Effect of Overexpression of Hes1 Factor in Human Umbilical Cord Mesenchymal Stem Cells on the Expression of IL-6 Gene

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Abstract

Objective: To investigate the effect of Hes1 overexpression on IL-6 gene expression in human umbilical cord mesenchymal stem cells.Methods: huc-msc was isolated by collagenase digestion, and identified as mesenchymal stem cells by flow cytometry and induction of adipogenesis, osteogenesis and chondrogenesis. Construction of Hes1 overexpression plasmid and packaging of lentivirus. Then, we infected huc-msc cells with lentivirus. The expression of IL-6 in huc-msc cells was detected by real-time PCR, apoptosis and cell cycle were detected by flow cytometry, cell proliferation was detected by CCK-8, and the expression of IL-6 in supernatant was detected by ELISA. Results: in vitro experiments showed that HUC MSc possessed the characteristics of mesenchymal stem cells in morphology, cell surface phenotype and induced differentiation ability. After overexpression of Hes1 in huc-msc, IL-6 gene expression increased significantly, cell cycle and apoptosis did not change significantly, cell proliferation was not inhibited, and IL-6 in cell supernatant increased significantly.. Conclusion: Hes1 can promote the expression of IL-6 in huc-msc cells.

Keywords

Cord mesenchymal stem cells, Lentivirus, Notch signal, Hes1.

1. Introduction

The biological activity of umbilical cord mesenchymal stem cells (ucmscs) is good, and the amount of material taken is large and there is no ethical limit. Ucmscs have the potential of self-renewal and differentiation into mature cells such as bone, cartilage, fat, cardiac muscle and muscle fiber. In addition, it also has the following biological characteristics of MSC, mainly including:[1] immunoregulation: low immunogenicity, less rejection after transplantation. [2]Paracrine function: MSCs can produce cytokines, chemokines, exosomes, etc., and participate in immune regulation. [3]Homing characteristics: with the effect of chemokines, targeted migration to damaged tissue. Therefore, hucmscs are commonly used as seed cells in the treatment of diseases, tissue engineering, regenerative medicine and other research. In vivo and in vitro, cell tracing is necessary. Notch signaling is a classical signaling pathway in many animals, which plays an important role in regulating the growth, differentiation and regeneration of various tissues. Mammalian expression of four Notch receptors (notch 1-4) and five ligands (Jagged 1, jag 2, delta 1, delta 3, delta 4) through cell-cell contact activation, through two proteolysis, activate downstream target gene Hes1, downstream target gene expression is notch. [4]Previous studies showed that LPS could activate the Notch signaling pathway of rat bone marrow mesenchymal stem cells and promote the expression of Hes1 and hey1 protein downstream. According to other literature, [5]Hes1 can promote the expression of IL-6 in mouse chondrocytes. The aim of this study is to overexpress Hes1 in cord mesenchymal stem cells infected with lentivirus and to verify its effect on L-6.

2. Experimental Materials

1.1 the cord blood and cord were taken from full-term newborns and normal parturients in overseas Chinese hospital. DMEM / F12 culture medium is GIBCO company, Percoll (1.073 g / ml) is sigma

company's product; Ficoll (1.077 g / ml) is purchased from Institute of Hematology, Chinese Academy of Medical Sciences; fetal bovine serum is GIBCO company's product; induction reagent: dexamethasone, β - glycerin phosphate, ascorbic acid phosphate, 3-isobutyrate-1-methylxanthine (IBMX), insulin, indomethacin are sigma company's products; Flow cytometric monoclonal antibodies (mAbs) against human including PE labeled CD14, CD29, CD31, CD34, CD73, CD105 and FITC labeled CD44, CD45, CD90, HLADR are all products of Becton Dickinson, alkaline phosphatase test kit is sigma, reverse aidtm firststrand cDNA synthesis kit, Taq DNA polymerase , dNTP mix (Takara), SYBR... Green realtime PCR Master Mix Plus, plus solution (Toyobo); the primers used were designed by ourselves according to GenBank sequence and synthesized by Beijing Qingke biological company.

3. Methods

3.1 Isolation and culture of HUC MSc

Take the umbilical cord within one hour after delivery place it in a sterile 50ml centrifuge tube and quickly take it to the ultra clean bench. Wash the umbilical cord with sterile physiological saline for three times. Cut it into 1.5ml EP tube and make it paste with naked eyes. Add DMEM / F12 culture medium containing 20% FBS (250-500 μ L, according to the amount of tissue homogenate), gently blow the pipette to the full suspension, evenly spread it to the bottom of 25cm2 culture bottle, and put it in 37 ℃ and CO2 incubator for 16-18h Take it gently to the inverted microscope to observe the growth characteristics and morphological characteristics of the cells, and replenish the fluid properly. Every 2-3 days, use DMEM / F12 medium of 10% FBS to change the medium. Pay attention to observe the growth of cells. When the cells around the tissue block gather, they can be subcultured for the first time. When they are subcultured, they can be subcultured in a flask with a bottom area of 75 cm2 or larger. When they are fused for about 3-5 days, 80% of them can be subcultured. The third generation of HUC MSCs were collected for the next experiment. If there were more, they would be frozen.

3.2 Flow cytometry

Flow cytometry was used to detect the cell surface markers. The adjusted density of the third generation of cells was 1 × 106 / ml; PBS was washed twice; PE labeled CD14, CD29, CD31, CD34, CD73, CD105 antibodies and FITC labeled CD44, CD45, CD90, HLA-DR antibodies were added 10 μ l each, and a tube was set as the blank control; room temperature dark response 30 min, PBS washing once, 200 μ l polyformaldehyde fixation, after repeated PBS washing for 2-3 times, FACS caliber was used for detection, and cell quest software was used for data analysis.

3.3 Induction experiment

In the induction experiment, the cultured MSCs were inoculated into the 6-well plate according to 1 × 104 / cm2. After the cells were attached to the wall, the fat induction system was added: insulin 10 μ g / ml, dexamethasone 1 μ mol / L, IBMX 0.5 mmol / L, indomethacin 60 μ mol / L, 100ml / L FBS, high glucose DMEM. The fluid was changed in full every 3 days. After 2 weeks, the oil red staining was carried out to observe the formation of fat droplets in the cells under the microscope. At the same time, the cells were seeded on the 6-well plate with 5 × 103 / cm2, and added into the osteogenic induction system: 10 mmol / L β - glycerophosphate, 50 μ mol / L ascorbic acid phosphate, 0.1 μ mol / L dexamethasone. After 4 weeks, von Kossa staining was performed. Add chondrogenic induction system: insulin, transferrin, sodium selenite 6.25 μ g / ml, BSA 1.25 μ g / ml, sodium pyruvate 1 mmol / L, ascorbic acid phosphate 37.5 μ g / ml, TGF β 150 ng / ml, change fluid in half every 3 days, culture continuously for 4 weeks, alcian blue staining, microscopic observation.

3.4 Qpcr

Real time PCR was used to detect the notch ligands on the surface of uc-msc and the expression of Hes1 gene in CD34 + cells to extract the total RNA of cells. Using oligo (DT) as the primer, M-MuLV reverse transcriptase was used to reverse transcribe into cDNA at 42 ℃. The target gene was amplified by PCR using cDNA as template and β - actin as internal reference. The primers used were

designed according to the sequences found on GenBank (Table 1). Fluorescence the quantitative PCR reaction system was 20 μ L, in which SYBR Green 10 μ L, cdna2 μ g, upstream and downstream primer mixture (10 pmol / μ L) 2.4 μ L, ddH2O were supplemented; the reaction parameters were set as follows: pre denaturation of 95 ℃ for 30 s; (95 ℃ for 5 s; 56 ℃ for 10 s; 72 ℃ for 15 s to collect fluorescence at the end of extension) 40 cycles; fusion analysis (50 ℃ - 99 ℃).

3.5 Western blot

Western blot was used to detect the protein expression protein lysate to treat each group of samples. The samples were cleaved at low temperature for three times, each time for 30 s, centrifuged (4 ℃, 12000 × g, 10 min). The protein concentration was measured, the protein concentration of each group was balanced, the protein denaturation was carried out. After SDS-PAGE electrophoresis, the membrane was transferred to PVDF membrane, 5% skimmed milk was sealed for 2 h, nestin (1:500), cyclin D1 (1:1000) , Smad4 (1:1000), BMP2 (1:500) 4 ℃ refrigerator overnight, discard the first reactant, wash the membrane with tbst for 5 min, 3 times; turn the membrane to the second antibody (1:10000) was incubated in the hybrid bag at room temperature for 2 h, and then discarded. Tbst was washed for 5 min for 3 times. ECL system was developed and β - actin was used as the internal parameter to calculate the relative gray value, and the experiment was repeated for 3 times.

3.6 Statistical analysis

statistical analysis the experimental data were expressed by mean ± SD, and the significant level of the difference was detected by bilateral students' t-test. $P < 0.05$, the difference was statistically significant.

4. Experimental results

4.1 Morphological observation of HUC MSCs

As can b e seen from Fig. 1, it can be seen from (a) of Fig. 1 that the cell morphology of P1 generation becomes slender, more similar to the long fusiform of MSCs, and tends to be consistent (b); the cell morphology of P2 and P3 generation has been roughly the same, showing a typical long fusiform, vortex like growth (C, d); the morphology of P4 and P5 generation of HUC MSCs is stable (E, f).

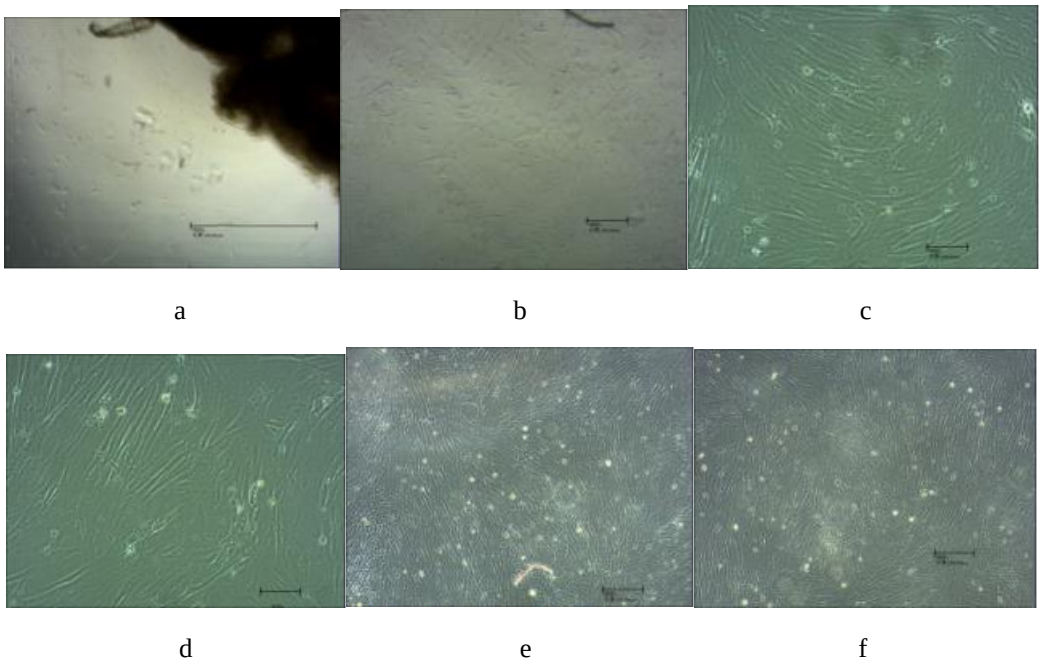


Figure 1. The morphologies of different passage hUC-MSCs.

a. p0 hUC-MSCs; b. p1 hUC-MSCs; c. p2 hUC-MSCs;

d. p3 hUC-MSCs;e. p4 hUC-MSCs; f. p5 hUC-MSCs.

4.2 Cell cycle of HUC MSCs

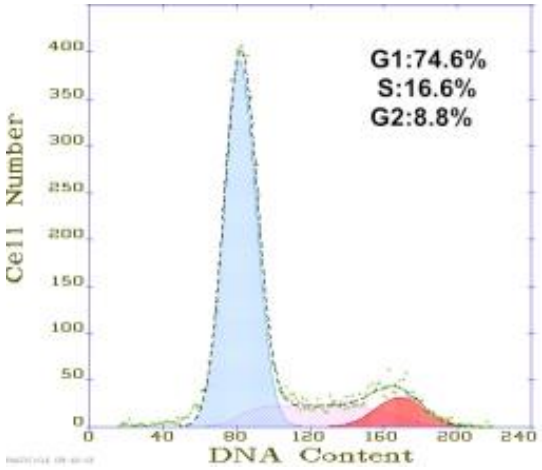


Figure 2 The cell cycles of p3 hUC-MSCs.

According to Figure 2, about 74.6% of the cells in the third generation of HUC MSCs are in the G1 phase of still division, while the proportion of cells in G2 / S phase of cell division is about 25.4%, indicating that the proliferation activity of HUC MSCs is very good.

4.3 Apoptosis detection of HUC MSCs

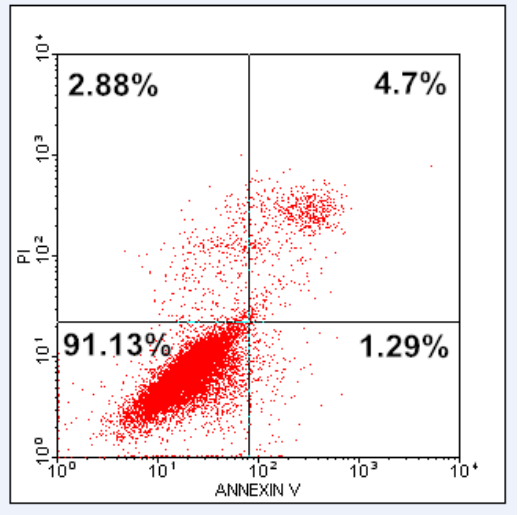
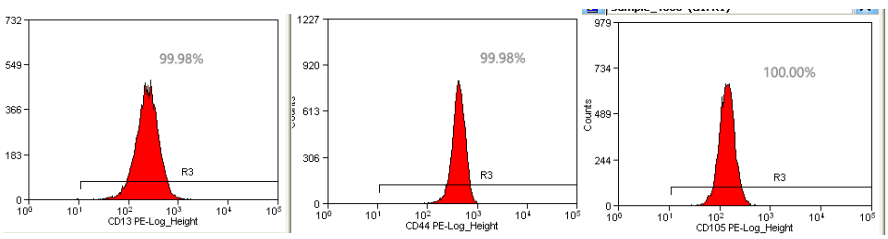


Figure 3 Apoptosis of hUC-MSCs in the third Generation

As can be seen from figure 3, about 94.1% of the cells in the third generation of HUC MSCs are in normal state, while the proportion of cells in early and late stage of apoptosis is 5.99%, indicating that most of the HUC MSCs are in good state and have high activity.

4.4 Detection of cell surface markers in HUC MSCs



a b c

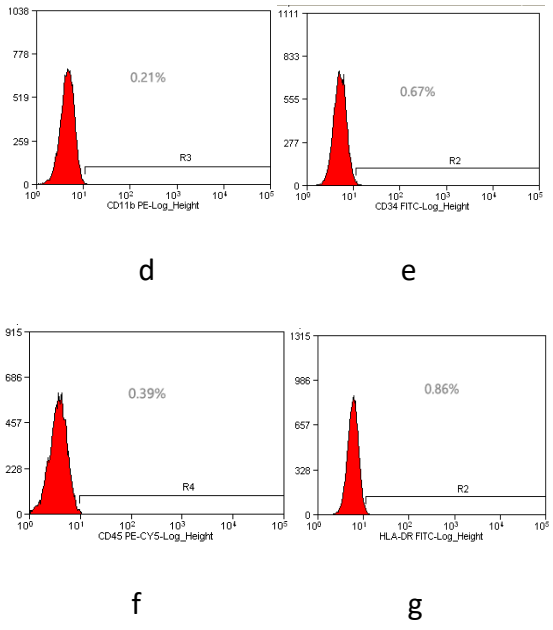


Figure 4 The surface markers of hUC-MSCs.

It can be seen from figure 4 that the positive rates of CD13, CD44 and CD105 of the third generation of HUC MSCs are 99.98%, 99.98% and 100% respectively, while the negative rates of CD11b, CD34, CD45 and HLA-DR are 0.21%, 0.67%, 0.39% and 0.86% respectively, indicating that the purity of HUC MSCs is very high.

4.5 Detection of multi-directional differentiation ability of HUC MSCs

As can b e seen from figure 5, compared with the control group (a), alizarin red staining was positive after osteogenesis induction, and the cytoplasm was stained orange red (b); compared with the control group (c), toluidine blue staining was positive after chondrogenesis induction, and the cytoplasm was stained purple blue (D); compared with the control group (E), oil red O staining was positive after lipogenesis induction, and red lipid droplets (f) appeared in the cytoplasm. The above results indicate that HUC MSCs have the ability of multidirectional differentiation.

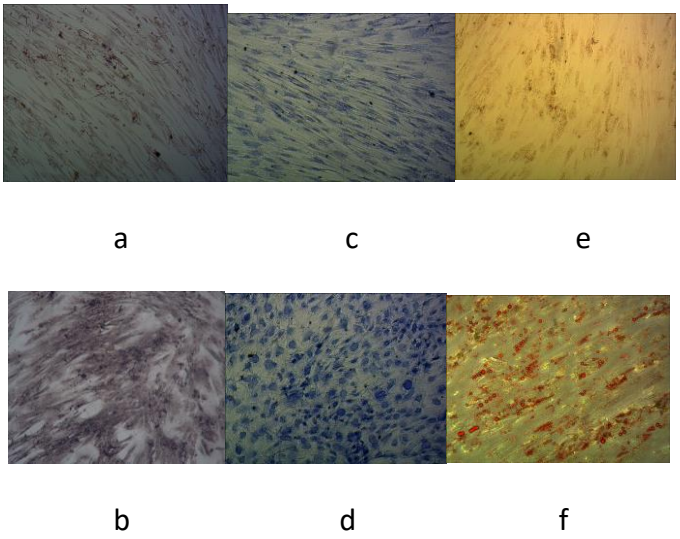
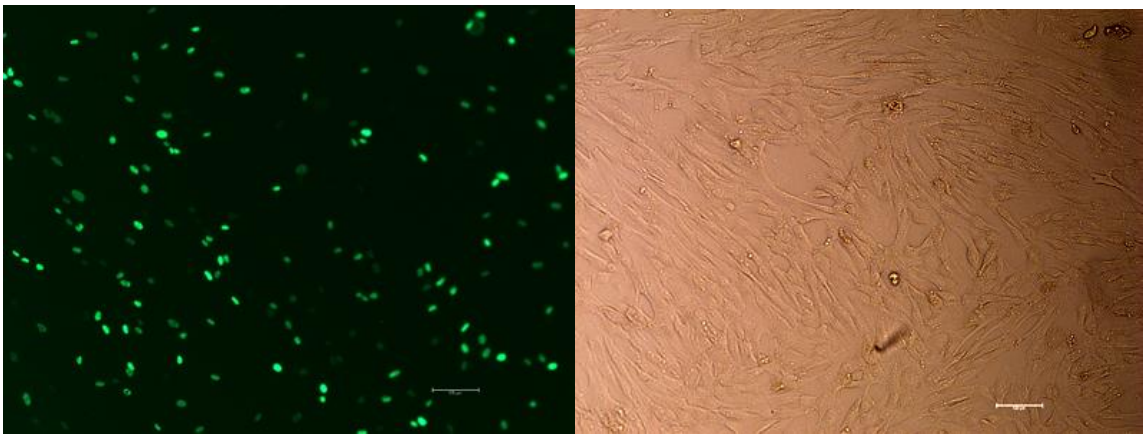


Figure 5. Multidirectional differentiation of hUC-MSCs

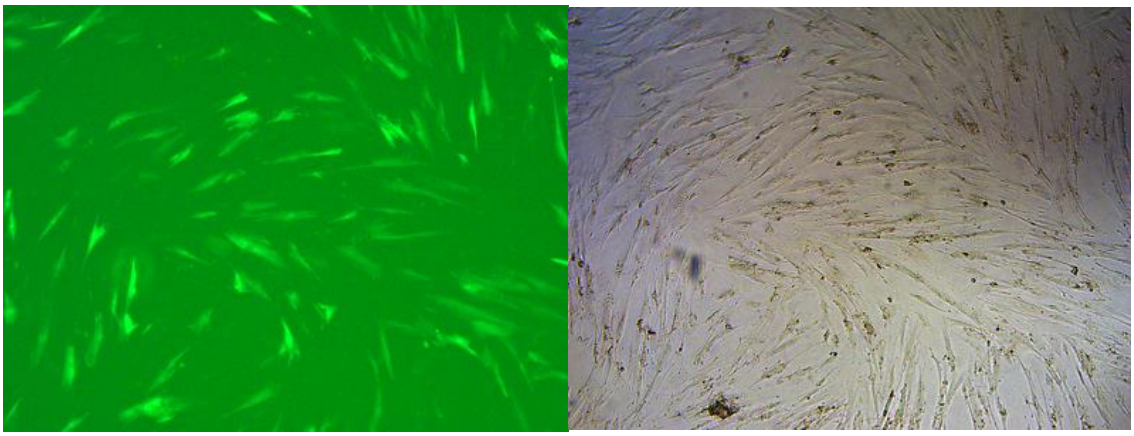
a. Alizarin red staining control group; b. alizarin red staining experimental group;
c. toluidine blue staining control group;d. Toluidine blue staining experimental group;
e. oil red O staining control group; f. oil red O staining experimental group.

4.6 Observe the green fluorescence of HUC MSCs in fluorescence microscope

48 hours after chronic virus infection with HUC MSCs, as shown in Figure 6, green fluorescence was observed in both experimental and control groups under inverted microscope. In Hes1 group, the green fluorescence was small and concentrated in the nucleus. In the control group, the fluorescence was dispersed in the whole cell, indicating that the lentivirus had successfully infected the HUC MSCs cells. In the experimental group, the location of green fluorescence coincided with that of nucleus, indicating that Hes1 transcription factor fused with green fluorescence entered into nucleus of HUC MSCs.



a b



c d

Fig. 6 lentivirus infection with hUC-MSCs

a Hes1 group virus infection hUC-MSCs fluorescence field of vision;

b Hes1 group virus infection hUC-MSCs bright field of vision;

c control group virus infection hUC-MSCs fluorescent field of vision;

d control group virus infection hUC-MSCs bright field

4.7 Analysis of fluorescence positive rate, cycle and apoptosis results

According to figure a of 3-2, D, we can see that the infection efficiency of lentivirus Hes1 and control group is 25.75% and 23.07% respectively. It can be seen from Fig. 3-2 B and f that in the control group, the G1 phase is 66.42%, while in the experimental group, the G1 phase is 72.09%. From figure 3-2, the apoptosis rate of C and G experimental group is 5.03%, and that of control group is 5.47%.

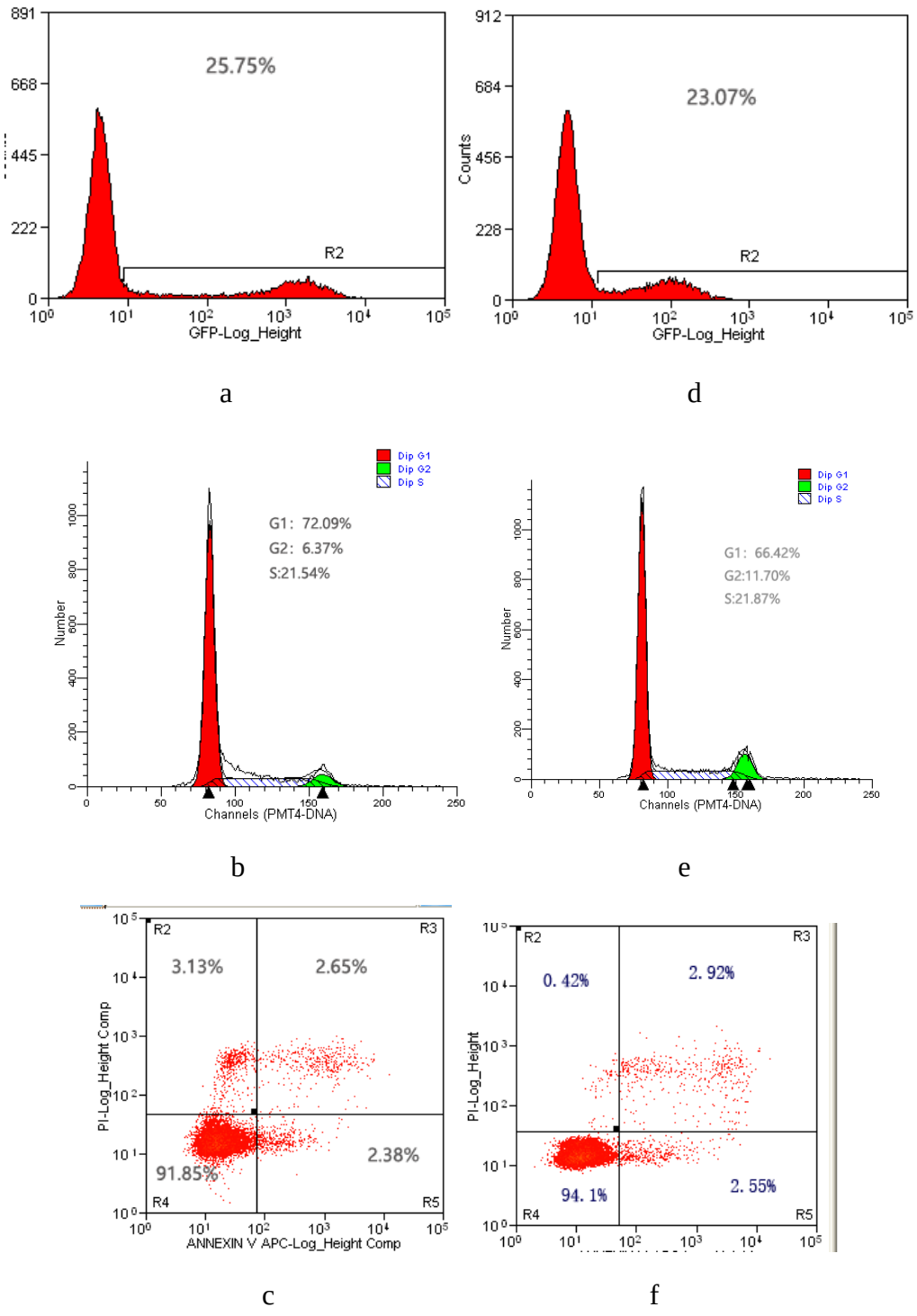


Fig. 7 flow detection**.** In Hes1 group, the positive rate of hUC-MSCs fluorescence, cell cycle and apoptosis were observed. The positive rate of hUC-MSCs fluorescence, cell cycle and apoptosis were found in GFP group.

4.8 Detection of cell proliferation ability of HUC MSCs and uninfected HUC MSCs after chronic virus infection

According to the OD value detected by CCK-8, the proliferation curve was drawn. According to Fig. 3-3, there was no significant difference between the three groups 24 hours and 48 hours after inoculating 96 well plates with HUC MSCs, and the OD value increased, indicating that the cells proliferated slowly in this period of time. At 72 hours, the OD value of the uninfected group was

significantly higher than that of the two groups infected with lentivirus, indicating that the cells in the uninfected Group continued to grow, but the proliferation of cells in the experimental group and the control group was inhibited.

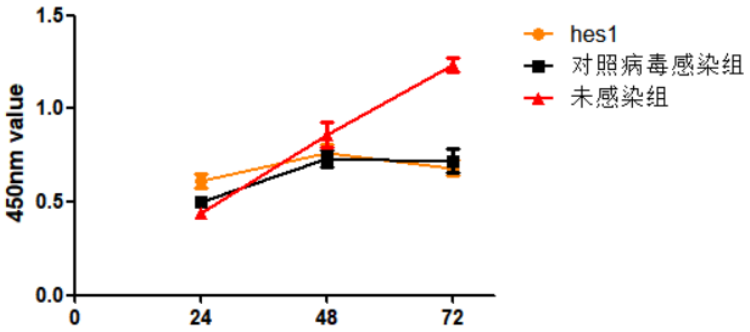


Fig. 8 Proliferation of hUC-MSCs cells in each group

Proliferation curve of Hes1 group, control virus infected group and uninfected group

4.9 Expression level of Hes1 protein fused with GFP after chronic virus infection with HUC MSCs

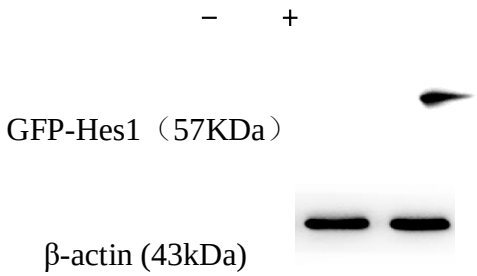


Fig. 9 Hes1 protein expression level

In Figure 9, it can be seen that the Hes1 overexpression group fused with GFP has very obvious bands, while the Hes1 protein (30kda) expressed by HUC MSCs itself in the control group does not fuse with GFP, so there is no band in the same molecular weight. The above results indicated that Hes1 transcription factor fused with GFP was overexpressed in HUC MSCs.

4.10 Analysis of qPCR results after infection of lentivirus by HUC MSCs

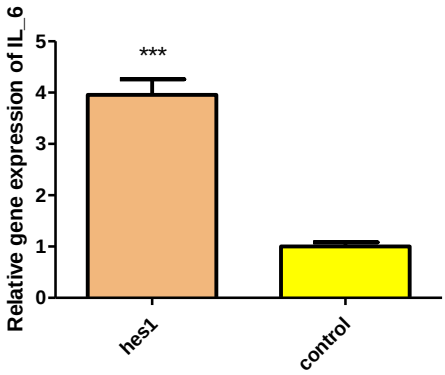


Fig. 10 Expression of IL-6 gene after lentivirus infection with hUC-MSCs(n=3)

***p < 0.0001,compared to control group.

4.11 Detection of IL-6 in supernatant of UUC MSCs after chronic virus infection

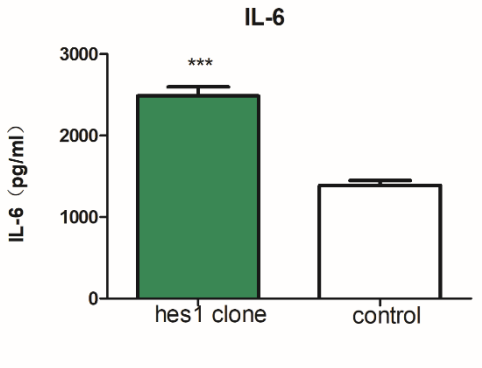


Fig. 11 content of IL-6 in hUC-MSCs supernatant after lentivirus infection (n=3)

***p < 0.0001, compared to control group

From the results of figure 11 it can be seen that compared with the control group, the content of IL-6 in the cell supernatant increased significantly 48 hours after infection with lentivirus. According to the statistical method, the result was p < 0.001, the difference was very significant, with statistical significance.

5. Discussion

The results of cell cycle detection showed that the cells in the experimental group decreased slightly compared with the G2 / S phase of the control group, but there was no significant change in the G2 / S phase of cell division and the identification group of uninfected virus in the experimental group, indicating that Hes1 and lentivirus had no significant effect on cell proliferation 48 hours after infection with HUC MSCs, and the cell proliferation ability was still strong. The results of apoptosis showed that the apoptosis rate of the experimental group and the test group were basically the same, all of which were about 5%, indicating that neither the lentivirus nor the Hes1 factor carried by the virus had significant effect on the apoptosis.

Notch signaling pathway regulates cell proliferation. CCK-8 detection shows that the cell proliferation ability of overexpression group, control group and uninfected chronic disease group is significantly lower than that of uninfected chronic disease group, which is inconsistent with the research of Tang Li et al. [6]. The possible reason is that different lentivirus systems are used, which have different toxicity to cells. It is also possible that the lentivirus vector used in this experiment has purinomycin resistant gene, and long-term effect in the cells may produce toxicity to HUC MSCs. There is also a potential reason for the relative vulnerability of HUC MSCs, which become very sensitive after infection with lentivirus. At this time, the trypsin used in cell passage will produce certain damage to it, thus affecting its growth state.The results of qPCR showed that the expression of IL-6 was up-regulated in the experimental group, which was consistent with the results of shurei Sugita [5] et al. The results of ELISA showed that the content of IL-6 in the extracellular supernatant increased significantly, and the cells were buried in the plate at the same time, infected with chronic diseases, changed the culture medium, collected the supernatant, and the positive rate of infection was similar through flow cytometry, so these results showed that Hes1 could promote the level of IL-6 protein.IL-6 cytokines have a variety of biological functions and are one of the key proteins regulating the complex cytokine network of the body [7]. In the case of inflammation, necrosis or the increase of IL-6 secreted by immune cells stimulated by tumor cell antigens, IL-6 in serum is increased, and overexpression of IL-6 is often related to some diseases [7]. Wang Jirong et al. [8] found that the expression of Hes1 and hey1 was up-regulated in LPS induced secretion of IL-6 and CXCL1 by BMSCs, while inhibition of Notch signaling pathway by inhibitors reduced the secretion of IL-6 and CXCL1. In this study, it was found that overexpression of Hes1 could promote the

expression of IL-6 in HUC MSCs, which suggested that Hes1 might be one of the direct regulators of LPS induced IL-6 expression.Microenvironment has an obvious effect on gene expression and cell differentiation of mesenchymal stem cells, thus changing their original characteristics. Exosomes derived from gastric cancer can promote the high expression of TGF - β, IL-6 and N-cadherin in HUC MSCs, thus promoting the occurrence and growth of SGC7901 cells subcutaneously transplanted in nude mice [9]. Overexpression of IL-6 in tumor microenvironment can promote the malignant transformation of MSCs [9]. Microenvironment also plays a role in the aging of mesenchymal stem cells. When MSCs are induced to senescence and differentiation under different microenvironment, LKB1 expression is gradually increased, and the expression of senescence related genes is also high. LKB1 promotes MSCs senescence through AMPK phosphorylation [10]. Therefore, in the treatment of arthritis by transplantation of umbilical cord mesenchymal stem cells, there may also be changes in gene expression of mesenchymal stem cells caused by OA microenvironment. In the typical Notch ligand, Jag1 expression in mouse OA tissue is very rich and increases with the deterioration of arthritis [11]. Literature studies show that IL-6 is highly expressed in mesenchymal stem cells [35], and our experimental results show that Hes1 can promote the expression of IL-6 in hcmscs. Therefore, we speculate that the microenvironment of arthritis may activate the Notch signaling pathway of hcmscs to up regulate Hes1 and promote the expression of IL-6. The synovium of knee joint contains mesenchymal stem cells [12], so the microenvironment of arthritis may also promote the secretion of IL-6 by the mesenchymal stem cells. IL-6 is associated with pain in arthritis, so mesenchymal stem cells may have adverse effects on patients after injection into the arthritis site. But the secretion of IL-6 can promote the proliferation of MSC and prevent its apoptosis. IL-6 is necessary for the survival of MSC cells. IL-6 plays different roles in different diseases during the treatment of mesenchymal stem cells. Zhao, JL and others found that IL-6 is a key protein in the induction and repair of Alzheimer's disease by HUC MSCs [13]. At the same time, IL-6 secreted by HUC MSCs can promote the migration and proliferation of osteosarcoma cells [14]. In a word, how to control the expression of IL-6 in the treatment of different diseases by mesenchymal stem cells is the premise of effective use of mesenchymal stem cells to solve patients' pain.

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