Effect of lentivirus-mediated growth and differentiation factor-5 transfection on differentiation of rabbit nucleus pulposus mesenchymal stem cells

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Research

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Abstract

Background：Intervertebral disc degeneration (IDD) is a natural progression of age-related process. Degenerative disc disease (DDD) is a pathologic condition associated with IDD that has been one of the most common causes of chronic low back pain, which can have a severe impact on patients' quality of life. The purpose of this study is to observe the biological and cytological characteristics of rabbit nucleus pulposus mesenchymal stem cells (NPMSCs), and to determine the effect of growth and differentiation factor-5 (GDF-5) on the differentiation of rabbit NPMSCs transducted with lentivirus vector.

Methods: In vitro culture model of rabbit NPMSCs was established and NPMSCs were identified by flow cytometry (FCM) and quantitative real-time PCR (qRT-PCR). Subsequently, NPMSCs were randomly divided into three groups: the lentiviral vector carrying GDF-5 gene used to transfect NPMSCs was recorded as transfection group; the NPMSCs transfected with an ordinary lentiviral vector was recorded as control virus group; the NPMSCs alone was normal group. FCM, qRT-PCR and Western Blot (WB) were used to detect the change of NPMSCs.

Results: The transfected NPMSCs by GDF-5 gene displayed elongated shape, the cell density decreased, and the positive rate of GDF-5 in the transfected group was significantly higher than that in the other two groups ($P < 0.05$). The mRNA expression of KRT8, KRT18, and KRT19 in the transfected group was significantly higher in comparison with the other two groups ($P < 0.05$), and the result of WB was consistent with that of qRT-PCR.

Conclusions: GDF-5 can induce the differentiation of NPMSCs and repair degenerative intervertebral disc. Lentiviral vector carrying GDF-5 gene can be integrated into the chromosome genome of NPMSCs and promote differentiation of NPMSCs into nucleus pulposus cells (NPCs).

Background

Intervertebral disc degeneration (IDD) is a natural progression of age-related process. Degenerative disc disease (DDD) is a pathologic condition associated with IDD that has been one of the most common causes of chronic low back pain, which has a severe impact on patients' quality of life. Apoptosis of nucleus pulposus cells (NPCs) and decrease of cell viability are widely recognized as key factors for disc degeneration, which leads to decrease in extracellular matrix synthesis such as type II collagen and glycoprotein [1, 2]. Prevention or reversal of IDD is a promising treatment approach for DDD. However, current treatment methods for IDD, including surgical and conservative options such as medications, steroid injection, and physical therapy, are mainly focused on alleviating the sysmptoms rather than restoring the disc structure and biomechanical function of IDD. If the number of NPCs can be supplemented by artificial intervention, we may fundamentally reverse the process of IDD. It has been reported that there is a type of stem cell in the nucleus pulposus (NP) with strong proliferation and differentiation capabilities, which is nucleus pulposus mesenchymal stem cells (NPMSCs) [3, 4]. In this study, GDF-5 gene was used to intervene NPMSCs of rabbits in vitro, and the effect of GDF-5 gene on

differentiation of NPMSCs was observed. Despite limited data, biological intervention is a promising treatment approach for DDD that could impact our future management of low back pain.

Materials And Methods

Adult healthy rabbits (purchased from Experimental Animal Feeding and Management Center of Bengbu Medical College).

Main materials and instruments

Cells: rabbit NPMSCs wereisolated and cultured in our laboratory.

Instruments: ultra-clean working table (Bengbu HVAC Purification Equipment co., LTD.), cell culture box (American Saimofei Technology co., LTD.), micro-centrifuge (Hangzhou Aosheng Instrument co., LTD.), PCR instrument (Hangzhou Lattice Scientific Instrument Co., LTD.), flow cytometry (Becton Dickinson, USA).

Reagents: DMEM (Gibco, C11995500CP), RPMI 1640 (Gibco, C11875500BT) fetal bovine serum (Bio IND, 04-002-1 a), Antibiotic-Antimycotic (Lifetechnologies, 15240-112); PBS, pH7.4 (Gibco, 10010-023); Trypsin-edta (0.05%) (Lifetechnologies, 25300-054); Bovine serum albumin (Lifetechnologies, 15561012); Lipofectamine® 2000 Transfection Reagent: Lifetechnologies, 11668-019; Opti-mem ® I Reduced Serum Medium: Lifetechnologies, 31985-062; Western blot and IP cell lysis solution; Predye protein Marker (Fermentas, Lithuania); Poluvinylidene membrane (Millipore, USA).

Antibody: CD90antibody (ABCAM, ab225); CD105antibody (ABCAM, ab221675); CD34antibody (Bioss, bs-0646R); CD45antibody (Bioss, bs-0522R); Fitc-labeled sheep anti-mouse IgG (Bioss, bs-0296g-fitc); Fitc-labeled sheep anti-rabbit IgG (Bioss, bs-0295g-fitc); GDF5 wereantibody (ABCAM, ab93855); Anti-actin antibody (Sigma, USA).

Kit: Ultrapure RNA Kit RNA extraction Kit, kangwei century (CW0581S); SuperRT cDNA Synthesis Kit, kangwei century (CW0741S); UltraSYBR Mixture (High ROX), kangwei century (CW2602M); BCA protein quantitative kit (Shanghai biyuntian institute of biotechnology).

Cell isolation culture and identification

Acquisition of NPMSCs Briefly, the rabbit NP tissues were harvested, digested by collagenase, centrifuged, resuspended, and NPCs were obtained. Then the NPCs suspension was centrifuged. Subsequently, the supernatant fluid was removed and the complete medium of mesenchymal stem cells were added. Finally suspension was performed to obtain NPMSCs. When the cell fusion rate reached up to 80%-90%, they could be digested by the trypsin and re-inoculated in the culture bottle for subculture of NPMSCs. The morphological and biological characteristics of cells were observed under an inverted microscope.

The surface immunophenotype of NPMSCs in rabbits was identified by flow cytometry (FCM). The third generation of NPMSCs cells were digested with trypsin, and then centrifuged. Subsequently, the precipitated cells were collected, washed with PBS, and diluted to cell suspension. The anti-rabbit CD90, CD105, CD34, and CD45 were added. The suspension was incubated at room temperature, washed with PBS, incubated with FITC-labeled sheep anti-mouse/sheep anti-rabbit IgG second antibody away from light, and washed with PBS. After mixing procedure, the mixture was detected by FCM (six times).

Quantitative Real-time PCR (qRT-PCR) Samples were collected to extract RNA, and the mRNA level of the target gene was detected by qRT-PCR. RNA was extracted from the NPMSCs, and then cDNA was synthesized. Subsequently, qRT-PCR was performed, including internal reference GAPDH gene, and three parallel holes was performed for each of the genes per sample. After adding each component to the PCR tube, we carefully seal the plate membrane, mix it evenly, and simply centrifuge the solution to the bottom of the tube. PCR amplification conditions were based on the instructions of the qRT-PCR kit. According to the instructions of the instrument, PCR experiment was carried out (six times). Data were collected and analyzed.

Cell transfection and observation

NPMSCs were divided into three groups: lentiviral vector carrying GDF-5 gene used to transfect NPMSCs was recorded as transfection group; the NPMSCs transfected with an ordinary lentiviral vector was recorded as control virus group; the NPMSCs alone was normal group. Transfection methods: the lentivirus plasmids were diluted to 25 L of opti-mem ® I Reduced Serum Medium, and 0.5 L of Lipofectamin2000 was diluted to 25 L of opti-mem ® I Reduced Serum Medium. The solutions were mixed gently, followed by keeping static for a while, and the mixture were added into the three groups of cell culture wells for culturing.

The gene transfection rate was identified by FCM. All groups of cells were digested with trypsin and centrifuged. The precipitated cells were collected followed by being washed with PBS, and cell suspension was diluted. Anti-rabbit GDF-5 was added and then incubated for 30 minutes, respectively. The cells were incubated with FITC-labeled sheep anti-rabbit IgG second antibody and washed with PBS. After mixing, the positive rate of GDF-5 in all groups was determined by FCM (six times in each of the three groups).

qRT-PCR was used to detect the mRNA expression of keratin 8, 18, 19 (KRT8, KRT18, and KRT19) in all groups. Cell samples were collected, RNA was extracted and then cDNA synthesis was carried out. The samples were detected by qRT-PCR (six times in each of the three groups). The procedures were identical with previously described.

The protein expression levels of KRT8, KRT18 and KRT19 in all groups were detected by Western Blot (WB). Steps include: the extraction of total protein of cells, quantification of protein, protein electrophoresis, film transferring, sealing and incubation, PVDF membrane chemiluminescence, followed by developing and fixing (six times in each of the three groups). Finally the image analysis and statistics

were performed: the developing strip was analyzed and the optical density was scanned by Image J. The ratio of the optical density of the target band and the corresponding value of β-actin was the relative expression.

Statistical methods

The mean values obtained were compared by analysis of variance (ANOVA). Data were presented as mean ± standard deviation.One-way ANOVA was used for comparisons between groups, and independent sample t test was used for pairwise comparison. Differences were statistically significant at *P* value < 0.05.

Results

Culture and identification of NPMSCs

Most of the NPMSCs screened by the complete culture medium of mesenchymal stem cells (MSCs) were single after digestion (**Figure 1A**), some of the cells were adherent to the wall after primary culture for 4 × 6 days, and the morphology of the cells was spindle-shaped or polygonal (**Figure 1B**). After 12 days, cell colony formation was observed, and 3-4 weeks later the cell fusion rate reached 90%. After passage, the cells proliferation increased obviously and the following passage takes only about one week, and the numbers of spindle cells began to increase. After passaging to the third generation, most of the cells were fusiform (**Figure 1C**).

FCM showed that the surface immunophenotype CD34, CD45 were negative (**Figure 2B**), the surface immunophenotype CD90, CD105 were positive in detected cells (**Figure 2C**). The expression rate of CD molecule of surface immunophenotype of NPMSCs is shown in **Figure 2D**.

qRT-PCR results: NPMSCs expressed stem cell gene SOX2, Nanog (**Figure 1D**).

Detection of GDF-5 gene transfected NPMSCs

The transfected NPMSCs displayed enlongated shape, and the cells density decreased (**Figure 4A**).

FCM showed that the GDF-5 positive expression rate (%) of the transfected group was significantly higher than that of the normal group and the control virus group (P < 0.001) (**Figure 3D**). The positive rates (%) of GDF-5 expression of the three groups were shown (**Figures 3A**, **3B**, **4C**).

The results of qRT-PCR in the three groups showed that the mRNA expression levels of KRT8, KRT18, and KRT19 in transfected group were significantly higher than that in the other two groups, and the difference was statistically significant (P < 0.05) (**Figure 4B**).

WB results: the protein expression levels of KRT8, KRT18 and KRT19 in transfected group significantly increased compared to the other two groups (P < 0.001) (**Figure 4C**, **4D**).

Discussion

Lumbar degenerative disease, which is caused by lumbar disc degeneration, has severely affected the quality of life of patients and has become a serious social problem. However, current treatment approaches including surgical and conservative measures could not fundamentally reverse disc degeneration [5, 6]. Fortunately, with the growing number of researchers working on elucidating the molecular mechanism and genetic engineering of disc degeneration, it may be feasible to treat or reverse disc degeneration at the gene level.

Mesenchymal stem cells (MSCs) are pluripotent stem cells originated from the mesoderm, which can differentiate into bone, cartilage, fat, muscle, ligament, tendon and other tissues [7, 8]. In 2006, the International Association for Cell Therapy proposed a common standard for the definition of MSCs [9]: A. In vitro culture, such cells grow adherently to the wall; B. Some specific antigens (markers) are expressed on the cell surface; C. The cells possess the ability to differentiate into adipocytes, osteoblasts and chondrocytes. Many studies have shown that there is a type of cell in the intervertebral disc tissue, and the cell has the ability to differentiate into osteoblasts, chondroblasts and lipid cells. Additionally, the cell also characteristically expresses the surface protein molecules of MSCs and can complete the three-line induced differentiation. Therefore, such cells are named as NPMSCs[10-12].

NPMSCs is the precursor of NPCs, which exists in healthy and degenerative IVD tissues and has the potential to proliferate and differentiate into NPCs, and can be applied to regenerative and repairing sciences [3, 4]. Li XC et al successfully isolated human NPMSCs by fluorescence activated cell sorting and the cells expressed tyrosine kinase receptor 2 (Tie-2) and ganglioside 2 (GD-2). The cells had significant proliferation and differentiation potential and could differentiate into osteoblasts, adipocytes, and chondrocytes [13]. Erwin WM et al demonstrated that NPMSCs possessed a powerful capability to differentiate and proliferate in animal studies, and played an important role in IVD repair, nerve repair and other renewable medicine [14].

Lin et al cultured NPMSCs of rats in vitro and induced differentiation, and found that NPMSCs could express stem cell genes, such as SOX2, Oct4 and Nanog [15]. Hui Zhang et al compared rat NPMSCs and bone marrow MSCs by in vitro culture, and observed that both of them could differentiate into bone, cartilage and fat, and could express stem cell genes Nanog, Oct-4 and SOX-2, with no obvious difference [16]. In this study, FCM was used to detect surface immunophenotype CD molecule of NPMSCs. The immunophenotypes CD90 and CD105 showed positive, and the immunophenotypes CD34 and CD45 were negative. qRT-PCR was performed to determine the positive expression levels of SOX-2 and Nanog, showing that NPMSCs expressed the stem cell genes SOX2 and Nanog.

Scientists have found that human notochord cells (NCs) gradually disappear with the growth of age and disc degeneration, and the NCs are namely NPMSCs [17]. Rodrigues-pinto R et al found that KRT8, KRT18 and KRT19 were specific markers of human NCs, and were expressed in all stages of NCs [18]. Minogue et al detected the mRNA levels of KRT8, KRT18 and KRT19 in bovine NPCs and NCs by RT-PCR, and found that both of them expressed KRT8, KRT18 and KRT19, and the expression level was slightly higher than

that in NPCs [19]. Meanwhile, other researchers have found that NPCs express KRT8, KRT18, KRT19 and other gene phenotypes, and the expression level of NPCs is higher than that of articular chondrocytes and ring fibroblasts [20-22].

GDF-5, also known as BMP14, is a member of the bone morphogenetic protein (BMP) family. BMP was originally thought to be a component of the mineralized bone matrix, and when fractures or ectopic ossification occurred, it can induce the formation of new bone tissue [23, 24]. Current studies have shown that GDF-5 can repair degenerative intervertebral discs and promote the proteoglycan and collagen type II protein expression [25, 26]. GDF-5 was transfected into human-induced pluripotent stem cells to promote disc regeneration in rats[27]. Animal studies have found that the central region of intervertebral disc of GDF-5 knockout mice showed low signal in MRI T2 weighted and the normal lamellar structure of the fibrous ring disappeared, leading to atrophy and disorder of the NP tissues, and the content of proteoglycan decreased significantly. The expression of proteoglycan and type II collagen mRNA decreased, indicating that the deletion of GDF-5 gene was closely related to IDD. Meanwhile, GDF-5 also promoted the differentiation of stem cells [28]. In current study, mRNA and protein expression levels of KRT8, KRT18 and KRT19 in transfected group were significantly increased compared to the other two groups, suggesting that GDF-5 gene could promote the differentiation of NPMSCs.

Regarding the mechanism of GDF-5 acting on NPMSCs, Liu W found that GDF-5 could inhibit the transcription and expression of RNA fragment microrna-34a, reduce the generation of IL-1 β, and increase the expression of proteoglycan and collagen II type. These findings indicated that GDF-5 could delay or stop the degeneration of the disc [29].

Conclusion

In this study, an in vitro culture model of NPMSCs was established, and gene transfection, qRT-PCR, WB and other techniques were used to demonstrate the existence of NPMSCs in rabbit nucleus pulporeal tissue. GDF-5 was demonstrated to promote the differentiation of rabbit NPMSCs into NPCs in vitro, but the mechanism was not identified. Thus, further studies will be required in the near future. Although a significant gap exists between the research findings and clinical practice, based on currentdata, it helps uncover the pathogenesis of IDD and will advance the research and development of feasible and effective therapies for IDD.

Declarations

Ethics approval and consent to participate

The experimental protocol was established, according to the ethical guidelines of the Helsinki Declaration and was approved by the Ethics Committee of The First Affiliated Hospital of Bengbu Medical College. Written informed consent was obtained from individual or guardian participants.

Consent for publication

Written informed consent for publication was obtained from all participants.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

Gang Xu and Changchun Zhang designed this project. Yuchen Ye conducted literature review. Kun Zhu is responsible for laboratory implementation and paper writing. Zhao Rui did the article polishing and statistical analysis. All authors read and approved the final manuscript.

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None

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Figures

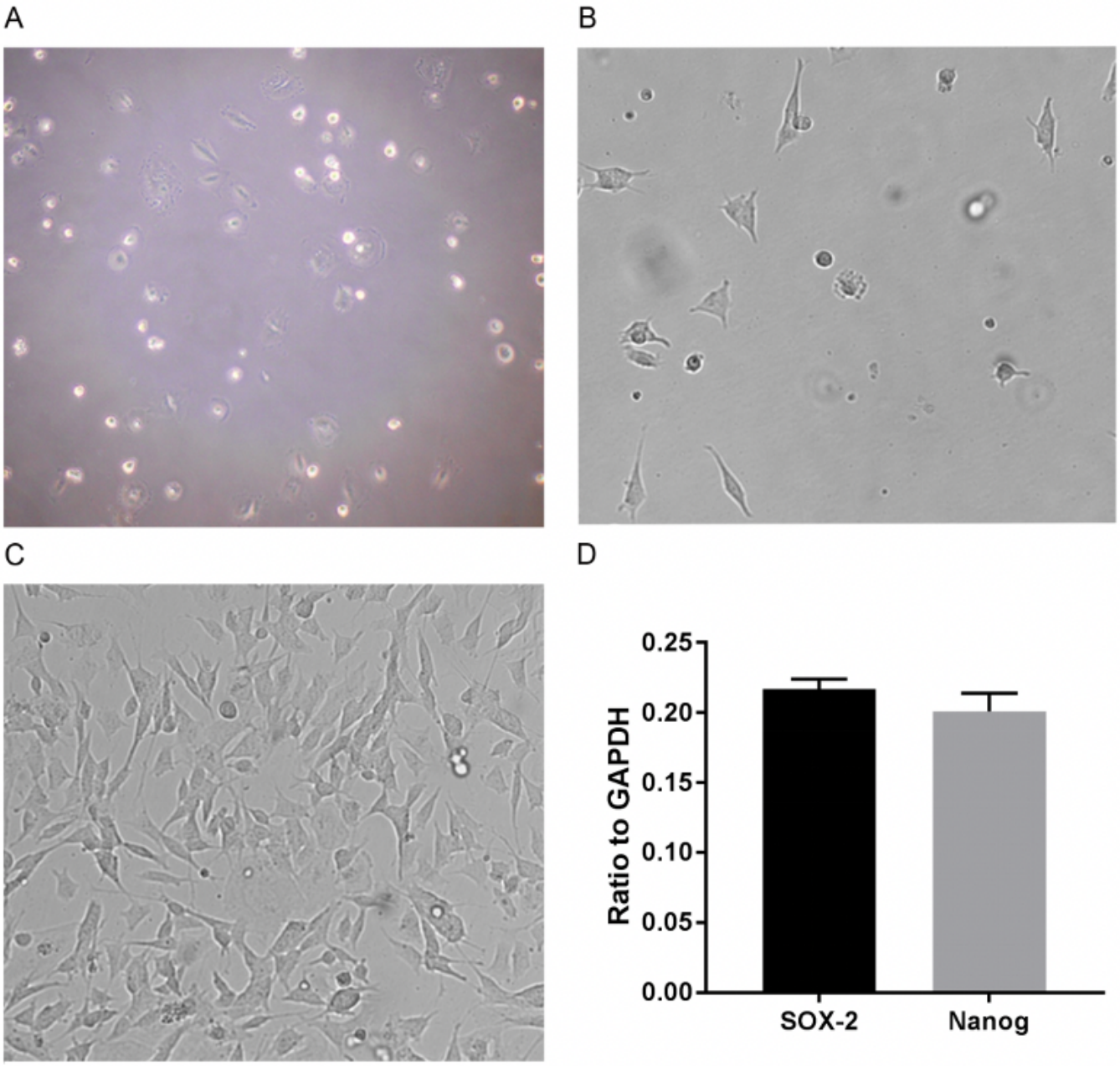

Figure 1

Primary, passage and transfected NPMSCs. A. Completely digested NPMSCs (magnification, ×200); B. Primary NPMSCs (magnification, ×200); C. Third generation (magnification, ×200); D. qRT-PCR results: NPMSCs could express stem cell gene SOX2, Nanog.

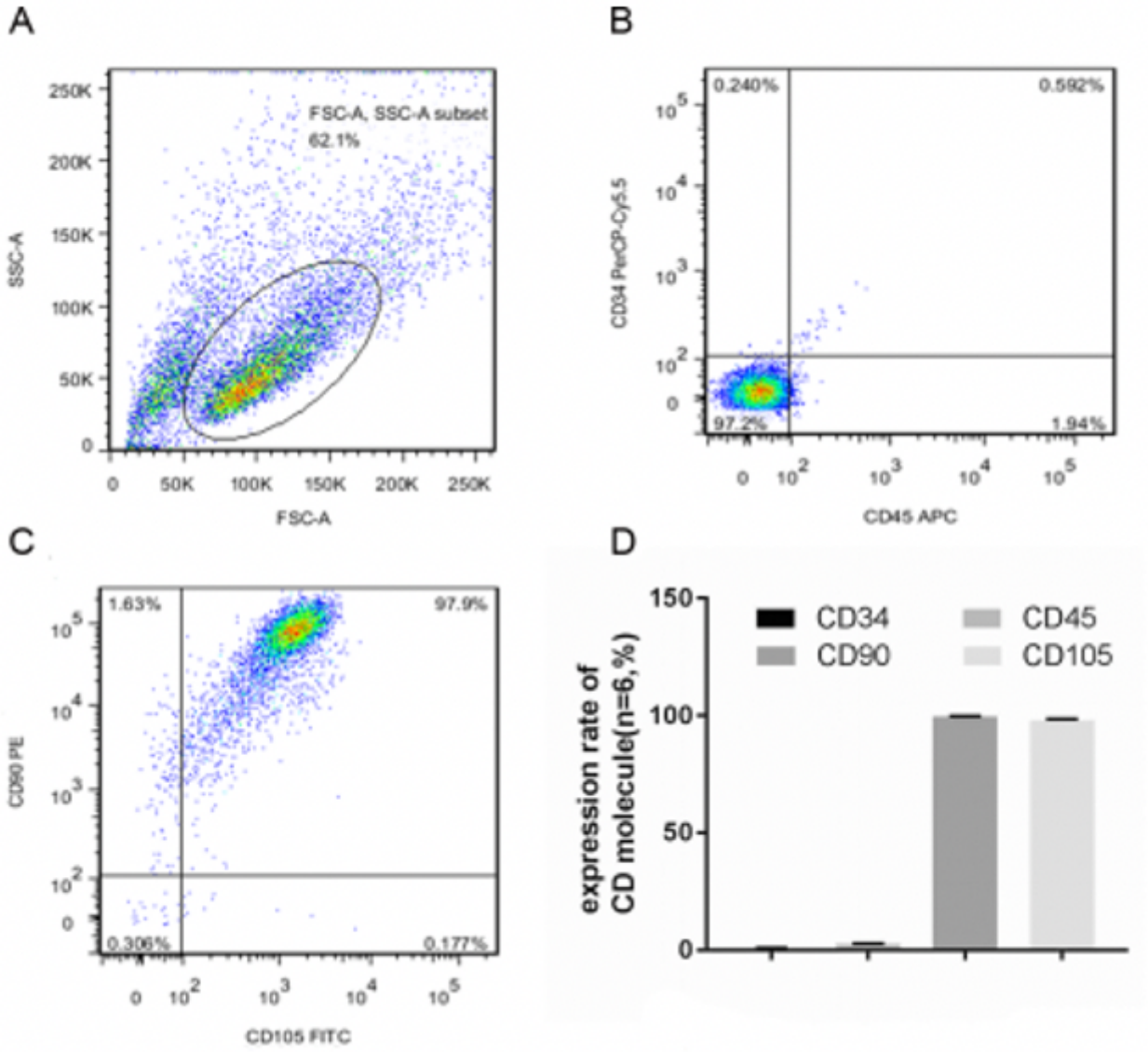

Figure 2

Identification of NPMSCs by FCM. A. Remove cell fragments by FSC/SSC scatter plot, and circle NPMSCs. B. The immunophenotypes CD34 and CD45 of hematopoietic stem cells were negative. C. The immunophenotypes CD90 and CD105 expressed positive. D. The expression rate of CD molecule of surface immunophenotype of NPMSCs.

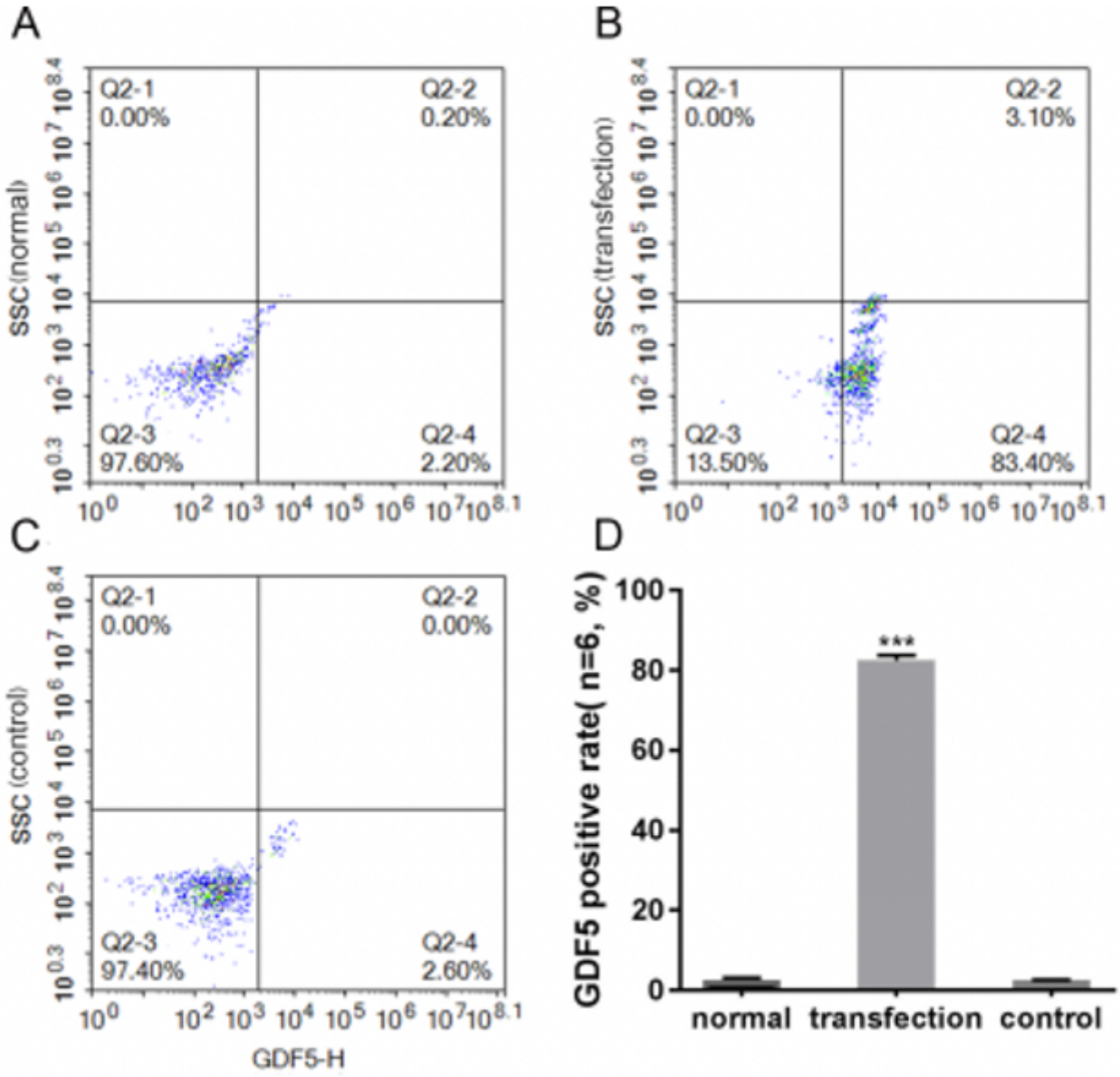

Figure 3

FCM cell transfection rate. A. GDF5 positive rate in normal group; B. GDF5 positive rate (%) in the transfection group; C. GDF5 positive rate of control group; D. The GDF5 positive rate of the transfection group was significantly higher than that of the normal group and the control group, and the difference was statistically significant ($P < 0.001$).

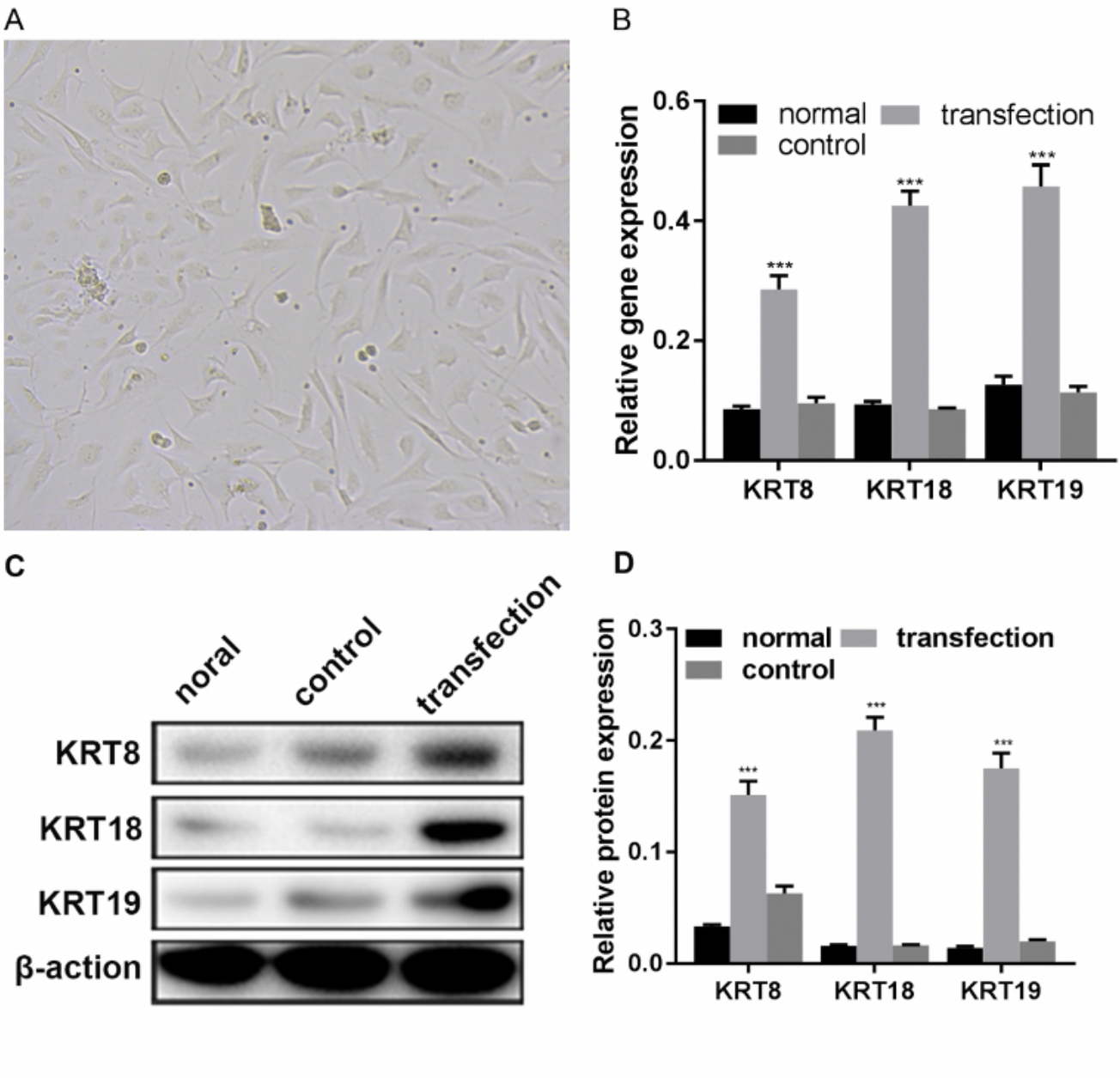

Figure 4

A. Recombinant NPMSCs after transfection (magnification, ×200); B. qRT-PCR results: mRNA expression levels of KRT8, KRT18 and KRT19 significantly increased in the transfection group compared to the other two groups ($P < 0.001$). C. WB detected the protein expression levels of KRT8, KRT19 and KRT19 in the three groups. D. WB results: protein expression levels of KRT8, KRT18 and KRT19 significantly increased in the transfection group compared to the other two groups ($P < 0.001$).