results showed that the lentiviral plasmid was successfully transfected into 293T cells.

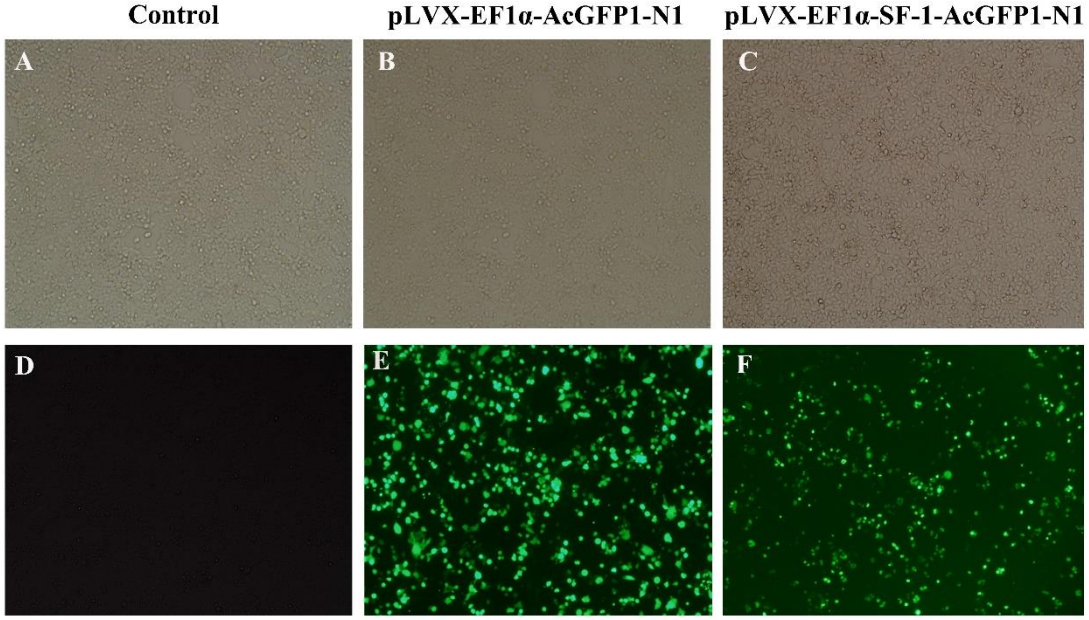

Figure2.Expression of the fluorescent protein AcGFP1 in 293T Cells. Control group(A, D, no vector), pLVX-EF1α-AcGFP1-N1 group(B, E) and pLVX-EF1α-SF-1-AcGFP1-N1 group(C, F) were observed at 48 h following transfection; (A) and (D) were observed in the same field for the white light and fluorescent view, respectively; (B) and (E)were respectively observed in the same field of view of white light and fluorescent lamp; (C) and (F) were observed in the same field of view with white light and fluorescent lamp, respectively.

3.3 Transfection and differentiation of BM-MSCs

The lentivirus particles pLVX-EF1α-AcGFP1-N1 and pLVX-EF1α-SF-1-AcGFP1-N1 were added to BM-MSCs respectively at a MOI of 100. The transfected BM-MSCs were cultured in steroidogenic differentiation medium and fluorescence was detected on the fourth day after lentiviral transfection (Figure 3). As a result, fluorescence signals were detected in the pLVX-EF1α-AcGFP1-N1 group and the pLVX-EF1α-SF-1-AcGFP1-N1 group, whereas no fluorescence was detected in the normal group. The fluorescent signals indicate that lentivirus particles were successfully transfered into BM-MSCs.

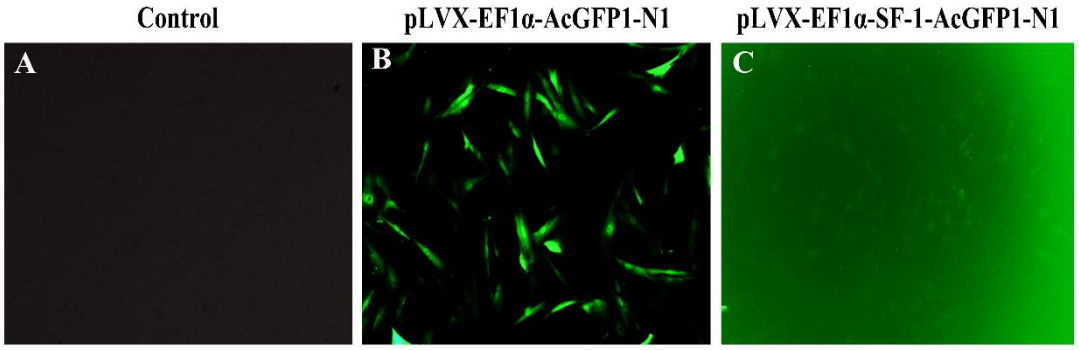

Figure3. Expression of the fluorescent protein AcGFP1 in human BM-MSCs. Control group (A, no vector), pLVX-EF1α-AcGFP1-N1 group (B) and pLVX-EF1α-SF-1-AcGFP1-N1 group (C) were observed on the fourth day following transfection.

3.4 Testing mRNA expression

CYP17A1 and CYP11A1 are two genes closely related to hormone synthesis. Therefore, the mRNA expression levels of these two genes was detected by q-PCR to verify the effect of SF-1 gene on BM-MSCs hormone. The results of q-PCR showed that the mRNA expression of CYP17A1 and CYP11A1 of pLVX-EF1α-AcGFP1-N1 group was significantly higher than that of the control group (Figure 4).

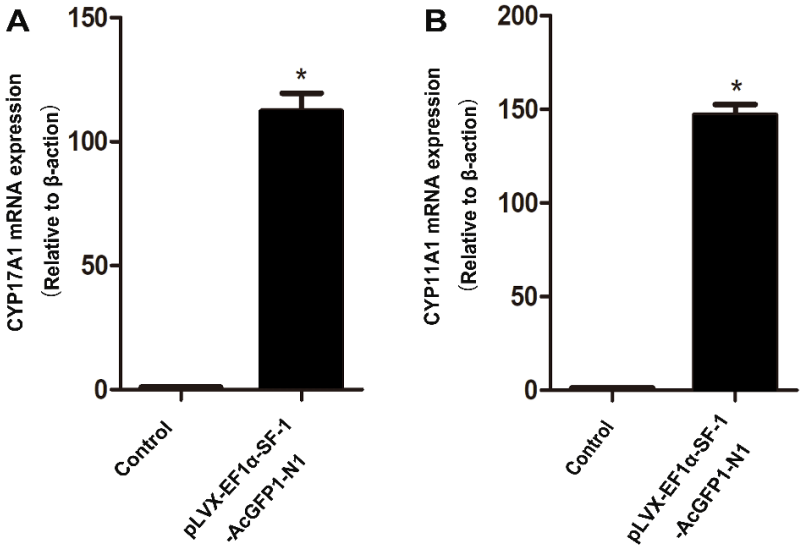

Figure4. Analysis of the mRNA expression of steroidogenic genes (CY17A1 andCYP11A1) by real-time RT-PCR. The mRNA expression levels of the CYP17A1gene (A) and CY11A1 gene (B) were detected by RT-PCR. Relative mRNA expression levels were calibrated to β-actin. Values are means + SD (n=3). *p<0.05 compared to control group.

3.5 Measurements of steroid hormones

After transfection of lentivirus, the expression of steroid hormone after BM-MSCs differentiation was detected. We collected the cell culture supernatant of BM-MSCs on the 7th day and then tested the contents of testosterone and cortisol. The results showed that testosterone and cortisol in pLVX-EF1α-SF-1-AcGFP1-N1 group were significantly higher than those in control group (Figure 5).

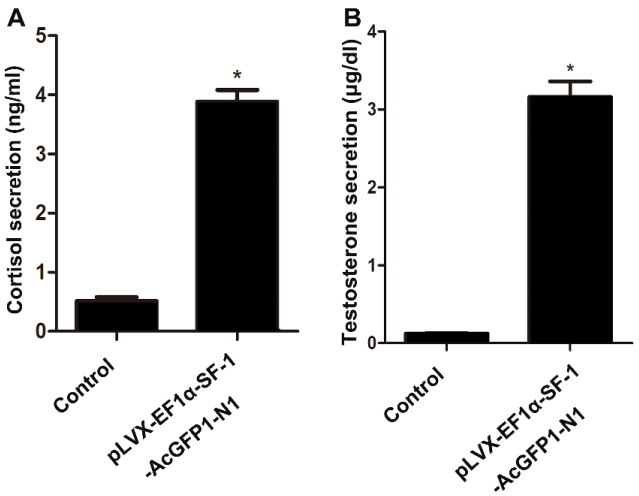

Figure 5. Detection of steroid hormone secretion. Secretion of cortisol (A) and testosterone (B) in medium were detected by ELISA after BM-MSCs were infected with lentivirus containing SF-1 cDNA for 7 days and were induced to differentiate into steroidogenic cells in the presence of cAMP. Control: no lentiviral vector. Values are means ± SD (n = 3). *P < 0.05 relative to respective control.

4. Conclusion

In this study, the SF-1gene was successfully transferred into BM-MSCs and resulted in the high expression of testosterone and cortisol. The results showed that we successfully induced BM-MSCs to differentiate into steroidogenic cells. Our study lays the foundation for using BM-MSCs to treat PADAM.

Acknowledgments

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