Differentiation of umbilical cord mesenchymal stem cells into Leydig cells using a new lentiviral vector

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Abstract

It is common to see that male serum testosterone levels are low, or not detectable, which is caused by a variety of factors such as drug, heavy metals and age. Steroid generation factor-1 (SF-1, also known as Ad4BP or NR5A1), a member of the nucleus receptors superfamily, plays a key role in pituitary development, steroid production process and adrenal carcinogenesis. SF-1 participates in the regulation of the expression of a variety of genes including CYP11A1、CYP17A1 and 3β-HSD. Now, stem cell transplantation has emerged as a promising, alter¬native treatment for testosterone supplementation and has many advantages. In this study, we constructed recombinant lentiviral vector by Gateway technology. An optimal lentiviruse multiplicity of infection (MOI) of 100 was determined. The results showed that SF-1 expression with genetically modified umbilical cord mesenchymal stem cells (UC-MSCs) compared to UC-MSCs control culture. The mRNA expression levels of 3β-HSD, CYP11A1 and CYP17A1 and contents of steroid hormones were significantly higher in the steroidogenic differentiation group than control group. Our study has laid a significant foundation for using a human mesenchymal stem cells of overexpression of SF-1 as a treatment tool for low testosterone level diseases.

Keywords

SF-1; testosterone; stem cell transplantation; lentiviral vector.

1. Introduction

Steroid generation factor-1 (SF-1, also known as Ad4BP or NR5A1), a member of the nucleus receptor superfamily, plays a key role in pituitary development, steroid production process and adrenal carcinogenesis [1-3]. Besides, owing to having different structure features compared to other nucleus receptors, SF-1 is regarded as an orphan nuclear receptor gene. SF-1 participates in the regulation of the expression of a variety of genes such as CYP11A1 [4]、CYP17A1 [5] and 3β-HSD [6], müllerian inhibitory substances [7], luteinizing hormone β subunit [8]. According to previous report, SF-1 gene completely knockout can lead to no formation of adrenal glands and gonads, showing a reversal of male to female sex, pituitary function damage of pituitary gonadotropin secretion cell and nucleus loss of hypothalamic ventral [8-9]. Therefore, SF-1 plays a significance impact on human health.

It is common to see that male serum testosterone levels are low or not detectable, which is caused by a variety of factors such as drug, heavy metals and age [10-11]. That patients were not only hypogonadism but also sufferred from the disease [12]. Testosterone supplementation have many side-effects and can cause many health problems. Cell transplantation could be a future direction for treatment of this disease. Stem cell transplantation has emerged as a promising, alternative treatment

for testosterone supplementation. There are many advantages of the available stem cells that have the advantages for self-renewal, a multilineage differentiation potential, low immunogenicity, immunosuppressive properties and low tumorigenicity [13-14].

This study mainly focused on the construction of recombinant lentiviral vector by Gateway technology [15-17]. The results showed that SF-1 expression with genetically modified UC-MSCs compared to UC-MSCs control. Our study has laid a significant foundation for using a stable human mesenchymal stem cell line of overexpression of SF-1 as a treatment tool for the low testosterone level diseases.

2. Materials and methods

2.1 Materials

The BP and LR Clonase Enzyme Mixes were purchased from Thermofisher. The human SF-1 gene was isolated from the plasmid pUC57-SF-1. The lentiviral plasmid pCDH-CMV-Venus, donor vector pDONR201 and lentiviral packaging plasmids psPax2 and pMD2.G were purchased from Addgene. The transfection reagent polyetherimide (PEI) was purchased from Polyscience.

2.2 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 ℃ incubator, saturated humidity and 5% CO2. At approximately 90% confluence, cells were harvested using 0.25% trypsin (Invitrogen) and passaged for expansion. Cells in the logarithmic phase of growth were used for all experiments.

2.3 Recombinant lentiviral vector construction

The Full-length SF-1 cDNA was cloned into the pCDH-CMV-Venus by Gateway technology. The following primer sequences were used for amplification of SF-1 cotaining attB sites. The forward and reverse primers were 5'-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC AAT GGA CTA TTC GTA CGA CGA GGA-3' and 5'- GGG GAC CAC TTT GTA CAA GAA AGC TGG GTT CAA GTC TGC TTG GCT TGC A-3', respectively. Perform a BP recombination reaction with attB-SF-1 PCR product and the donor vector pDONR201 to generate an entry clone. Perform an LR recombination reaction with above entry clone and the lentiviral vector pCDH-CMV-Venus of choice to generate the recombination lentiviral vector pCDH-CMV-Venus/SF-1. The entire SF-1 cDNA sequence was verified by DNA sequencing and recombinant lentiviral vector.

2.4 Lentiviral preparation and infection of UC-MSCs

Lentiviruses containing SF-1 were packaged and amplified by co-transferring pCDH- CMV-Venus/SF-1 vector, together with two plasmids psPAX2 and pMD2G into 293T cells with the help of PEI. Lentiviral particles were collected in 24, 48, and 72 h after transfection from the supernatant of 293-T cells, and then concentrated using Amicon Ultra Centrifugal Filter Devices (Millipore), the virus solution was stored at -80 ℃ until use. Meanwhile, set up a normal culture MSCs group as a negative control.

Human UC-MSCs were cultured at a density of 5×10^5 cells/well overnight in a six-well plates. The next day, cells were infected with lentiviruses at multiplicity of infection (MOI) of 20, 50, 100, 200 or 300 in the presence of polybrene (10μg/ml final concentration). Venus expression was observed under a fluorescence microscope on the 4th day. Through the conparison of different concentrations of MOI, an optimal MOI of 100 was selected for subsequent experiments. Lentiviruses pCDH- CMV-Venus/SF-1 and pCDH-CMV-Venus were each added separately into the culture medium at an MOI of 100. Transfections were performed for 24 h at 37˚C, and then cells were cultured in steroidogenic differentiation medium containing DMEM-F12 supplemented with 10% (v/v) FBS, 500μM bdcAMP, 100U/ml penicillin and 100g/ml streptomycin. Medium was changed every 2 days for 7 days. Meanwhile, set up a normal culture MSCs group as a negative control.

2.5 Reverse transcription-quantitative (real-time) PCR (RT-qPCR)

RT-qPCR was performed to analyze mRNA expression levels of 3β-HSD, CYP11A1 and CY17A1. HiPure Total RNA Micro Kit (Magen, Guangzhou, China) was used to extract the total RNA 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 (real-time) PCR analysis.

The primer sequences used in this experiment were:

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

Real-time RT-PCR was conducted as previously described, PCR conditions including 95 ℃ for 15s, 60 ℃ for 15 s and 72 ℃ for 30 s for 40 cycles usingThe Bio-Rad CFX connect Real-Time system (Bio-Rad Laboratories).The PCR-amplified cDNA 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.

2.6 Measurements of steroid hormones

The cells culture media of both differentiated MSCs group and undifferentiated MSCs group as negation control were collected. Then, steroid hormones including testosterone and cortisol secreted from the two groups were measured using ELISA kits, according to manufacturer's instruction (Diagnostics Biochem Canada Inc, London, Ontario, Canada). The OD value at 450 nm was detected using an ELISA reader (BD).

2.7 Statistical analysis

Data are expressed as means ± SEM. And data were analyzed using GraphPad Prism 6.0 software. Statistical comparisons were performed using Student's t-test. **P*-values<0.01 were considered statistically significant.

3. Result and discussion

3.1 Recombinant lentiviral vector construction

First, we cloned the SF-1 gene containing attB sites from pDONR201 by PCR. The agarose gel electrophoresis of the PCR amplification showed that thecorrect size of target gene fragments (SF-1) (Figure 1A). Next, the recombination lentiviral vector pCDH-CMV-Venus/SF-1was generated by Gateway technology. The enzyme-digested DNA fragments of recombination lentiviral vector pCDH-CMV-Venus/SF-1 by BSrGI (Figure 1B) and the sequencing of SF-1gene fragments were performed. The result revealed that the SF-1 gene fragments were correctly subcloned into the vector. The lentiviral vectors were successfully constructed. This is worthy mentioning that it is faster and more efficient approach to construct recombination vector using Gateway technology.

3.2 Lentivirus preparation

There are three groups, (1) control group: normal culture 293T cells as a negative control; (2) pCDH-CMV-Venus group: co-transferring pCDH-CMV-Venus vector, together with two plasmids psPAX2 and pMD2G into 293T cells with the help of PEI; (3) pCDH-CMV-Venus/SF-1group: co-transferring pCDH-CMV-Venus/SF-1 vector, together with two plasmids psPAX2 and pMD2G into 293T cells with the help of PEI. Lentiviral particles were collected from the supernatant of 293T cells at 24, 48, and 72 h following transfection. Venus expression was observed under a fluorescence microscope after 48 h transfection (Figure 2). Venus fluorescence was observed in both pCDH group and pCDH-

SF-1 group, and the result showed that the lentiviral vectors were successfully transferred into 293T cells.

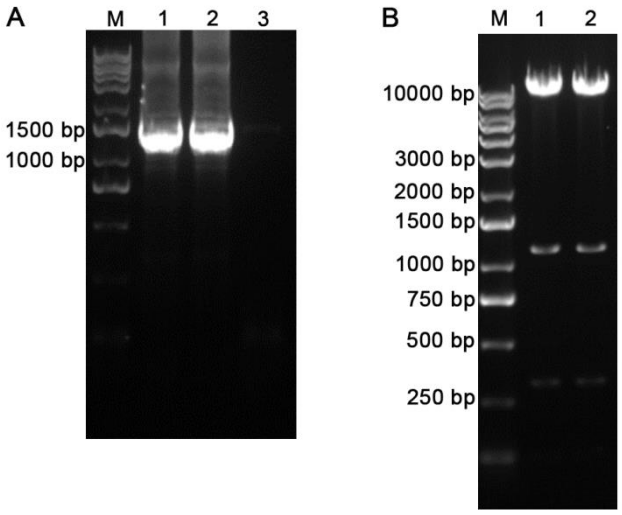

Figure 1. PCR of the target gene fragments. (A) Lane M is DNA marker; lane 1 and 2 are PCR products of SF-1 gene fragments containing attB sites from pDONR201; lane 3 is negation control (no templates). (B) Lane M is marker; lane 1 and 2 are enzyme-digested DNA products of recombination lentiviral vector pCDH-CMV-Venus/SF-1 by BSrGI.

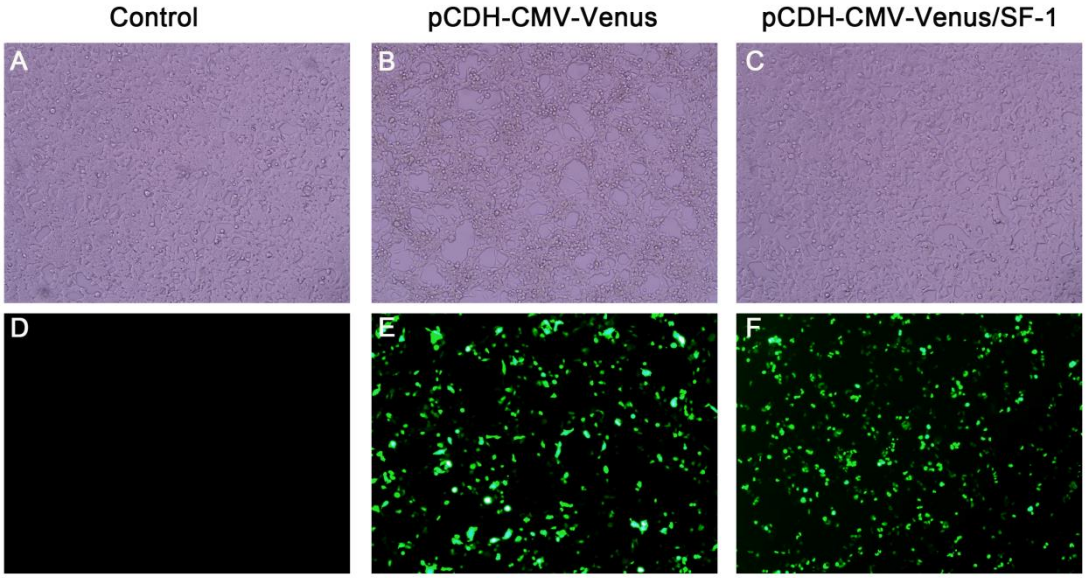

Figure 2. The Lv-Venus fluorescent protein expression in 293T cells. Control group: normal culture 293T cells as a negative control; pCDH-CMV-Venus group: co-transferring pCDH-CMV-Venus vector, together with two plasmids psPAX2 and pMD2G into 293T cells with the help of PEI; pCDH-CMV-Venus/SF-1 group: co-transferring pCDH-CMV-Venus/SF-1 vector, together with two plasmids psPAX2 and pMD2G into 293T cells with the help of PEI; Control group (A, D), pCDH-CMV-Venus group (B, E) and pCDH-CMV-Venus/SF-1 group (C, F) were observed at 48 h following transfection; A, B and C were observed with white-light; D, E and F were observed with fluorescent lamp

3.3 UC-MSCs transfection and differentiation

Lentiviruses pCDH-CMV-Venus/SF-1 and pCDH-CMV-Venus were each added separately into the culture medium at a MOI of 100. Transfections were performed, and then cells were cultured in steroidogenic differentiation medium. Control group, pCDH-CMV-Venus group and pCDH- CMV-Venus/SF-1 group were observed at the fourth day following transfection (Figure 3). The

fluorescence signal was not observed in normal group, while fluorescence signal was observed in both pCDH-CMV-Venus/SF-1group and pCDH-CMV-Venus group. However, fluorescence signal showed difference in its distribution inside the cells. Fluorescence signal was observed in the whole cell in pCDH-CMV-Venus group, while fluorescence signal was only observed in cell nucleus in pCDH-CMV-Venus/SF-1 group. Because SF-1 is a nucleus receptor, SF-1 could carry the Venus (fluorescent protein) into cell nucleus since Venus gene and SF-1 gene were expressed as a fusion protein.

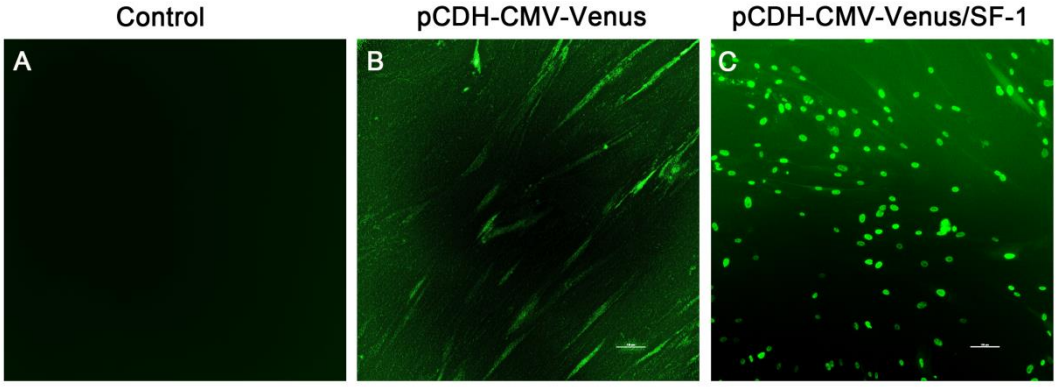

Figure 3. The Lv-Venus fluorescent protein expression in human UC-MSCs. Control group: normal culture human UC-MSCs as a negative control; pCDH-CMV-Venus group: co-transferring pCDH-CMV-Venus vector, together with two plasmids psPAX2 and pMD2G into UC-MSCs with the help of PEI; pCDH-CMV-Venus/SF-1group: co-transferring pCDH-CMV-Venus/SF-1 vector, together with two plasmids psPAX2 and pMD2G into UC-MSCs with the help of PEI. Control group (A), pCDH-CMV-Venus group (B) and pCDH-CMV-Venus/SF-1 group (C) were observed at the fourth day following transfection; A,B and C were observed with fluorescent lamp .

3.4 mRNA expression

There are some key genes involved in synthesis of hormones such as 3β-HSD, CYP11A1 and CYP17A1. To determine the differentiation of MCSs by lentivirus containing SF-1 induction, the mRNA expression levels of 3β-HSD, CYP11A1and CYP17A1 were detected by RT-qPCR. The result indicated that the mRNA expression levels of 3β-HSD, CYP11A1 and CYP17A1 in the pCDH-SF-1 group were markedly higher than control group (Figure 4).

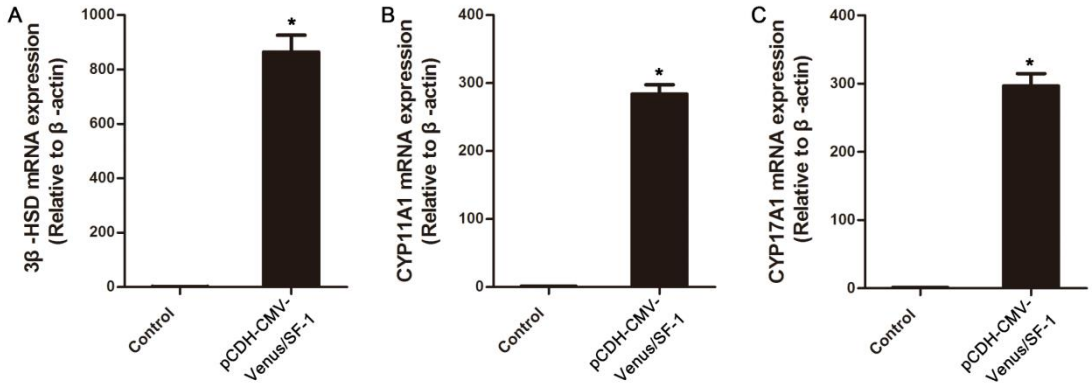

Figure 4. The gene expression by SF-1 regulation. RT-qPCR detected the mRNA expression levels of the 3β-HSD gene (A), CYP11A1 gene (B) and CY17A1 gene (C). Control group: normal cultureMSCs is used as a negative control; pCDH-CMV-Venus/SF-1group: MSCs were infected with pCDH-CMV-Venus/SF-1 at multiplicity of infection (MOI) of 100 in the presence of polybrene (10μg/ml final concentration). Relative mRNA expression levels were calibrated to β-actin. Values are means + SD. *$p<0.05$ compared to control group; Each of the experiments was repeated 3 times (n=3).

3.5 Measurements of steroid hormones

In order to detect the differentiation potential of UC-MSCs by lentivirus transfection, we collected the culture medium after human UC-MSCs were infected with lentivirus containing SF-1 cDNA for 7 days. Then testosterone and cortisol secreted by infected UC-MSCs were measured. The two hormones were detected in culture medium of cells infected with lentivirus containing SF-1, but not detected in culture medium of normal culture cells as negative control. Hormone levels of pCDH-SF-1 were significantly higher than control (Figure 5). The results of measurements of steroid hormones was consistant with the results of mRNA expression of steroidogenic genes.

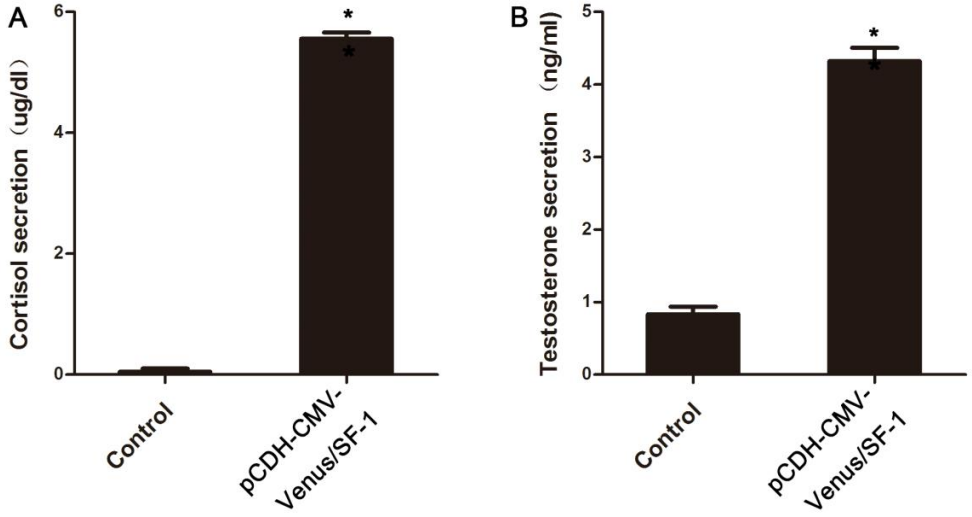

Figure 5. Measurement of steroid hormones including testosterone and cortisol secreted. Control group: normal culture MSCs is used as a negative control; pCDH-CMV-Venus/SF-1group: MSCs were infected with pCDH-CMV-Venus/SF-1 at multiplicity of infection (MOI) of 100 in the presence of polybrene (10μg/ml final concentration). The medium solution were collected from the two groups and were measured by ELISA. The OD value at 450 nm was detected using an ELISA reader. Values are means + SD (n = 3). *$p<0.05$ compared to control group.

4. Conclusion

We constructed recombinant lentiviral vector using a novel vector by Gateway technology. An optimal lentivirus multiplicity of infection (MOI) of 100 was determined. The result showed that SF-1 expression with genetically modified MSCs compared to MSCs control culture. The mRNA expression levels of 3β-HSD, CYP11A1 and CYP17A1 and contents of steroid hormones were significantly higher in the differentiation group than the control group. Our study has laid a significant foundation for using a human mesenchymal stem cells of overexpression of SF-1 as a treatment tool for low testosterone level diseases.

Acknowledgments

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