Original article

Over-expression of $VEGF_{165}$ in the adipose tissue-derived stem cells via the lentiviral vector

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Keywords: *adipose tissue-derived stem cells; $VEGF_{165}$; lentivirus; over-expression*

Background Many researchers studied the possibility of using stem cells as gene therapeutic vector. But few related reports on the adipose tissue-derived stem cells (ADSCs) are available. Therefore we intended to construct a lentiviral $VEGF_{165}$ expression vector and then infect the ADSCs to produce therapeutic seed cells.

Methods EHS1001-68950485313912 clone was mutated by PCR method to produce consensus fragment of $VEGF_{165}$ transcript (NM_001025368). Lentivirus was enveloped with pGC-FU, pHelper 1.0 and pHelper 2.0 plasmids in 293T cells. And then the ADSCs (multiplicity of infection=20) were transfected with the vectors after titer determination. Stable expression of $VEGF_{165}$ in ADSCs was confirmed by immunofluorescence staining, enzyme-linked immunosorbent assay (ELISA) and Western blotting analysis.

Results DNA sequencing and 293T transfection verified $VEGF_{165}$ was linked to the GFP fused vector. The virus titer is up to 2×10^8 determined by quantitative PCR. $VEGF_{165}$ transduced cells could show green fluorescence confirmed by immunofluorescence staining (almost 95%). ELISA analyses could detect out the density of VEGF was 850.86–1202.13 pg/ml (mean (923.00±31.22) pg/ml) in the supernatant of $VEGF_{165}$-transduced cells but not detected in the GFP-transduced cells (P <0.001) and the Western blotting analyses also confirmed $VEGF_{165}$ expression in $VEGF_{165}$-transduced cells.

Conclusions The $VEGF_{165}$ over-expression ADSCs were obtained and may be used as a cell therapeutic tool and may be applied for vascular regeneration, especially in the treatment of erectile dysfunction.

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Adipose tissue-derived stem cells (ADSCs) is a hotspot in stem cell therapeutic research recently, and multi-direction division of ADSCs is not affected by genetic modification and posses high proliferation rate, gene transduction susceptibility *in vitro* and long-term expression endurance. Hence it is the optimal gene therapeutic vectors.[1-6] Introduction of exogenous gene into stem cells is a vital procedure and relied on transduction vector on large extent. Adenovirus is acknowledged the most widely used media for high infect efficiency, but its deficiency is obvious: foreign genes are not integrated thus expression was transient and possessed cell toxicity. Letivirus, derived from HIV-1 virus overwhelmed adenovirus with its DNA integration mechanism triggered by Rev.[7] In addition, coat protein of HSV dramatically alleviats immunogenicity.[8,9] Lentiviral vectors introduce permanent and high expression in ADSCs to produce seed cells for damaged replace and long term treatment which overwhelm traditional therapeutic vectors by its endurance and efficiency.[10]

Vascular endothelial growth factor (VEGF) plays a critical role in vascular regenesis.[11-13] There are 5 subtypes of VEGF family and $VEGF_{165}$ is most active and widely distributed.[11] Nevertheless, for its short half life *in vivo*, drugs targeting at $VEGF_{165}$ are costliness and difficult to supply. Consequently, the clinical application of these drugs is considerable hindered. Attribute to the development of molecular biology and genetic modification, marvelous progress in gene therapy improves the research in vascular regenesis of ischemia tissue.[14] Additionally, the VEGF signaling system would work less well in diabetic erectile dysfunction (DMED) penile tissues as a result of the reduced expression of VEGF and its two receptors. The decrease of VEGF would lead to diminished endothelial production of NO and apoptosis-related erectile tissue damage. The abnormalities of the VEGF signaling system in the penis may lead to the pathophysiology of diabetic erectile dysfunction (ED).[15] And the forthcoming therapy was not fully effective.[16,17]

Therefore, we herein intend to construct a lentiviral

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vector which can over-express $VEGF_{165}$ and transfect into rat ADSCs to obtain the therapeutic cells for potentially use in the DMED.

METHODS

Vectors and cells

EHS1001-6895048 5313912 clone was purchased from OPEN BIOSYSTEMS. Lentiviral expression vector pGC-FU, and envelope helper vectors pHelper 1.0 and pHelper 2.0 were produced by Genechem (Shanghai, China). *E. Coli* DH5a used in subclone, 293T cells used in envelope were conserved by Genechem. And we have previously described the isolation of rat ADSCs.[18] Rat tissues were obtained with approval from our Institutional Animal Care and Use Committee.

Reagents

AgeI was purchased from New England Biolabs (USA), Taq polymerase and Sybr Green Master were products of TAKARA (Dalian, China) and plasmid extraction kit supplied by QIAGEN (USA). Lipo2000 kit used in tansfection and TRIZOL used in RNA extraction were produced by Invitrogen (USA). Antibodies were from Santa Cruz (Canada). Western blotting adopted ECL kit, Amersham (USA). M-MLV reverse transcriptase produced by Promega (USA) were used in qPCR analysis.

Vector construction

EHS1001-6895048 5313912 clone was mutated by PCR method to produce consensus fragment of $VEGF_{165}$ transcript (NM_001025368). Primers $VEGF_{165}$-N-R (5′-CTGACGGACAGACAGACAGACACCGCCCCCA GCCCCAGCTACCACCTC-3′), $VEGF_{165}$-N-F(5′-CTG-ACGGACAGACAGACAGACACCGCCCCCAGCCCC AGCTACCACCTC-3′), $VEGF_{165}$-N2-F (5′-CCTCCC-CGGCCGGCGGCGGAC-3′), $VEGF_{165}$-N2-R (5′-G-AGTCTGTGTTTTTGCAGGAACATTTACACGTC-3′), $VEGF_{165}$-C1-F (5′-GTTCCTGCAAAAACACAGACT-CGCGTTGCAAG-3′), were used to modify sequence and obtain 94 bp, 1002 bp and 105 bp fragments. The mixture of mutated fragments was amplified by $VEGF_{165}$-AgeI-F (5′-GAGGATCCCCGGGTACCGGTCGCCACCATGCT GACGGACAGACAGACAGAC-3′) and $VEGF_{165}$-AgeI-R (5′-TCACCATGGTGGCGACCGGCCGCCTCGGCTTG TCACATC-3′) by PCR to produce $VEGF_{165}$ cDNA clone. Entire fragment was digested by *AgeI* and link to pGCFU vector. DH5a transform ant was examined by PCR test using $VEGF_{165}$-SEQF (5′-GTGGTGAAGTTCATGGAT-G-3′) and EGFP-N-R (5′-CGTCGCCGTCCAGCTCGA-CCAG-3′). Positive clones were extracted and sequenced.

$VEGF_{165}$ expression in 293T cell

Construction verified by sequencing was designated pGC-FU-$VEGF_{165}$. Then 293T cells were transfected with purified plasmid of pGCFU-$VEGF_{165}$ and pGC-FU which was harbouring a 3*tag fragment. The transfection followed the protocol of Lipo2000 kit and non-treatment cells as control. For Western blotting approximately 2×10^6 cells were harvested and prepared total protein. Samples separated by polyacrylamide gel electrophoresis (PAGE), were blotted to polyvinylidene fluoride (PVDF) under 200 mA, 4°C for 2 hours. First antibody for GFP was added in hybridization solution and incubated for 2 hours. Then wash membrane with Tris-buffered saline containing 0.1% Tween-20 (TBST) and incubated with second antibody. Blot visualized by enhanced chemiluminescence (ECL) blotting system.

Lentivirus envelope and titer determination

Purified pGC-FU-$VEGF_{165}$, pHelper 1.0 and pHelper 2.0 plasmid transfected 293T cells were used to produce lentivirus. The supernatant of cell culture were harvested after 48-hour cultures, debris was dumped by 4000 *g* centrifugation for 10 minutes at 4°C. Then virus was purified with plus-20 kit (Millipore, USA) and storage at –80°C. The production of control virus enveloping pGC-FU followed the same protocol, meanwhile. For titer determination, virus was diluted 10 times in serum free culture consistently to prepare 8 concentrations of solution. 293T culture (App. 500 μl) planted in 24 wells plate were added with 10 μl virus solution. Cells were harvested after 4 days for qPCR test. Total RNA was extracted with TRIZOL and cDNA were obtained by M-MLV reaction. To determine the expression rate of GFP, SYBR-Green method was employed. The primers for GFP were upstream: 5′-TGCTTCAGCCGC-TACCC-3′, downstream: 5′-AGTTCACCTTGATGC-CGTTC-3′, amplified 211–496 bp fragment. And for actin were upstream: 5′-GTGGACATCCGCAAA-GAC-3′, downstream: 5′-AAAGGGTGTAACGCAA-CTA-3′, amplified 932–1233 bp fragment. Reaction followed the two-step protocol: 95.0°C for 15 seconds, 1 cycle, then 95.0°C for 5 seconds, 60.0°C for 30 seconds, 40 cycles. The titer of virus was then illustrated by mRNA expression rate.

Lentivirus infection and expression analysis

For rat ADSCs infection, lenti-$VEGF_{165}$ storage solution was diluted to appropriate concentration and added into cell culture. We used the lenti-GFP as the positive control and the no-transduced ADSCs as the negative control. These constructs were transduced into ADSCs overnight at a multiplicity of infection (MOI) of 20. After 48 hours, cells were examined under a microscope. For $VEGF_{165}$ expression analysis, we performed the enzyme-linked immunosorbent assay (ELISA) of the supernatant. And cells were harvested after 3 days to prepare total protein. Antibody for $VEGF_{165}$ was used in Western blotting, and procedure followed the steps described previously.[10]

Statistical analysis

Data were analyzed with SPSS 13.0 (SPSS Inc., IL, USA) and expressed as means ± standard deviation. Student-*t* test was used to compare difference between two groups. Differences with P <0.05 were considered statistically

significant.

RESULTS

Vector construction

Entire $VEGF_{165}$ cDNA sequence was obtained by PCR reaction with three mutated fragments as template (Figure 1). *AgeI* digested DNA was linked to pGC-FU and produced pGC-FU-$VEGF_{165}$ vector, positive clones were examined by colony PCR test. Eight clones were examined in colony PCR test and DNA sequencing demonstrated that $VEGF_{165}$ cDNA was introduced into the GFP-fusion expression vector (Figure 2).

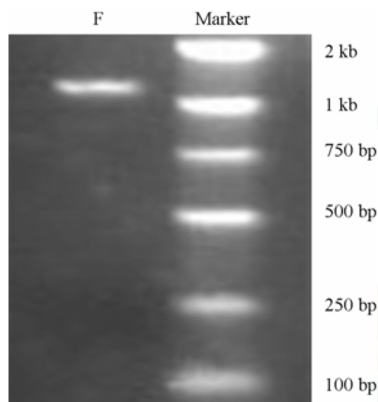

Figure 1. Fragment containing entire $VEGF_{165}$ cDNA sequence. F: Products of PCR reaction with three mutated fragments as template and $VEGF_{165}$-Age I-F, $VEGF_{165}$-Age I-R as primers.

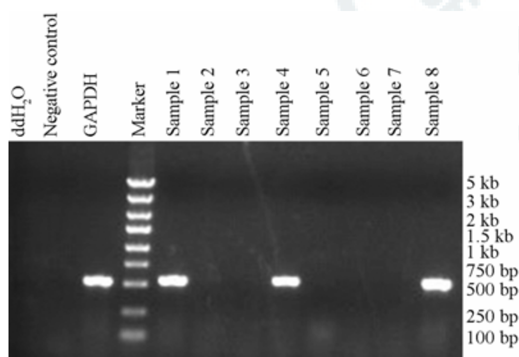

Figure 2. Colony PCR tests of transformed DH5a. Samples 1–8: Products of PCR reaction with boiled DH5a cells as templates. The samples 1, 4, 8 were positive.

$VEGF_{165}$ expression in 293T cells

pGC-FU-$VEGF_{165}$ and pGC-FU vectors were transfected into 293T culture, after 24 hours more than 70% cells were observed GFP expression. Western blotting presented ~48 kD band for pGC-FU and a ~50 kD band for pGC-FU-$VEGF_{165}$ (Figure 3). Taking the IRES included in $VEGF_{165}$ sequence into consideration, the molecular weight of the fusion protein is 49 kD. Therefore the blot verified the expression of $VEGF_{165}$ under Ubi promoter driving in 293T cells.

Lentivirus envelope and titer determination

In quantitative test, 1 in 20 μl cDNA sample was applied

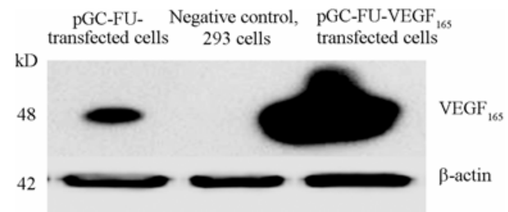

Figure 3. Western blotting analysis of pGCFU-$VEGF_{165}$ transfected 293T cells. The 293T cells positively showed the $VEGF_{165}$ after the pGC-FU-$VEGF_{165}$ transfected.

in real time PCR, thus read out value was 1/20 titer of virus solution. GFP expression rate illustrated by Table demonstrated that 10^{-4} (v/v) virus infected cells evidently expressed GFP. Therefore, titer of virus solution is $20/(10^{-4}\times10^{-3})= 2\times10^{8}$ TU/ml.

Letivirus infection and $VEGF_{165}$ expression in ADSCs

In the present study we chose rat ADSCs as candidates for transduction with $VEGF_{165}$. Transduction with GFP served as a negative control as well as for the determination of transduction efficiency, which was found to be greater than 95% (percentage of cells displaying green fluorescence). Afete 48-hour transduction, the $VEGF_{165}$ and the GFP transduced cells can show green fluorescence confirmed by immunofluorescence staining (Figure 4A), and there was no significant difference of the transduced ratio between $VEGF_{165}$ and the GFP transduced cells (P >0.05). The Western blotting analyses confirmed $VEGF_{165}$ expression in $VEGF_{165}$-transduced cells but not in GFP-transduced cells (Figure 4B). And the ELISA analyses could detect out the density of VEGF was 850.86–1202.13 pg/ml (mean (923.00±31.22) pg/ml) in the supernatant of $VEGF_{165}$-transduced cells but not in the GFP-transduced cells (P <0.001).

DISCUSSIONS

VEGF stimulates proliferation and migration of endothelial cells and plays a key role in cell division, apoptosis, basal layer hydrolysis, invasion and regenesis.[11] These adjustments performed considerable specificly, which effectively contribute to physiological or pathological procedure as embryonic development, repairing in trauma and collateral circulation formation etc.[19] Within 5 variants of VEGF, $VEGF_{165}$ is the most widely distributed and with the strongest activity.[11] Therefore we constructed a $VEGF_{165}$ expression vector from a commercial clone by PCR mutation method. Sequence was blasted by Genebank online and confirmed consistently with registered transcripts NM_001025368 (NCBI). Because clinical application of VEGF is largely hindered by ephemerality and costliness, gene therapy is supposed to be optimal method in vascular regenesis research. Aligned with ADSC cells, the effects of $VEGF_{165}$ will fulfilled in target tissue treatment. Indeed, we construct a vector including Ubi promoter and GFP fusion to enhance the VEGF expression in stem cell and ease the expressing location. The Western blotting test

Table. Quantitative PCR analysis of lentivirus infected 293T cells

Samples	Ct $_{Actin}$	Ct $_{GFP}$	Average Ct$_{GFP}$	△Ct	△Ct $_{Con}$-△Ct$_{i}$
Control	14.2	34.46	35.035	20.835	0
		35.61			
10 µl	14.67	17.62	17.610	2.940	17.895
		17.60			
1 µl	14.62	20.60	20.625	6.005	14.830
		20.65			
10^{-1} µl	14.89	23.91	24.080	9.190	11.645
		24.25			
10^{-2} µl	14.38	26.96	26.975	12.595	8.240
		26.99			
10^{-3} µl	15.03	31.27	31.095	16.065	4.770
		30.92			
10^{-4} µl	14.67	33.22	33.000	18.330	2.505
		32.78			
10^{-5} µl	14.56	33.84	33.295	18.735	2.100
		32.75			

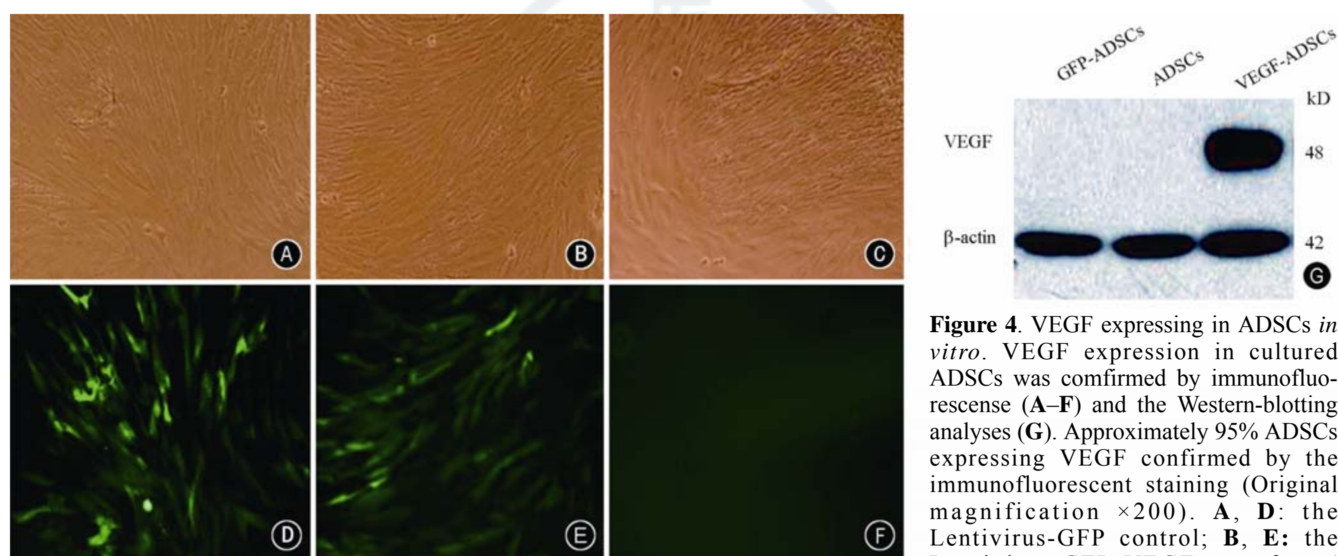

Figure 4. VEGF expressing in ADSCs *in vitro*. VEGF expression in cultured ADSCs was comfirmed by immunofluorescence (**A–F**) and the Western-blotting analyses (**G**). Approximately 95% ADSCs expressing VEGF confirmed by the immunofluorescent staining (Original magnification ×200). **A**, **D**: the Lentivirus-GFP control; **B**, **E:** the Lentivirus-GFP-VEGF transferred ADSCs; **C, F:** the negative ADSCs. The Western-blotting analyses (**G**) have also confirmed the expression of VEGF in ADSCs.

presents that GFP fused VEGF$_{165}$ expressed in 293T cells; thus, construction was verified at both DNA and protein level.

Moreover, produce lentivirus is crucial to obtain permanent VEGF expressing ADSC cells. Compared to traditional adenovirus, lentivirus takes advantages of wide infection range and long term expression endurance. As gene is integrated into host genomic DNA by lentivirus, it will not be dumped during division or differentiation. Besides, lentivirus is more protective than adenovirus in practice, as a pseudotype.[20,21] We adopted three vectors envelope system and 293T cells to produce virus. Quantitative PCR test illustrated that titer of solution reached 2×10^{8} TU/ml which is sufficient for ADSCs infection.

To obtain a therapeutic seed cells, efficient infection and stable expression of VEGF$_{165}$ is essential. One of the major disadvantages of adipose derived stem cell is that ADSCs are not a completely homogeneous cell population in addition to complicated isolating process. Tolerance of ADSCs to gene modification is high, meanwhile, the cells possess high division potency.[22,23] On the other side, the genotoxity of lentvirus is low, which supposes that ADSCs align with lentiviral expression vector would produce powerful therapeutic tool for related treatments. To our results, we successfully detected the VEGF$_{165}$ expression in the ADSCs but not in the control group, confirmed by the ELISA and Western blotting analyses. It illustrated that the VEGF$_{165}$ had successfully transfected the ADSCs and may suit to do the followed research.

In colclusion, we have constructed a VEGF$_{165}$-GFP fused expression lentivral vector and verified in 293T cells. Lentivirus was produced and titer is up to 2×10^{8} TU/ml, determined by quantitative analysis of GFP mRNA. ADSCs were successfully infected and stable expression of VEGF$_{165}$ was confirmed by Western blotting. It suggested this cell may be widely introduced to vascular regenesis research, especially on ED treatment.

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