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Full Length Research Paper

Construction of retrovirus vector taking MDR1/ACBC1 and its transfection into human placenta derived mesenchymal stem cells

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In the study, we used both the methods of perfusion and density gradient centrifugation to isolate and purify mesenchymal stem cells (MSC_S) from placenta tissue, and constructed a retroviral vector with multiple drug resistant genes, and the green fluorescent protein (GFP) has been used as an indicative mark. The 293T cell was transfected by the retroviral vector PMX-flag-MDR1-GFP together with its peripheral membrane protein gene. After the infective and replication–defective retrovirus were acquired, we transfected them into human placenta-derived mesenchymal stem cells (HPMSCs). We successfully observed the expression of the reporter gene-GFP by using the green light fluorescence microscope and the p-glycoprotein (P-gp) expressed by exogenous gene MDR1 by Western Blotting. All these facts indicated that the retroviral vector PMX-flag-MDR1-GFP had successfully been transfected into HPMSCs and the exogenous gene multidrug resistance (MDR)1 was detected as normally expressed. The daunorubicin (DNR) pump experiment proved that P-gp of HPMSCs transfected with PMX-flag-MDR1-GFP was of biological activity. The result indicates that MDR1 retroviral vector can transfect the HPMSCs. Not only can the exogenous gene be expressed, but also the expression protein had the biological activity. The conclusion lays a solid foundation of the clinical application of MDR1 genetic therapy.

Key words: Transfect, human placenta-derived mesenchymal stem cells, multidrug resistance (MDR)1 gene.

INTRODUCTION

The chemotherapy occupies an extremely important position in the treatment of gynecology malignant tumor (Randall et al., 2009). Some familiar malignant gynecology tumors, such as malignant trophoblastic tumor, ovary cancer and endometrial cancer, are sensitive to chemotherapy, among these tumors; malignant trophoblastic tumor can be cured only by the methods of chemotherapy (Osborne et al., 2011). However, frequentative and high dose of chemotherapy usually leads to the occurrence of bone marrow depression and drug resistant. It is of great value to find an effective way to protect hematopoietic cells under chemotherapy; meanwhile, it can improve the therapeutic efficacy of malignant tumors.

Transfecting the multidrug resistance gene (MDR) into haemopoietic stem cells of the bone marrow would make the haemopoietic stem cells and peripheral blood leucocytes able to tolerant high dose of chemotherapy,

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Abbreviations: $BMSC_S$, Bone marrow mesenchymal stem cells; **HPMSCs,** human placenta-derived mesenchymal stem cells; **RT-PCR,** reverse transcription polymerase chain reaction; **MDR,** multidrug resistance; **P-gp,** p-glycoprotein; **DNR,** daunorubicin; **MSC_S,** mesenchymal stem cells; **GFP,** green fluorescent protein.

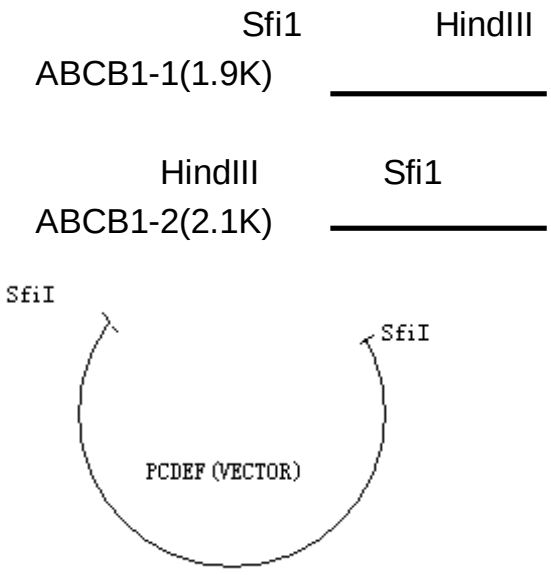

Figure 1. Full-length gene ABCB1 in PCDEF carrier.

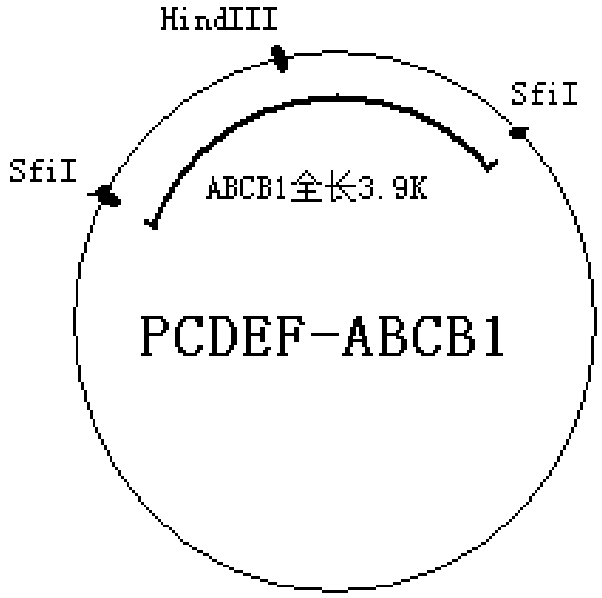

Figure 2. PCDEF-ABCB1 incompletely digested by EcoR I and BamHI

while tumor cells can be eliminated as many as possible. But the haemopoietic stem cell's transfect efficiency, which is rather poor, limits the development of this technique (Niethammer et al., 2005). Human placenta-derived mesenchymal stem cells (HPMSCs) as a significant source of adult stem cells (Lee et al., 2011), has obtained numerous attention in recent years. Studies have shown that HPMSCs are basically consistent with BMSCs in morphological and biological trait aspects.

Currently, numerous studies proved that the mesenchymal stem cells (MSC_S) from humans and animals can be transfect with exogenous gene by many kinds of vector; the exogenous gene itself is of high expression, and it does not affect the autogenous gene of the stem cells. MSCs have many biological specifics, such as multiple differentiations, modulation of immune function, improvement of hematopoietic microenvironment, and the ability of hematopoietic reconstruction. In this study, we chose $HPMSC_S$ because it is easily obtained, does not involve ethical issues (patients agreement and signed) and will not cause secondary trauma to the patient. Due to the development of the origin, it has less immunogenic than other mesenchymal stem cells, so allograft does not cause rejection; tumorigenic and teratogenic experiments were negative. All these show that it is a good source of seed cell bank with industrial development prospects.

In this study, we used the retroviral vector carrying exogenous gene MDR1 and reporter gene green fluorescent protein (GFP) to transfect the HPMSCs *in vitro*, and explored the expression and function of exogenous gene MDR1, trying to find out a solid theoretical basis of the clinical application of MDR1 genetic therapy. If HPMSCs could be transfect with MDR-1 gene, combined with its function of reconstruction bone marrow microenvironment, the HPMSCs transfected with MDR-1 will support hematopoietic stem cell transplantation, and be able to more effectively improve the therapeutic role of high-dose chemotherapy in gynecological tumors.

MATERIALS AND METHODS

Expand high fidelity polymerase chain reaction (PCR) system kit (ROCHE), QIAgen Plasmid MiDi Kit (QIAGEN), 293T (ATCC), daunorubicin (DNR, Italian), anti—flag (sigma F3165), polybrene (sigma USA), restriction endonuclease EcoRI, BamHI (Takara), deoxyribonucleic acid (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), $HPMSC_S$ [cultured and identified in our laboratory (Liu zhihui et al., 2009)], were used. The abandoned placenta was from the Department of Obstetrics and Gynecology, Second Hospital, Jilin university; maternal agreement was used for this study. $HPMSC_S$ belonged to adult stem cells, (did not involve ethical issues). SMART library (SMART cDNA library construction Kit), Fluorescence microscopy (NIKON), inverted microscope (Olympus), Flow cytometric FACS Calibur (B.D Co USA) were used also.

Construction of PMX-flag-GFP-mdr1

ABCB1-1 (1.9 K) and ABCB1-2 (2.1 K) fragment were amplified separately from SMART library and cloned into T-Vector. We got PKT-ABCB1 and PKT-ABCB2 correct sequence and connected these two sequences into PCEDEF carrier by restriction enzyme digestion (Stitching) Figure 1). The full-length gene ABCB1 in PCDEF carrier is shown in Figure 1. PCDEF-ABCB1 was incompletely digested by EcoR I and BamHI (Figure 2), and then ABCB1 was connected into the PMX carrier (Figure 3).

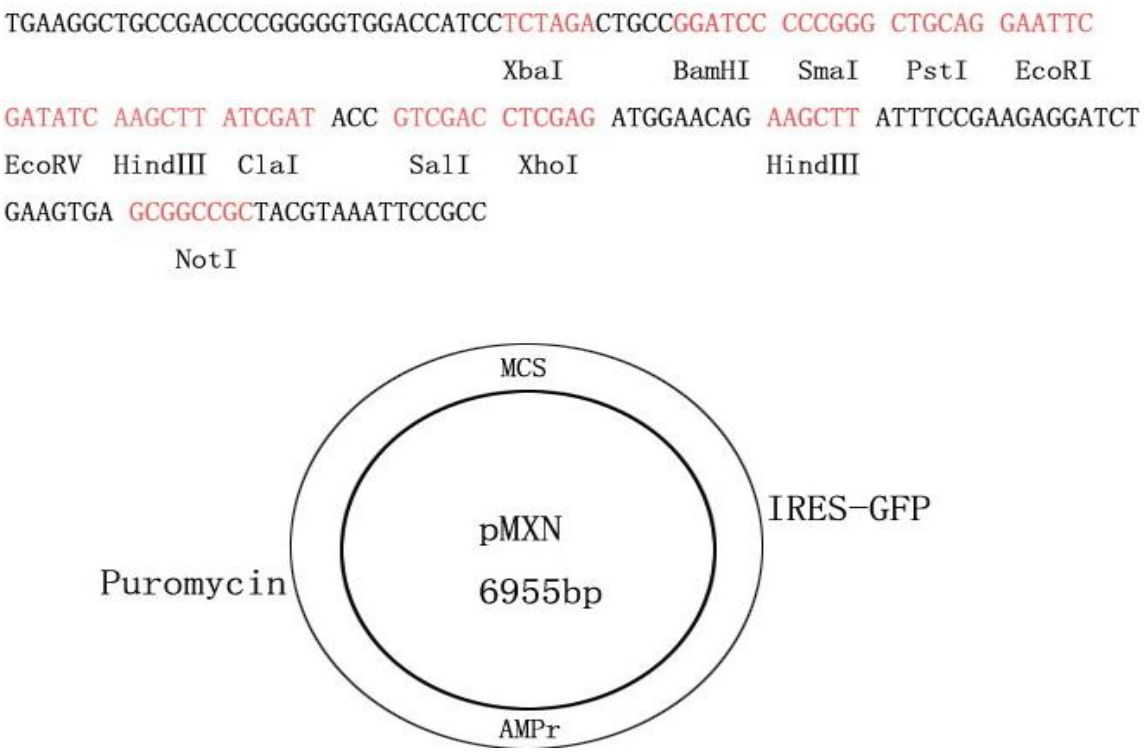

Figure 3. PMX carrier.

Retroviral taking PMX-flag-GFP-mdr1 transfected HPMSCs

PMX-flag-GFP-MDR1, pCIVSV and pCIPGB1 three plasmid were transfected into HEK293T cells according to 1:1:1 ratio by calcium phosphate method. Then, HEK293T cells were cultured 48 h and supernatant liquid was filtered and collected; the retroviral supernatant containing MDR1 gene was stored at -80°C. When the infected HPMSCs were more than 80% bus, HPMSCs was used for transfection. When the supernatants was clear, infected HPMSCs by retrovirus supernatant was collected and 4 ug/ml polybrene was added. After learing retrovirus supernatant after 24 h, adding fresh medium, and puromycin (2 ug/ml) screening after 24 h, observed fluorescence after 3 days.

Identification of MDR1 activity expressed by HPMSCs transfected with PMX-flag-GFP-MDR1

Western blot analysis of MDR1 protein obtained from $HPMSC_S$ transfected with PMX-flag-GFP-MDR1 was done and also, in order to verify whether the p-glycoprotein (P-gp) produced by exogenous MDRI gene transfected into HPMSCs is a normal function, DNR pump test was conducted for non-transfected cells and cells transfected with 3 days. The tested cultured cells were made of 2 × 106 ml^{-1} suspension, and each tube was added with daunorubicin; the final concentration was 7.5 ug/ml, was incubated at 37°C, 5% CO_2 for 30 min. The culture medium was removed, the daunorubicin outside the cell membrane was washed away by fresh medium without fetal calf serum and was incubated at 37°C, and 5% CO_2 for 10 min. The pump function was terminated by verapamil (the final concentration of 10 ug/ml), washed twice with PBS, and the machine was put on for the test. For each sample collected (1.5 × 10^4 cells), flow cytometry determination was done by FRCS calibur detector. Statistical test was done by F test.

RESULTS

Amplification of target gene fragments

ABCB1-1 gene sequence was 1.9 kb in length and ABCB1-2 was 2.1 kb in length. Reverse transcription polymerase chain reaction (RT-PCR) products were obtained for electrophoresis on agarose gel, and target strips appeared on about 2.0 kb of the DNA marker (Figure 4), which indicate that the amplification of the target gene fragments succeeded.

Double digestion identification of PCDEF-ABCB1

The electrophoresis results of restricted incision enzyme EcoRI and BamHI double digestion of PCDEF-ABCB1 show that a strip appeared on nearly 3.9 kb of DNA marker with the same size of the target fragment (Figure 5), which indicate the successful connection between the segment and the vector.

293T cells transfected with PMX-flag- GFP-MDR1

293T cells transfected with the plasmid after 48 to 72 h became round, adherent of variation (Figure 6), and almost most of the cells expressed fluorescent protein observed by fluorescence microscopy (Figure 7), which

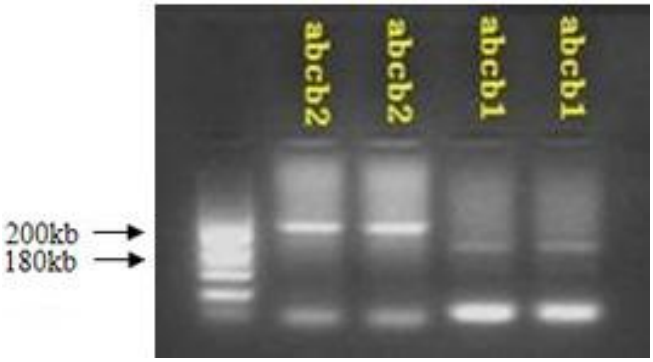

Figure 4. PT-PCR products.

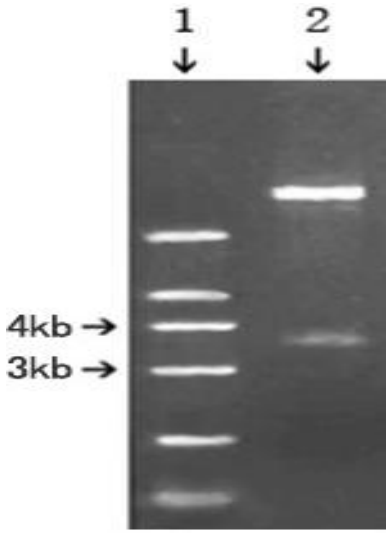

Figure 5. Electrophoresis of PCDEF-ABCB1 double digestion. Lane 1, marker; lane 2, PCDEF-ABCB1.

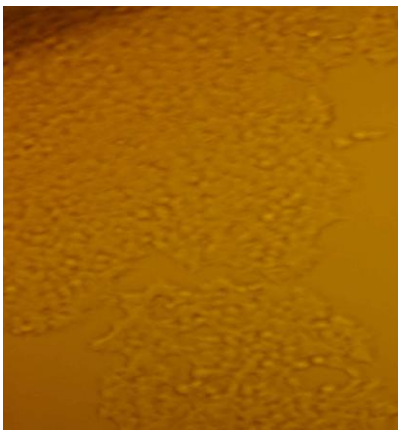

Figure 6. Morphology of 293T cells transfected with the plasmid cultured 48 to 72 h observed under light microscope (×200).

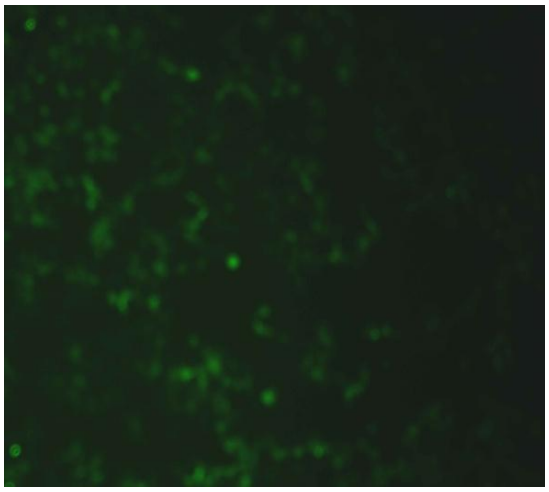

Figure 7. 293T cells transfected with the plasmid cultured 48 to 72 h observed by fluorescence microscopy (×200).

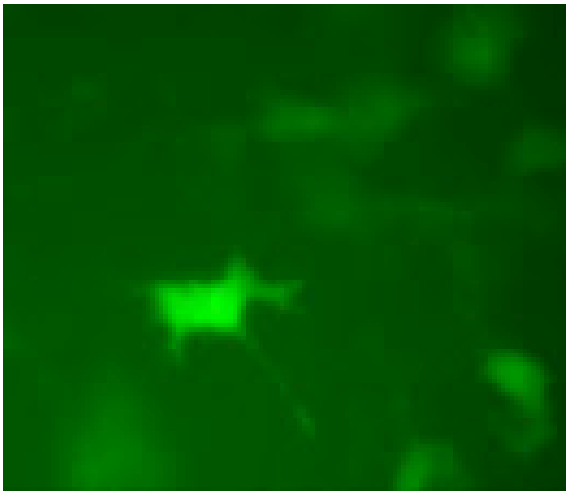

Figure 8. $HPMSC_S$ transfected with retroviral supernatant taking PMX-flag-GFP-MDR1 cultured 3 days observed by fluorescence microscopy (×200).

showed that calcium phosphate precipitation method has higher transfection efficiency.

$HPMSC_S$ transfected with retroviral supernatant taking PMX-flag-GFP-MDR1

$HPMSC_S$ transfected with retroviral supernatant taking PMX-flag-GFP-MDR1 expressed fluorescent protein observed by fluorescence microscopy (Figure 8), however, $HPMSC_S$ transfected with empty vector did not express fluorescent protein (Figure 9), which showed that PMX-flag-GFP-MDR1 was transfected into $HPMSC_S$ successfully.

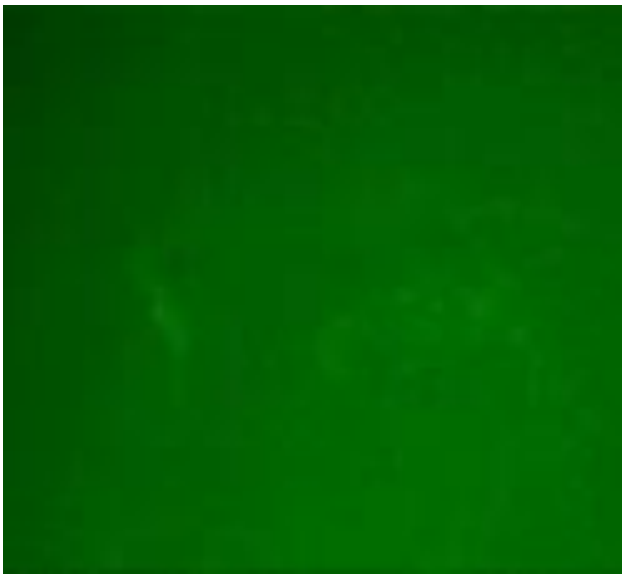

Figure 9. HPMSC$_S$ transfected with retroviral supernatant taking PMX-flag-GFP cultured 3d observed by fluorescence microscopy (×200).

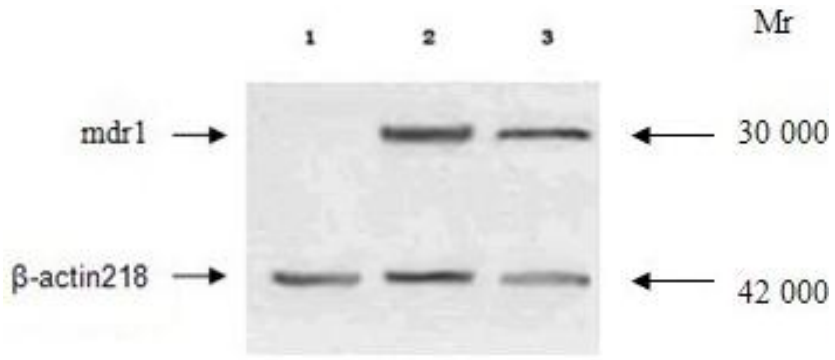

Figure 10. MDR1 protein in HPMSC$_S$ was detected by western-blot, and the result was adjusted by β-actin218. 1: blank 2: 293T pMX—ABCB1. 3: HPMSCs pMX-ABCB1

Western blot analysis of MDR1 protein obtained from HPMSC$_S$ transfected with PMX-flag-GFP-MDR1

A strip was seen in groups 2 and 3; β-actin218 gene transcript was used as a reference template (Figure 10).

DNR pump test

DNR-positive rate of non-transfected cells was 95.34%, and the DNR-positive rate of transfected cells was 67.65%. F test statistical methods was used ($\alpha = 0.05$, $P < 0.05$), which showed that there was a significant difference of P-gp protein function expression between non-transfected cells and transfected cells. The results show that the P-gp protein of transfected cells had biological activity.

DISCUSSION

The transmembrane glycoprotein (p-gp) encoded by Multidrug resistance gene (MDR1) is an energy-dependent efflux pump (Tufan et al., 2007). It can conduct the excretion of MDR1 related medicines, and reduce the drug's intracellular accumulation. In that way it can avoid toxic drugs from damaging the cell (Hesdorffer et al., 1994). In order to avoid the mutation in researching the exogenous MDR1 genes, we got ABCB1-1(1.9 K) and ABCB1-2(2.1 K) respectively from the SMART Lib, and obtained the MDR1 full-length gene by restriction enzyme digestion and splicing such fragments. As the normal cell

also has the expression of the MDR1 multidrug resistance pump (P-gp), we added flag label into the PMX vectors to distinguish the expression between autochthonous and exogenmdrous MDR1 gene.

The flag tag, which is a peptide fragments composed of eight hydrophilic amino acids (Asp-Tyr-Lys-Asp-Asp-Asp-As-of Lys), has one binding site of specific monoclonal antibody and fused to recombinant protein. Because of its hydrophilic characteristics, the flag tag is fixed on the surface of fusion protein, and therefore, it is more likely to combine with antibodies and catalyzed by enterokinase. As the flag tag is composed of only eight amino acids, it does not occupy other epitopes or binding domains, so it would not change the function, secretion and transmission of the fusion protein (Huebner et al., 2009). We can detect the glycoprotein expressed by exogenous gene MDR1 through anti-flag antibody in western blotting, and avoid the interference of the P-gp pump induced by autochthonous multidrug resistance gene.

In recombinant PMX-flag-MDR1-GFP vectors, the GFP gene locates on the downstream fragment of MDR1, so the GFP gene would express only if MDR1 has finished its expression, that is, MDR1 should be expressed as long as we observe the GFP expression. The detection of GFP is easy and feasible, so it is suitable for GFP to be traced during MDR1 exogenous expression. In the research, using fluorescence microscope, we detected that the transfected $HPMSC_S$ had already expressed GFP, which proved that MDR1 was successfully transfected and expressed. For further experiment of western blotting, we used the 293-PMX-ABCB1 as a positive control, which showed that MDR1 was expressed at marker 140 Kda, which proved successfully transfected MDR1 into $HPMSC_S$.

P-gp can be used as drug pumps, and its function can be proved by RH-123 or DNR pump experiment. Both Rh-123 and DNR can release AF auto fluorescence which can be easily detected, and they both can be substrate of P-gp. RH-123 and DNR can accumulate inside the sensitive cells that do not present P-gp; the more RH-123 and DNR they have, the stronger auto fluorescence they release (Balcerczak et al., 2010). We managed to detect the strength of the AF by FACS, and we analyzed the residual rate of intracellular drugs by the software of cell quest; in these ways we can detect the P-gp's function. Our research chose the non-transfected cells and 3 days after transfected cells by managing DNR pump experiment, the result of FACS showed that the P-gp expression between the experimental group and control group had a significant difference. The research proved that transfected cells did have biological activity.

Placenta, a waste of obstetrics and gynecology, is easy to acquire, $HPMSC_S$ can be isolated from it, and it provides plenty of seed cells for genetic and tissue engineering. The constructing of MDR1 retroviral vector has great contribution to our further research towards MDR1's function and expression. The MDR1 retroviral vectors can effectively transfect $HPMSC_S$; the transfected genes cannot only be expressed, but also function normally. We further carried out multiple differentiation induction identification to HPMSCs transfected with PMX-flag-GFP-MDR1 in order to prevent it from generating genetic mutation, and losing multipotentiality. The results show that the transgenic HPMSCs still maintain the stem cell characteristics of multipotentiality. Next, we will build female rats' gynecological tumors animal model, and treat it with chemotherapy drug, discuss whether the HPMSCS transfected with MDR1 can improve the therapeutic role of high-dose chemotherapy by reconstructing the bone marrow microenvironment and support hematopoietic stem cell transplantation.

ACKNOWLEDGEMENTS

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