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Full Length Research Paper

Research on human placenta-derived mesenchymal stem cells transfected with pIRES2-EGFP-VEGF165 using liposome

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The experiment adopting reverse transcription polymerase chain reaction (RT-PCR) technology, amplified hVEGF165 gene fragments from human leukemia cells HL-60. hVEGF165 gene was reconstructed in pIRES2-EGFP and transferred into the human placenta-derived mesenchymal stem cells (HPMSCs) by liposome-mediated method successfully. The mRNA and protein of hVEGF165 in the transferred cells was detected by RT-PCR and Western blot, and the results showed that hVEGF165mRNA and the protein expressed by HPMSCs transfected with pIRES2-EGFP-hVEGF165 was significantly more than HPMSCs transfected with pIRES2-EGFP. EGFP expression was observed under fluorescence microscope, which proved that the report gene was successfully transferred to the target cells. hVEGF biology activity and cell proliferation activity of $HPMSC_S$ transfected with pIRES2-EGFP-hVEGF165 was detected by MTT array, which showed that hVEGF165 can promote the proliferation of $HPMSC_S$; however, hVEGF165 biology activity of $HPMSC_S$ transfected with pIRES2-EGFP-hVEGF165 was significantly more than HPMSCs transfected with pIRES2-EGFP. Identification of multipotentiality showed that $HPMSC_S$ transfected with pIRES2-EGFP-VEGF165 still maintained multipotentiality.

Key words: Transfect, human placenta-derived mesenchymal stem cells, hVEGF165.

INTRODUCTION

Vascular endothelial growth factor is a potent factor for promoting angiogenesis, and this can induce angiogenesis (Parati et al., 2003), increase vascular permeability (Jackson et al., 2001), maintain vascular function and play an important role in wound healing. The focus of the current research is to use growth factor in the treatment research of refractory wound (such as combined radiation and wound injury, etc.), whose family has five subtypes with VEGF165 as the most important phenotype. Since the vascular endothelial growth factor (VEGF) half-life is short (that is, about 30 to 45 min in normal tissues, and can be extended to 6 to 8 h in the ischemic area), it requires a delivery system to extend the time of vascular endothelial growth factor in wound repair parts.

It was discovered that the bone marrow mesenchymal stem cells (Halleux et al., 2001) and adipose-derived stem cells (Lei et al., 2007) had important application potential in the field of wound healing in recent years, in that they can locally extend homing to trauma (Kajstura et al., 2005) and directionally differentiate them into wound repair cells, endothelial cells and fibroblasts (Brittan et al., 2005). However, they can prompt the regeneration of

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Abbreviations: VEGF, Vascular endothelial growth factor; **BMSCS,** bone marrow mesenchymal stem cells; **ADSCs,** adipose-derived stem cells; **HPMSCs,** human placenta-derived mesenchymal stem cells; **RT-PCR,** reverse transcription polymerase chain reaction; **DMSO,** dimethylsulfoxide.

endogenous cells in tissues through creating and enhancing repaired microenvironment (Fathke et al., 2004; Badiavas et al., 2003), thereby accelerating wound healing. Stem cells are also good carrier cells for gene therapy and can carry target genes to target areas to play a treatment role (Badiavas et al., 2003). Human placenta-derived mesenchymal stem cell is a significant source of adult stem cells, which gradually attracted attention in recent years. The author shows that (Liu et al., 2009) HPMSCs are basically consistent with bone marrow mesenchymal stem cells (BMSCs) in morphological and biological trait aspects and can directly differentiate them into wound repair cells and endothelial cells in certain conditions through studies (Liu et al., 2010), which indicated that human placenta-derived mesenchymal stem cells (HPMSCs) can be used as seed cells for wound repair.

The study successfully constructed pIRES2-EGFP-VEGF165, and it was transfected into HPMSCs by liposome-mediated, so that vascular endothelial growth factor can be stably expressed for a long time. This not only plays the long effect mechanism of VEGF, but also exerts the function of HPMSCs to prompt trauma healing, thereby laying a prophase experiment foundation for studying the further application of vascular endothelial growth factor transgenic stem cell transplantation method in the treatment of refractory wound healing.

MATERIALS AND METHODS

Materials

Primer synthesis (cyagen biosciences), RT-PCR kit (Promega), agarose (Promega), T4 phage DNA ligase (Promega), restriction endonuclease EcoRI, BamHI (Takara), DNA gel extraction kit (Promega), DNA-Marker (Sigma USA), plasmid extraction kit (Promega), Trizol (Sigma USA), ethidium bromide (EB) (Sigma USA), IMDM medium (Sigma USA), fetal calf serum (Hyclone), pMD18 T vector (Clontech USA), pIRES2-EGFP (Clontech USA), human leukemia cells HL-60 (presented by Doctor Shen Weizhang of the Second Hospital of Jilin University), E. coli DH5α (Biochemical and Molecular Biology Lab of Bethune Medical School), $HPMSC_S$ (cultured and identified in our laboratory) (Liu et al., 2009) and the abandoned placenta (from the Department of Obstetrics and Gynecology, Second Hospital, Jilin University), were used for this study. $HPMSC_S$ belong to the adult stem cells, which do not involve ethical issues, such as lipofectin lipids body suspension (GIBCO), DEME medium (GIBCO), ECV304 (presented by the Department of Biochemistry, Basic Medical College) and G418 selection medium (Protec Company). Construction and identification of pIRES2-EGFP-hVEGF165

Acquisition, amplification and identification of target gene fragments hVEGF165

Trizol was used to extract HL-60 cell total RNA. The RNA concentration was determined, and it was best when A260/A280 ratio was 1.8. The first chain of cDNA was synthesized through using Oligo (dT) and M-MLV reverse transcriptase according to the instructions. hVEGF165 full-length gene sequences were searched from GenBank. A pair of primers starting from the initiation codon ATG on the upstream and terminating with the stop codon TGA on the downstream was designed. EcoRI and BamHI restriction endonuclease enzyme digestion site sequences were added on the upstream and downstream primers respectively, and they were sent to cyagen biosciences for synthesizing hVEGF165 primer sequences: upstream comprised 5'-CGGAATTCATGAACTTT CTGCTGTCTTGGGTG-3', containing EcoRI restriction endo-nuclease enzyme digestion sites; while downstream comprised 5'-CGGGATCCTCACCGCCTCGGCTTGTCACATCT-3', containing BamHI restriction endonuclease enzyme digestion sites.

Polymerase chain reaction (PCR) products passed through agarose gel electrophoresis, and the rubber of the target strips was cut, reclaimed and purified. The purified hVEGF165 PCR products were connected to pMD18-T vector, while the ligation product was transformed into competent cells DH5α. Colony growth conditions were observed, and the positive bacteria colony was screened by blue and white spot screening. PCR screening was carried out after culture, and finally, the pMD18-T-hVEGF165 enzyme digestion identification was carried out.

Construction and identification of pIRES2-EGFP-hVEGF165

EcoRI and BamHI double digests were identified for pMD18-T-hVEGF165 and pIRES2-EGFP, while T4DNA ligase was used for connecting stick ends of these products. pIRES2-EGFP-hVEGF165 was built and transformed into competent cells DH5α. However, reverse transcription polymerase chain reaction and double digest identification of pIRES2-EGFP-hVEGF165 was carried out.

pIRES2-EGFP-hVEGF165 transfected HPMSCs

About 1 ml serum-free DEME was used for diluting pIRES2-EGFP-hVEGF165 and pIRES2-EGFP, which was revolved for 1 s, and was added to Lipofectin liposome suspension, then scrolled again and placed at room temperature for 5 to 10 min so that the two parts could be combined, that is, pIRES2-EGFP-hVEGF165-Lipofectin and pIRES2-EGFP-Lipofectin were prepared. There were three groups: Group A was $HPMSC_S$ with an addition of pIRES2-EGFP-hVEGF165-Lipofectin, Group B was $HPMSC_S$ with an addition of pIRES2-EGFP-Lipofectin, and Group C was the control group (pIRES2-EGFP-Lipofectin without $HPMSC_S$). The cells were screened by G418 selection.

Identification of VEGF activity expressed by HPMSCs transfected with pIRES2-EGFP-hVEGF165

1. Western blot analysis of hVEGF165 protein obtained from bone marrow mesenchymal stem cells transfected with pIRES2-EGFP-hVEGF165.
2. The VEGF biology activity expressed by $HPMSC_S$ transfected with pIRES2-EGFP-hVEGF165 and MTT array human endothelial cell line ECV304 was seeded on 96-well plates, and added to each group of transfection supernatant with 1/2 volume. MTT solution was added and continuously cultured for 4 h, and then dimethylsulfoxide (DMSO) solution (100 µl) was added and shaken until the blue settlement was fully dissolved and the D_{480} value was measured. Subsequently, the statistical data were adopted as mean ± standard deviation (× ± S), and t test was adopted for group comparison.
3. Proliferation activity identification of $HPMSC_S$ transfected with hVEGF165 amid MTT array. Pass cells with excellent growth were respectively selected and seeded on 96-well plates. Each well was added to the cell suspension (200 µl) of each group and the 20 µl MTT solution (5 mg/ml). After culturing for 36 h, it was added to the150 µl DMSO oscillation solution. The wavelength of 490 nm

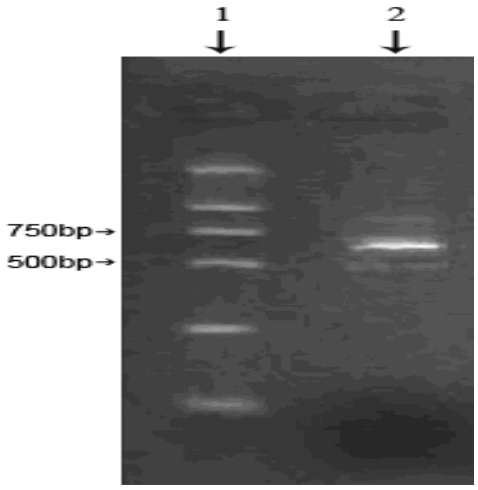

Figure 1. PT-PCR products. Lane 1: Marker; lane 2: Target gene (orso 600 bp).

was selected and the optical density was measured. Statistical data were adopted as mean ± standard deviation (× ± S), and t test was adopted for group comparison.
4. Observation of transfection result under fluorescence microscopy and flow cytometry analysis of transfection efficiency.

Identification of multipotentiality of HPMSC$_S$ transfected with pIRES2-EGFP-VEGF165

Lipoblast induction

It has a DMEM-HG induction system containing 10% FBS, dexamethasone (1 μM), anti-inflammatory pain (200 μM), IBMX (0.5 mM) and insulin (10 μg/ml). However, oil red staining was identified.

Chondrogenic induction

It has a 2.5% FBS DMEM-HG induction system added with insulin, transferrin, sodium selenite (6.25 μg/ml), BSA (1.25 μg/ml), sodium pyruvate (1 mM / L), ascorbic acid phosphate (37.5 μg/ml) and TGF-β1 (50 ng/ml). However, alcian blue staining was identified.

RESULTS

Construction and identification of pIRES2-EGFP-hVEGF165

Amplification of target gene fragment

hVEGF165 gene sequence has the full length of 576bp and the total length of 592 bp after adding enzyme digestion sequences and protective genes on the upstream and downstream. Reverse transcription polymerase chain reaction (RT-PCR) products were obtained for electrophoresis on agarose gel, and target strips appeared on about 600 bp of the DNA marker (Figure 1), which indicated that the amplification of the target gene fragment succeeded.

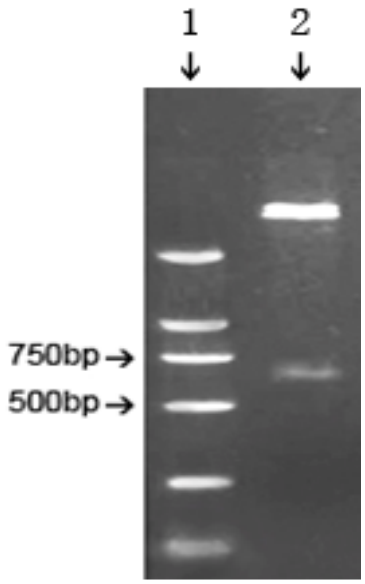

Figure 2. Electrophoresis of pMD18-T-hVEGF165 double digestion. Lane 1: Marker; lane 2: pMD18-T-hVEGF165.

Double digestion identification of pMD18-T-hVEGF165

The electrophoresis results of the restricted incision enzyme, EcoRI and BamHI double digestion recombinant cloning vector (Figure 3), showed that a strip appeared on nearly 600 bp of the DNA Marker with the same size of the target fragment, which indicated the successful connection between the segment and the vector.

Enzyme digestion identification of pIRES2-EGFP-hVEGF165

The electrophoresis result showed that recombinant comprised the fragment that is consistent with the target fragments, which indicated that the fragment was inserted into the expression vector successfully (Figure 2).

pIRES2-EGFP-hVEGF165 transfected HPMSCs

1. Groups A and B had a little survived cells at the seventh day through selection with about one to five cells in each low time field of vision. Group A generated a positive cell clone 15 days after screening by G418 selective medium, which indicated that both effects of HPMSCs transfected with pIRES2-EGFP-hVEGF165 and G418 selection are better (Figure 4).
2. RT-PCR verified expression of hVEGF165: It can be seen on gel image analysis system that hVEGF165

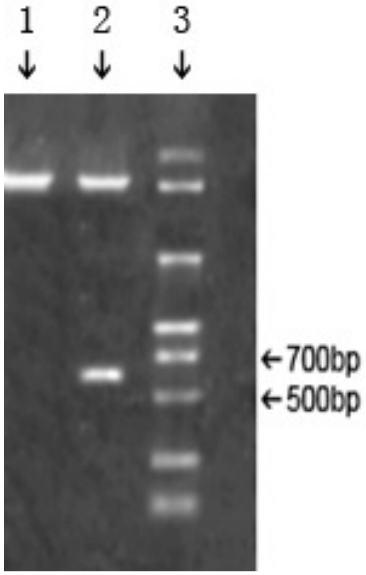

Figure 3. Electrophoresis result of EcoRI, BamHI double digestion pIRES2-EGFP-hVEGF165. Lane 1: pIRES2-EGFP; lane 2: pIRES2-EGFP-hVEGF165; lane 3: marker.

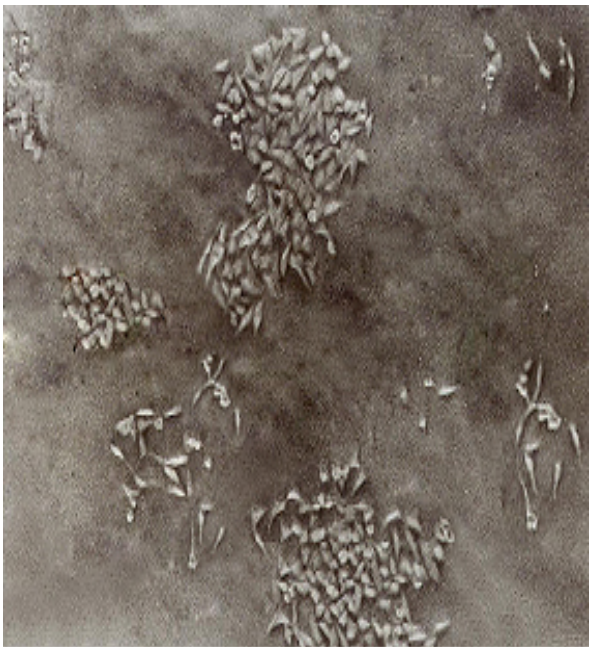

Figure 4. Fifteen days after $HPMSC_S$ were transfected with pIRES2-EGFP-VEGF165 through screening by means of G418 selective medium.

expression of group A is prominently intensified when compared with group B. Fragment expression being consistent with the size of the target fragment can also be seen on group B, β-actin218 gene transcript was used as a reference template (Figures 5 and 6).

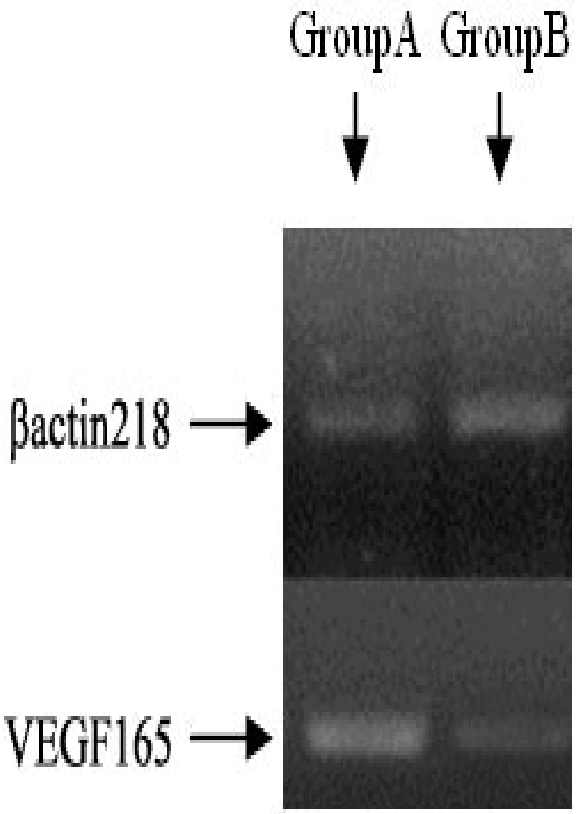

Figure 5. Detection of hVEGF165 mRNA in $HPMSC_S$ by RT-PCR, and the result was adjusted by β-actin218.

3. Western blot analysis of hVEGF165 protein expression: A strip with weak expression can be seen in group B, while a stronger strip can be seen in group A; however, β-actin218 gene transcript was used as a reference template (Figures 7 and 8).
4. Check of vascular endothelial growth factor biology activity with MTT array: Results showed that the VEGF biological activity expressed by $HPMSC_S$ of group A was prominently more than that of groups B and C, and that of group B was more than that of group C. Nonetheless, all the results were statistically significant (Table 1 and Figure 9).
5. Proliferation activity identification of human placenta-derived mesenchymal stem cells by MTT array: The results showed that the proliferation activity of HPMSCs in group A was significantly increased when compared with group B (Table 2 and Figure 10).
6. Observation of the transfection result under fluorescence microscopy and flow cytometry analysis of transfection efficiency: A large amount of human placenta-derived mesenchymal stem cells expressing green fluorescence can be seen in groups A and B after transfection for 48 h, and the 48 to 72 h was the expression peak period of green fluorescence. The expression decreased after 1 week, and the enhanced

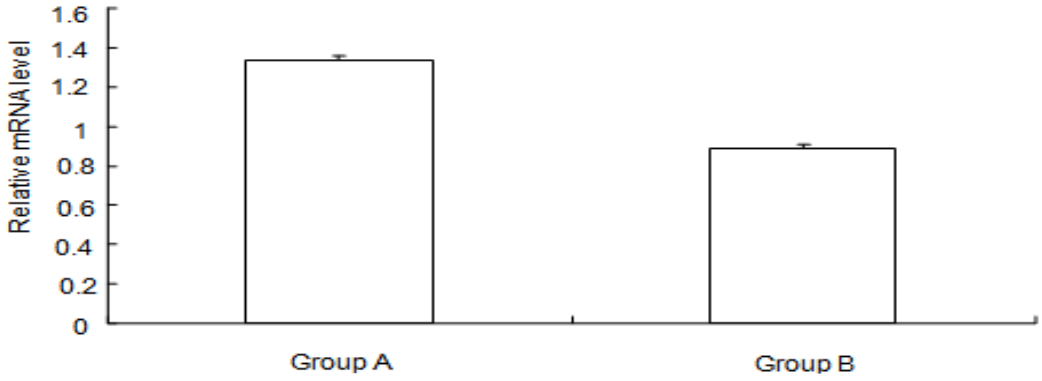

Figure 6. The ratio of hVEGF165 PCR product intensity to β-actin218 PCR product intensity (hVEGF165/β-actin218).

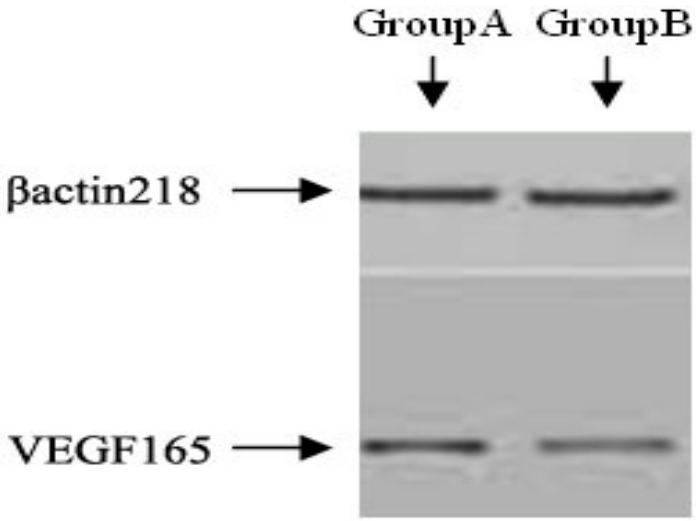

Figure 7. Detection of VEGF165 protein in $HPMSC_S$ by western-blot, and the result was adjusted by β-actin.

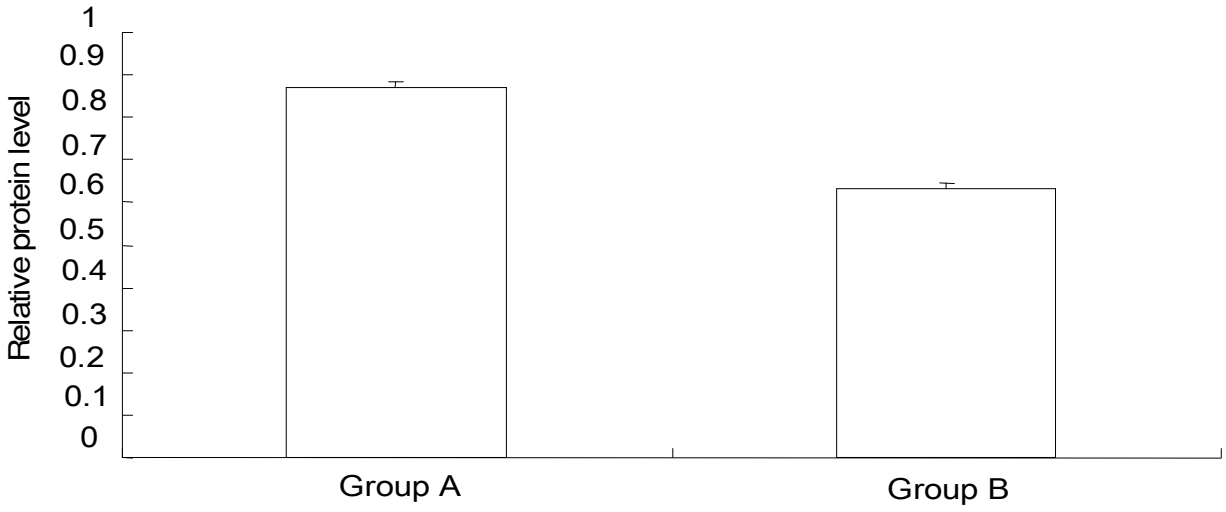

Figure 8. The ratio of hVEGF165 western blot product intensity to β-actin218 western blot product intensity (hVEGF165/β-actin218).

Table 1. Influence of $HPMSC_S$ culture supernatant on hECV304 proliferation activity.

Group	Optical density value (OD value)
C	0.54±0.0124
B	0.69±0.0876
A	0.87±0.0285

t tests of groups A and B were compared two by two ($P < 0.01$); t tests of group A and the control group were compared two by two ($P < 0.01$); t tests of group B and the control group were compared two by two ($P < 0.05$).

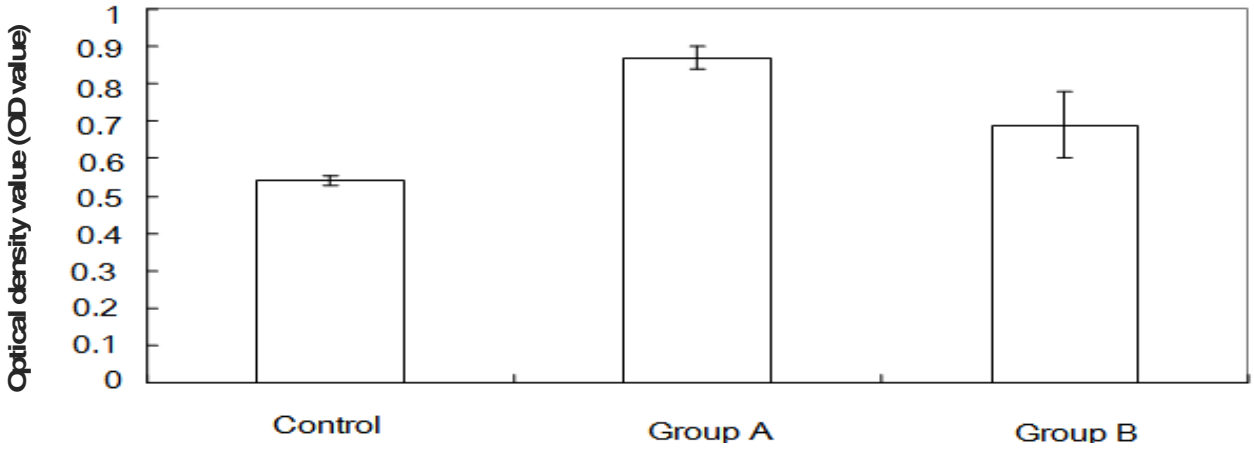

Figure 9. Influence of $HPMSC_S$ culture supernatant on hECV304 proliferation activity.

Table 2. Proliferation activity identification of $HPMSC_S$ by MTT array.

Group	Optical density value (OD value)
B	0.426±0.016
A	0.624±0.019

The t tests were compared two by two ($P < 0.01$).

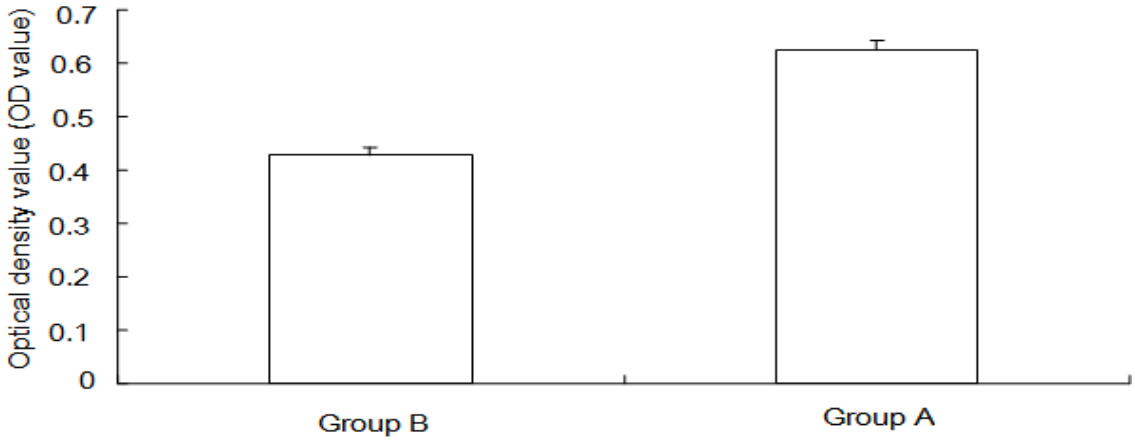

Figure 10. Proliferation activity identification of $HPMSC_S$ by MTT array.

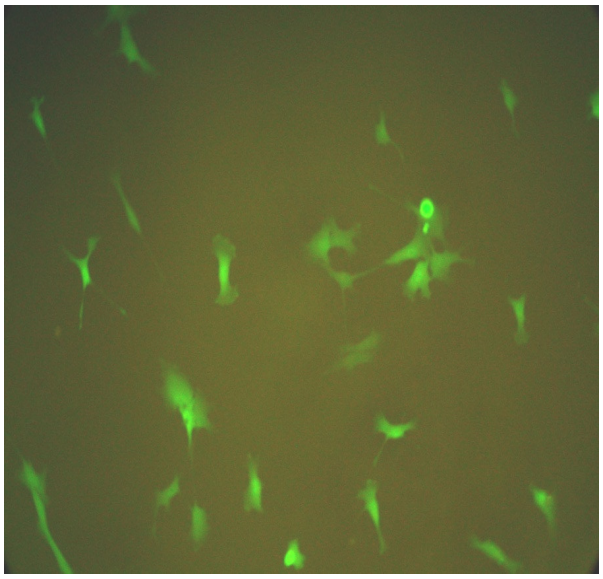

Figure 11. HPMSCs transfected with PIRES2–EGFP–hVEGF165 expressing green fluorescence *in vitro* (×400).

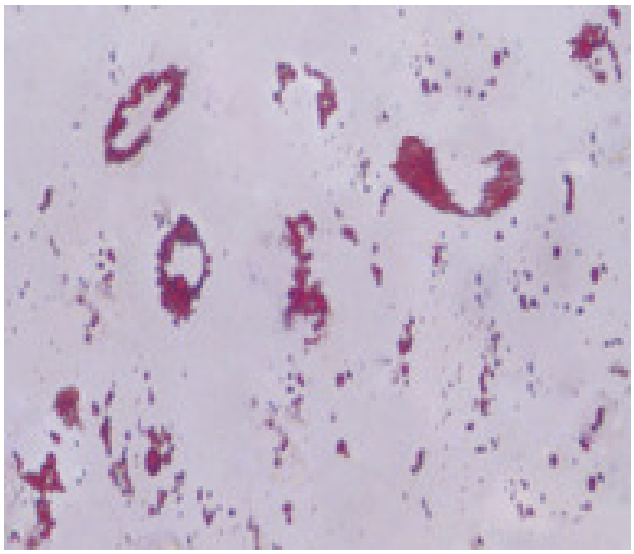

Figure 12. HPMSCs transfected with pIRES2-EGFP-hVEGF165 were induced into grease *in vitro* (oil red staining, ×100).

green fluorescent protein expression was still visible until the third week (Figure 11). The transfection efficiency was 46.8% after transfection for 72 h, detected by flow cytometry analysis.

Identification of the multipotentiality of HPMSCs transfected with pIRES2-EGFP-hVEGF165

It can be seen under adipose cell induction environment that fat generated in cells was specifically stained to be red (Figure 12), while in the cartilage induction environment, the stroma outside the cells formed by a type II collagen was specifically stained to be blue (Figure 13).

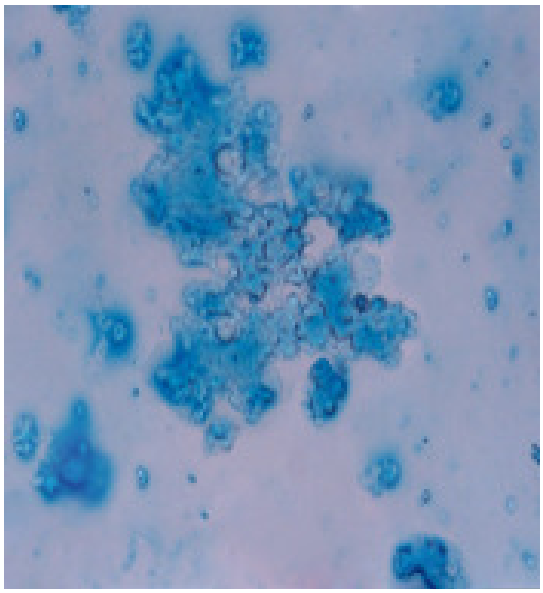

Figure 13. Stroma outside HPMSCs transfected with pIRES2-EGFP-hVEGF165 formed by a type of Ⅱ collagen was specifically stained to be blue *in vitro* (Alcian blue staining, ×200).

DISCUSSION

Gene therapy, which is currently the world's most popular field of research, has virtually pervaded various fields of medical research. The essential role of Vascular endothelial growth factor (VEGF) in promoting wounds' healing has commonly been permitted by scholars, but simply using VEGF has the disadvantages of short half-life, poor penetration, easy absorption of extracellular matrix and low biological utilization rate. Such negative factors greatly restricted its clinical application. Along with the rapid development of molecular biology, the transgenic technology makes vascular endothelial growth factor in the wounds efficient and long-lasting expression, which can overcome the direct application of exogenous VEGF165 faults (Vescovi et al., 1993). Using stem cells as a purpose of gene carrier, can not only play its role of wound healing, but can also carry target genes to target areas. Moreover, it has the great potential for refractory wounds healing. Based on these considerations, amplified target gene fragments hVEGF165 were acquired from leukemia cell HL–60 by RT–PCR. pIRES2-EGFP-hVEGF165 was constructed and the enzyme digestion identification of pIRES2-EGFP-hVEGF165 was used to evaluate the successful construction of the plasmid.

Human placenta-derived mesenchymal stem cells ($HPMSC_S$) was transfected with pIRES2-EGFP-hVEGF-165 by liposome-mediated. The liposome-mediated gene transfection method is one of the most commonly used methods, and the technology is mature, safe and reliable. The method has the advantages that: (1) it is simple, and can carry larger DNA molecules (Zhou et al., 1994); (2) it is more secure and can transfect more types of cells, and target cells do not need to be in the division state. Wherein the application of carrier system (plasmids) mediated with cationic liposome is the widest, it is non-immunogenic, non-toxic and non-carcinogenic, and can be easily prepared for large amount. The transfection efficiency is not as high as the viral vectors, but it has passed the United States National Institutes of Health (NIH) and the recombinant DNA Advisory Committee (RAC) for approval as a gene therapy vector to be introduced into the phase of II clinical trials, and is used for the treatment of certain cancers (Nabel et al., 1992). Therefore, the experiment adopted liposome-mediated pIRES2-EGFP-hVEGF165 to transfect HPMSCs from the comprehensive consideration on the aspects such as post animal experiment security and the likes of others. In so doing, VEGF can be expressed in transgenic $HPMSC_S$ efficiently and stably, which not only accords the requirements of gene therapy for wound healing, but also avoids the potential defects in the virus vector mediated gene therapy. Green fluorescence can be seen in groups A and B under fluorescence microscopy, which suggested that the reported genes were successfully transfected into target cells, and it was proved that it was feasible for human placenta-derived mesenchymal stem cells transfected with PIRES2–EGFP–hVEGF165 by liposome-mediated. RT-PCR and western blot analysis verified a stronger expression of vascular endothelial growth factor obtained from HPMSCs transfected with PIRES2–EGFP–hVEGF165 than from HPMSCs transfected with PIRES2–EGFP, which indicated that vascular endothelial growth factor had been successfully transferred to human placenta-derived mesenchymal stem cells on one hand, and HPMSCs had an endocrine function of VEGF on the other hand.

Influence of human placenta-derived mesenchymal stem cells culture supernatant on hECV304 proliferation activity was detected by MTT array, and the results suggested that vascular endothelial growth factor was expressed continuously and effectively after being transfected into human placenta-derived mesenchymal stem cells. The HPMSCs transfected with pIRES2-EGFP also had an expression of vascular endothelial growth factor, thereby suggesting that human placenta-derived mesenchymal stem cell had an endocrine function of vascular endothelial growth factor itself. Cell proliferation activity changes of human placenta-derived mesenchymal stem cells were detected by MTT array, and the results suggested that pIRES2-EGFP-hVEGF165 had been successfully transferred into HPMSCs and had a prominent prompting role to the proliferation of human placenta-derived mesenchymal stem cells.

We further carried out a multiple differentiation induction identification on HPMSCs transfected with PIRES2–EGFP–hVEGF165 in order to prevent it from generating genetic mutation and losing multipotentiality. The results showed that the transgenic human placenta-derived mesenchymal stem cells still maintain the stem cell characteristics of multipotentiality.

To sum up, the experiment successfully constructed the pIRES2-EGFP-hVEGF165 and utilized the liposome transfection method to successfully transfer it into human placenta-derived mesenchymal stem cells, where hVEGF165 had stronger expression in HPMSCs and was correctly decorated on one hand, which indicated that human placenta-derived mesenchymal stem cells can be used as carrier cells of hVEGF165 gene therapy. Human placenta-derived mesenchymal stem cells transfected with hVEGF165 gene can achieve a long-term release role of vascular endothelial growth factor, and it was also suggested that HPMSCs had endocrine hVEGF165 function on the other hand, but the effect was weak, in that VEGF has a significant promoting role to value adding of human placenta-derived mesenchymal stem cells. Conclusively, this experiment sets the former experiment's foundation for later application of HPMSCs carrying VEGF gene for treatment of refractory wound healing.

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