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DAPT inhibits the chondrogenesis of human umbilical cord mesenchymal stem cells

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Abstract: Notch signaling plays a key role in cell proliferation and differentiation, and is important in several biological processes, but its role in the chondrogenesis of human umbilical cord mesenchymal stem cells (UC-MSCs) is still unknown. N-[N-(3,5-difluorophenacetyl-L-alanyl)]-(S)-phenylglycinet-butyl ester (DAPT) is the inhibitor of Notch pathway. The aim of this study is to investgate the influence of DAPT on the chondrogenesis of UC-MSCs. In our study, UC-MSCs were isolated from human umbilical cord and their characteristics were identified. The UC-MSCs were induced to differentiate into chondrocytes *in vitro* and treated with 5 μM DAPT. Glycosaminoglycan (GAG) and collagen type II (COL-2A1) were analyzed qualitatively and quantitatively. The gene expression of Notch-1, Hes-1, GAG and COL-2A1 were analyzed by quantitative polymerase chain reaction (qPCR). The UC-MSCs separated from human umbilical cord, followed the characteristics of Mesenchymal Stem Cells (MSCs). The gene expression of Notch-1 and Hes-1 decreased after chondrogenic induction but the percentage in G1 period and the content of GAG and COL-2A1 increased. The expression of all tested Notch signaling and proliferation genes declined when 5 μM DAPT was added, also the content of GAG and COL-2A1 also decreased. Our study revealed that Notch signaling exists in UC-MSCs and it may remain the proliferative activity of UC-MSCs. Once the chondrogenesis begins, Notch signaling strength decline evidently. DAPT inhibits the chondrogenesis of UC-MSCs.

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Keywords: cartilage tissue engineering, chondrogenesis, COL-2A1, DAPT, GAG, Notch signaling

Abbreviations:

UC-MSCs, human umbilical cord mesenchymal stem cells; COL-2A1, collagen type II; DAPT, N-[N-(3,5-difluorophenacetyl-L-alanyl)]-(S)-phenylglycinet-butyl ester; GAG, Glycosaminoglycan; qPCR, quantitative polymerase chain reaction; MSCs, Mesenchymal Stem Cells; ECM, extracellular matrix; BMSCs, bone marrow mesenchymal stem cells; NICD, Notch intracellular domain; DMEM, Dulbecco's Minimum Essential Medium; FBS, fatal bovine serum; PBS, Phosphate Buffered Saline; IBMX, 3-isobutyl-1-methylxanthine; DMSO, dimethylsulfoxide.

1 Introduction

Articular cartilage is an important kind of connective tissue in our body, it consists of chondrocytes and extracellular matrix (ECM), and the major components of ECM are GAG and COL-2A1 [1,2]. Osteoarthritis is the main cause of injury and degeneration of articular cartilage, which is still one of the major medical challenges and brings enormous pain and economic burden to the patients [1,3]. Autologous chondrocytes transplantation is a traditional method to repair cartilage damage and has obtained satisfactory clinical effect. However, the limited self-repair capability of articular cartilage and the shortage of chondrocyte sources are still the main barriers of cartilage repair [4-6]. With the development of cytobiology and biomaterials, cartilage tissue engineering is regarded as one of the most possible methods to repair cartilage damage. Its principle is to culture and amplify specific seed cells in vitro, then the expanded cells are attached to a scaffold and transplanted into body to form a new organization. It involves seed cell, scaffold and cytokine, and it's critical to get enough number of efficient

seed cells [7-9].

MSCs are undifferentiated cells distributed in our body widely. They can differentiate into chondrocytes, osteoblasts, adipocytes and other kinds of histocyte in a suitable microenvironment. Therefore, they became the focus of tissue engineering and clinical applications [10,11]. In recent years, many scholars devoted to the study on bone marrow mesenchymal stem cells (BMSCs) and got certain achievements, but BMSCs need to be taken from autologous tissues, which does harm to the patients [12]. At the same time, it may lead to complications. In addition, BMSCs are in high risk of viral infection, and their abilities of amplification and differentiation will decline considerably with the increasing age. UC-MSCs are separated from human umbilical cord, some studies have suggested that they could differentiate into islet cells [13] and chondrocytes [14] *in vitro*, and relieved the symptoms of Parkinsonism in rodent [15]. Compared with the MSCs from other sources, there are many advantages of UC-MSCs: umbilical cord belongs to normal derelict from puerperae, so they have abundant source and they don't involve ethical debate; they have no negative effect on donors as well. More importantly, the requirement of human leukocyte antigen of UC-MSCs is low, which can expand the donor pool [16,17]. Previous study has also demonstrated that UC-MSCs can reduce the risk of graft-versus-host reaction [18]. Based on above information, UC-MSCs have the unique advantages in tissue engineering.

Notch signaling is a highly conservative pathway in the process of organismic evolution. In the mammals, there are four transmembrane Notch receptors (Notch 1-4), two Jagged-like ligands (Jag-1, Jag-2) and three Delta-like ligands (DLL1, DLL3, DLL4) of Notch signaling. Firstly the Notch ligands combine with the Notch receptors, next the Notch receptors are digested by γ-secretase and the Notch intracellular domain (NICD) transfers into nucleus, and then NICD interacts with downstream transcription factor CSL. Finally, it plays an important role in the cell proliferation and differentiation, organ development and diseases [19]. Previous evidence has also suggested that Notch signaling participates in the chondrogenesis and the proliferation and differentiation of chondrocytes [20,21].The Notch pathway exists in the differentitation and biological processes of multiple myeloma, including cell localization with the bone marrow, proliferation, survival and pharmachological resistance [22], Jag-1 and Notch-1 exist in mammalian cells [23], some studies have indicated that Notch signaling mediated by Jag-1 plays a role in the development of many tissues [24], and has an important effect on the chondrogenesis of BMSCs [25]. Multiple myeloma cells can activate the North pathway due to the over-express of Jad-1 ligands [22,26] thus activate osteoclastogenesis. In T cell acute lymphoblastic luekaemia, Notch-1 can enhance the proliferative advantage and specific chemotactic abilities through acting in concert with chemokine receptor pathways, therefore influencing tumor cell progression and localization [27]. Hes-1 is one of the target genes involved in chondrogenesis [28]. DAPT is the special blocker of Notch signaling. It inhibits the activation of γ-secretase, thus prevents the Notch signaling from activating [29]. In this study, UC-MSCs were induced to differentiate into chondrocytes *in vitro* and treated with DAPT to investigate the effect of DAPT on the chondrogenesis for the first time.

2 Materials and Methods

2.1 Isolation and culture of UC-MSCs

Human umbilical cord was taken from cesarean baby and cut into about 1mm^3 pieces under sterile conditions, then digested by 0.2% collagenase II (Sigma,America) at 37°C for 3 hours in a table concentrator. After that, the digestion product was filtered out by 200 mesh colander and centrifuged at 3000 rpm for 15 minutes. The cells were collected and resuspended with low-glucose Dulbecco's Minimum Essential Medium (DMEM) medium (Gibco, America) contained 10% fatal bovine serum (FBS) (Gibco, America) and 1% 100 U/ml penicillin-100 mg/ml streptomycin (Gibco, America). Finally, the cells were seeded at a density of 2.5×10^4 cells/cm^2 in T25 culture flasks (Corning, America) and put into a incubator with 37°C, 5% CO_2. Medium was changed for the first time after 3 days and the adherent cells were primary UC-MSCs. When the cells grew to about 80% confluence, digested with 0.25% trypsin (Sigma, America), the supernatant was discarded after centrifugation at 800 rpm for 5 minutes. Cells were resuspended with low-glucose DMEM medium (Gibco, America) and seeded at a density of 2.5×10^4 cells/cm^2 in new T25 culture flasks at 37°C, 5% CO_2, their images were captured by inverted microscope (Nikon, Japan).

2.2 Immunophenotype analysis

UC-MSCs at passage 3 were harvested and adjusted to 5×10^5 cells/tube, the mouse anti-human antibodies, including CD11b-PE, CD13-PE, CD34-FITC, CD44-PE, CD45-PC5, CD90-PC5, CD105-PE and HLA-DR-FITC (BD Bioscence, America) were put into the tubes respectively, then incubated in the dark at room temperature for 30

minutes. All samples were detected by flow cytometry (BD Bioscence, America) and analyzed by Cell-Quest software (BD Bioscence, America).

2.3 Osteogenic differentiation

UC-MSCs at passage 3 were harvested and cultured in a 6-well plate (Corning, America) at a density of 2×10^5 cells/well and incubated at 37°C, 5% CO_2. When the cells grew to about 80% confluent, they were transferred to osteogenic differentiation medium based on high-glucose DMEM medium (Gibco, America) and supplemented with 1 μM dexamethasone (Sigma, America), 50 μg/ml vitamin C (Sigma, America), 10 mM sodium β-glycerophosphate (Sigma, America), 5% FBS and 1% 100 U/ml penicillin-100 mg/ml streptomycin, the UC-MSCs of control group were cultured in high-glucose DMEM medium contained 5% FBS and 1% 100 U/ml penicillin-100 mg/ml streptomycin. Medium was changed every 3 days. After 21 days, medium was discarded, the cells were washed with Phosphate Buffered Saline (PBS) for 3 times and fixed with 4% triformol (Sigma,America) for 10 minutes, then washed with PBS for 3 times and stained with 0.1% Alizarin Red (Sigma,America) at room temperature for 30 minutes. Dye was discarded, the cells were washed with distilled water and their images were captured.

2.4 Chondrogenic differentiation

UC-MSCs at passage 3 were harvested and cultured in a 6-well plate at a density of 2×10^5 cells/well and incubated at 37°C, 5% CO_2. When the cells grew to about 80% confluent, they were transferred to chondrogenic differentiation medium based on high-glucose DMEM medium and supplemented with 0.1μM dexamethasone, 50 μg/ml vitamin C, 6.25 μg/ml insulin (Sigma, America), 6.25 μg/ml transferring (Sigma, America), 10 ng/ml TGF-β1 (Sigma, America), 5% FBS and 1% 100 U/ml penicillin-100 mg/ml streptomycin. Medium was changed every 3 days. After 21 days, medium was discarded, the cells were washed with PBS for 3 times and fixed with 4% triformol for 10 minutes, then washed with PBS for 3 times and stained with 0.1% Toluidine Blue (Sigma, America) for 30 minutes at room temperature. Dye was discarded, the cells were washed with distilled water and their images were captured.

2.5 Adipogenic differentiation

UC-MSCs at passage 3 were harvested and cultured in a 6-well plate at a density of 2×10^5cells/well and incubated at 37°C, 5% CO_2. When the cells grew to about 80% confluent, they were transferred to adipogenic differentiation medium A based on high-glucose DMEM medium and supplemented with 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) (Sigma,America), 10 μg/ml insulin (Sigma,America) and 1% 100 U/ml penicillin-100 mg/ml streptomycin. After 48 hours, the cells were changed to adipogenic differentiation medium B contained 10 μg/ml insulin, 10% FBS and 1% 100 U/ml penicillin-100 mg/ml streptomycin. 72 hours later, the cells were changed to adipogenic differentiation medium A again and changed to adipogenic differentiation medium B after 48 hours, then the adipogenic differentiation medium was discarded and changed to low-glucose DMEM medium contained 10% FBS and 1% 100 U/ml penicillin-100 mg/ml streptomycin. The UC-MSCs of control group were cultured in low-glucose DMEM medium contained 10% FBS and 1% 100 U/ml penicillin-100 mg/ml streptomycin all the time. Medium was changed every 3 days. After 21 days, medium was discarded, the cells were washed with PBS for 3 times and fixed with 4% triformol for 10 minutes, then washed with PBS for 3 times and stained with 0.3% Oil Red O (Sigma, America) at room temperature for 30 minutes. Dye was discarded, the cells were washed with distilled water and their images were captured.

2.6 Treatment of different groups

UC-MSCs at passage 3 were harvested and cultured in a 6-well plate at a density of 2×10^5 cells/well and incubated at 37°C, 5% CO_2. When the cells grew to about 80% confluent, the control group was changed to high-glucose DMEM medium supplemented with 5% FBS and 1% 100 U/ml penicillin-100 mg/ml streptomycin, induction group was changed to chondrogenic differentiation medium, dimethylsulfoxide (DMSO) group was changed to chondrogenic differentiation medium supplemented with 0.1% DMSO (Amrersco, America), 5 μM DAPT group was changed to chondrogenic differentiation medium supplemented with 5 μM DAPT (dissolved in DMSO) and the final concentration of DMSO was 0.1%. All groups were cultured for 6 days, 12 days and 18 days. The medium was changed every 3 days and their images were captured on the eighteenth day.

2.7 qPCR analysis of Notch-1, Hes-1, GAG and *COL-2A1*

On the sixth, twelfth and eighteenth day, total RNA of all groups were extracted by RNA extracting TRIreagent (Tiangen,China) according to the manufacturer's

instructions. cDNA was synthesized from 300ng RNA using a Reverse Transcription Kit (TOYOBO,Japan). Gene expression of Notch-1, Hes-1, GAG and COL-2A1 was detected by qPCR, and the gene expression of GAPDH was used as internal control. qPCR was performed by qPCR Mix Kit (TOYOBO, Japan) according to the manufacturer's instructions. Data was analyzed using ΔΔCt method, the relative copy number = $2^{-\Delta\Delta Ct} = 2^{-(\Delta Ct \text{ experiment group} - \Delta Ct \text{ control group})} = 2^{-[(Ct \text{ target-Ct GAPDH})-(Ct \text{ control}-Ct \text{ GAPDH})]}$, each sample was analyzed in triplicate. The primers (Invitrogen,America) were listed as Table 1.

2.8 Cell cycle analysis

On the sixth day, UC-MSCs of all groups were harvested and adjusted to 5×10^5 cells/ml. The cells were washed with cold PBS for 2 times and suspended with 70% (v/v) cold ethanol, then fixed in 4°C for 24 hours; The cells were suspended with Presidium Iodide (BD Bioscence, America) contained 10 μg/ml RNAase A and 50 μg/ml PI after discarding the suspernatant, and incubated for 20 minutes at 4°C in the dark, then detected by flow cytometry. 10^4 cells were obtained by the Cell-Quest software and the cell cycle was analyzed by ModiFit. These experiments were repeated for 3 times.

2.9 Toluidine Blue staining

On the eighteenth day, medium was discarded. The cells were washed with PBS for 3 times and fixed with 4% triformol for 10 minutes, then washed with PBS for 3 times and stained with 0.1% toluidine blue for 30 minutes at room temperature. Dye was discarded, the cells were washed with distilled water and their images were captured.

2.10 Immunofluorescence staining

On the eighteenth day, medium was discarded, the cells were washed with PBS for 3 times and fixed with 4% triformol for 10 minutes, then washed with PBS for 3 times and treated with 0.5% Triton X-100 (dissolved with PBS) (Amresco, America) for 10 minutes, then washed three times again, and incubated with 0.5% BSA (Amresco, America) for 30 minutes; washed twice, and incubated with primary antibody (Boster, China) overnight at 4°C (solubility of 1:120); washed three times and incubated with secondary antibody (Proteintech, America) in the dark at room temperature for 60 minutes (solubility of 1:80). Finally, the cells were washed thrice with PBS and captured by immunofluorescence microscope (Leica, Germany).

2.11 Quantitive analysis of GAG

On the sixth, twelfth and eighteenth day, 200 μl cell supernatant of each group was collected respectively, 40 μl papain solution (Amresco, America) was added to the supernatant for digesting for 24 hours. 100 μl 8 mol/L guanidine hydrochloride (Amresco, America) and 0.25% alcian blue solution (Sigma, America) were put into the digestion products at 4°C for 1 hour, then centrifuged at 12,000 rpm for 15 minutes. Supernatant was discarded and 150 μl isopropanol (Amresco, America) was added to the sediment for dissolving, and their absorbance values were acquired in the microplate reader at 600 nm. A series of concentration gradients of chondroitin sulfate standard solution (Amresco, America) and their corresponding absorbance values were used to draw a standard curve, the GAG content of all groups was obtained using this standard curve.

Table 1: Primers of qPCR.

Gene Name	NCBI No.	Primer Pairs	Product Length
NOTCH-1	NM_017617.3	Forward: 5'-GCCGCCTTTGTGCTTCTGTTC-3' Reverse: 5'-CCGGTGGTCTGTCTGGTCGTC-3'	300 bp
HES-1	NM_005524.3	Forward: 5'-AAGAAAGATAGCTCGCGGCA-3' Reverse: 5'-CCTCGGTATTAACGCCCTCG-3'	208 bp
COL-2A1	NM_001844.4	Forward: 5'-GCAGGATGGGCAGAGGTATAA-3' Reverse: 5'-GCACTCTCCGAAGGGGATCT-3'	151 bp
GAG	NM_013227.3	Forward: 5'- ACACTGGCGAGCACTGTAAC-3' Reverse: 5'- CCTTCGATGGTCCTGTCGTT-3'	235 bp
GAPDH	NM_002046	Forward: 5'- TGTTGCCATCAATGACCCCTT-3' Reverse: 5'- CTCCACGACGTACTCAGCG-3'	202 bp

2.12 Quantitive analysis of *COL-2A1*

On the sixth, twelfth and eighteenth day, 250 µl cell supernatant of all groups was collected respectively, 50 µl digestive solution and 2 ml reaction solution (Amresco, America) were added to the supernatant at 60°C for 15 minutes, then the mixture was centrifuged at 3500 rpm for 10 minutes after cooling. 150 µl supernatant was taken to detect their absorbance values at 560 nm. A series of concentration gradients of hydroxyproline standard solution (Amresco,America) and their corresponding absorbance values were used to draw a standard curve, the content of COL-2A1 was obtained using this standard curve.

2.13 Statistical analysis

The results are all presented as the mean (n = 3) ± standard deviation. Two tailed t tests were conducted to determine statistical significance.

3 Results

3.1 Morphology of UC-MSCs

UC-MSCs were extracted from human umbilical cord successfully by 0.2% collagenase II digestion. 80% of the cells were adherence after extraction for 3 days, they were mononuclear and spindle. At the same time, there were some impurities around the cells (Fig. 1 a). At passage 1, the morphology became coincident and grew equably (Fig. 1 b). At passage 3, the morphology began to be consistent (Fig. 1 c). The morphology at passage 5 tended to be stable and was nearly identical (Fig. 1 d).

3.2 Surface markers of UC-MSCs

UC-MSCs at passage 3 highly expressed CD13, CD44, CD90, CD105 and the positive percentages of these surface markers were nearly 100% (Fig. 2 a, b, c, d). On the contrary, they were negative for CD11b, CD34, CD45, HLA-DR and the negative percentages were close to zero (Fig. 2 e, f, g, h). All of these satisfied the characteristics of MSCs [30].

3.3 Pluripotent differentiation potential of UC-MSCs

After osteogenic induction, the result of Alizarin Red staining was positive and the chondrocyte were saffron (Fig. 3 b), while the color of control group was light (Fig. 3 a). Chondrocytes were dyed blue by Toluidine Blue staining after chondrogenic induction (Fig. 3 d), while the color of control group was undertone (Fig. 3 c). Compared with the control group (Fig. 3 e), there were a lot red lipid droplets in cytoplasm after adipogenic induction (Fig. 3 f). These results indicated that the cells extracted from

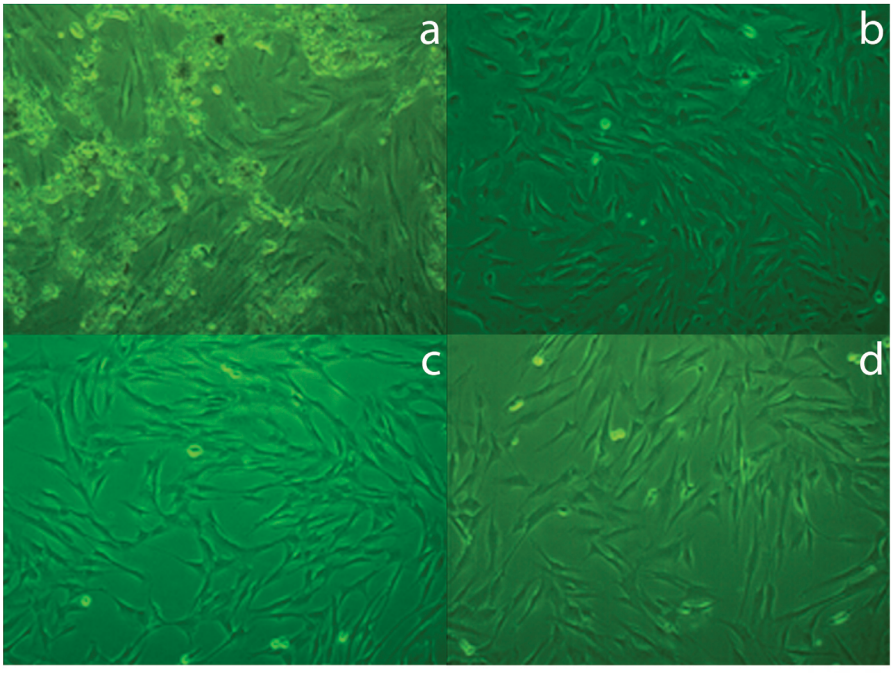

Figure 1: Morphology of UC-MSCs (×100): a. Primary UC-MSCs after extraction for 3 days, b. UC-MSCs at passage 1, c. UC-MSCs at passage 3, d. UC-MSCs at passage 5.

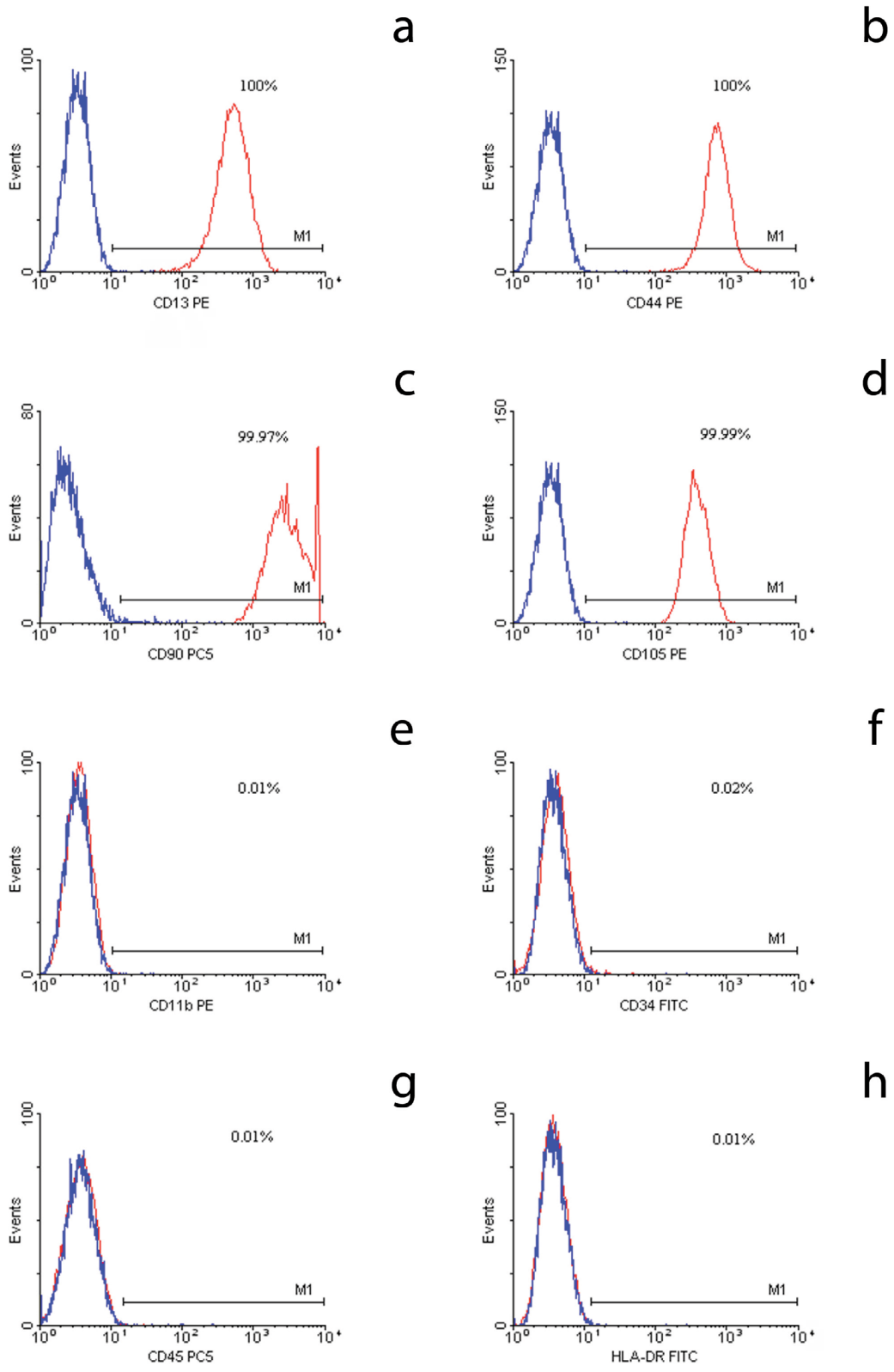

Figure 2: Surface markers of UC-MSCs.

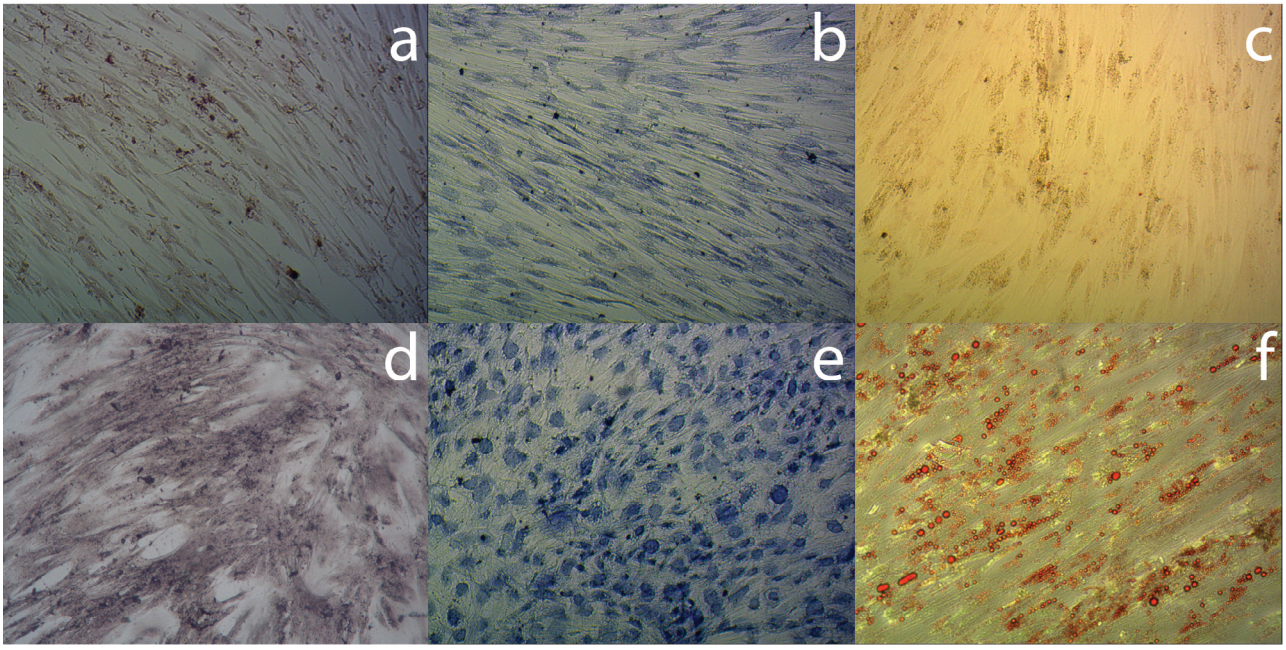

Figure 3: Pluripotent differentiation potential of UC-MSCs (×100): a. Control group of Alizarin Red staining, b. Induction group of Alizarin Red staining after osteogenic differentiation for 21 days, c. Control group of Toluidine Blue staining, d. Induction group of Toluidine Blue staining after chondrogenic differentiation for 21 days, e. Control group of Oil Red O staining, f. Induction group of Oil Red O staining after adipogenic differentiation for 21 days.

human umbilical cord could be induced to differentiate into osteogenic, chondrogenic and adipogenic cells.

3.4 Gene expression of Notch-1 and Hes-1 after chondrogenic induction

From Fig. 4, compared with the corresponding control group at all time points, the gene expression of Notch-1 and Hes-1 all decreased significantly after chondrogenic differentiation ($p < 0.01$). These results suggested that Notch signaling pathway exists in UC-MSCs, and Notch signaling strength declined evidently when UC-MSCs were induced to differentiate into chondrocytes.

3.5 Content and gene expression of GAG and COL-2A1 after chondrogenic induction

Alcian Blue method and hydroxyproline method were used to analyze GAG and COL-2A1 quantitatively. As showed in Fig. 5 a and b, the content of GAG and COL-2A1 increased markedly on the sixth day ($p < 0.05$). On the twelfth and eighteenth day, the content of GAG and COL-2A1 all increased very significantly ($p < 0.01$). These results suggested that the content of GAG and COL-2A1 increased gradually after chondrogenic induction. As showed in Fig. 5 c and d, the gene expression of GAG and COL-2A1 increased insignificantly on the sixth day ($p > 0.05$), but increased very significantly on the twelfth day ($p < 0.05$) and the eighteenth day ($p < 0.01$). These results showed that the gene expression of GAG and COL-2A1 increased gradually after chondrogenic induction.

3.6 Cell cycle analysis after chondrogenic induction and addition of DAPT

On the sixth day, the percentage of G1 period in the control group was 61.2 ± 1.51% (Fig. 6 a), which was approximately equal to the DAPT group (61.4 ± 3.26%) (Fig. 6 d), while the percentages of the induction group and the DMSO group were 70.9 ± 2.1% and 69.1 ± 2.1%, and they were higher than the control group ($p < 0.05$) (Fig. 6 b, c). These results suggested that chondrogenic induction surpressed the proliferation.

3.7 Morphology, Toluidine Blue staining and Immunofluorescence staining after addition of DAPT

On the eighteenth day, morphology of the control group was still spindle (Fig. 7 a-a). On the contrary, morphology of the induction group, DMSO group and DAPT group had changed from spindle to polygon and the cells grew intensively (Fig. 7 a-b, a-c, a-d), these results indicated that the morphology of UC-MSCs was closer to chondrocytes after chondrogenic induction. On the eighteenth day,

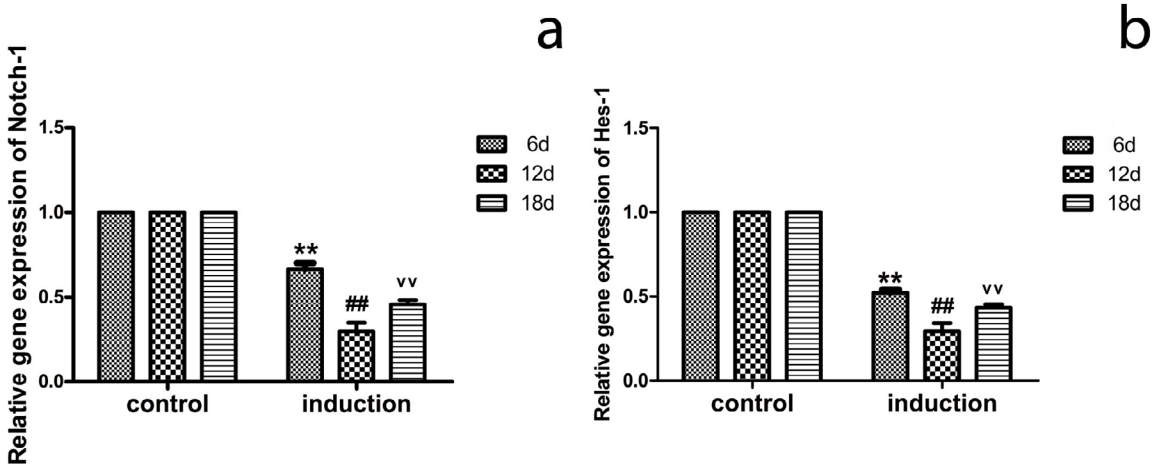

Figure 4: Gene expression of Notch-1 and Hes-1: a. Gene expression of Notch-1, b. Gene expression of Hes-1. Error bars indicate mean ± SD of 3 independent experiments. **, ##, vv represent $p < 0.01$, vs. corresponding control group, there were very significant differences.

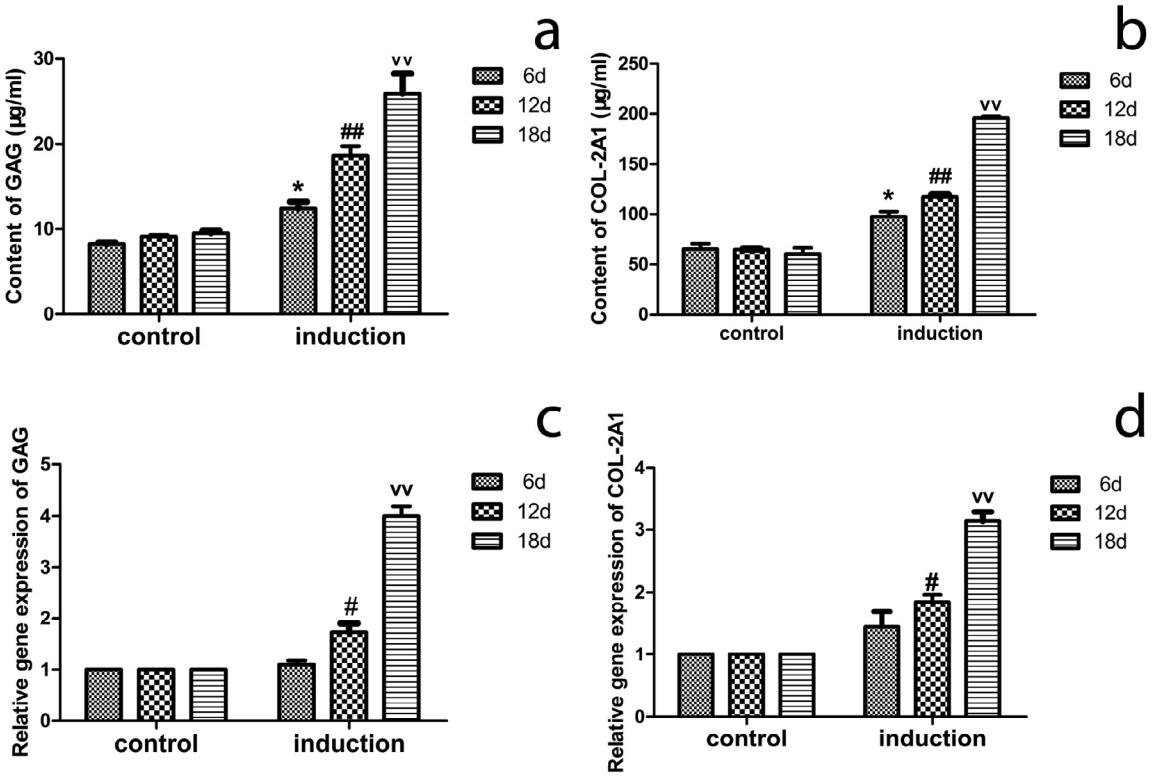

Figure 5: a. Content of GAG, b. Content of COL-2A1, c. Gene expression of GAG, d. Gene expression of COL-2A1. Error bars indicate mean ± SD of 3 independent experiments. *, # represent $p < 0.05$, vs. corresponding control group, there were significant differences. ##, vv represent $p < 0.01$, vs. corresponding control group, there were very significant differences.

Toluidine Blue staining was used to analyze the GAG qualitatively, colour of the control group was very light (Fig. 7 b-a), while color of the induction group, DMSO group and DAPT group became blue (Fig. 7 b-b, b-c, b-d). At the same time, the morphology of these groups had changed from spindle to polygon and the cells grew intensively, which was consistent with the morphological observation. These results suggested that GAG appeared after chondrogenic induction. On the eighteenth day, Immunofluorescence staining was used to analyze the COL-2A1 qualitatively. There was nearly no green fluorescence in the control group (Fig. 7 c-a), while a lot more green fluorescences appeared in the induction group, DMSO group and DAPT group (Fig. 7 c-b, c-c, c-d), especially in the induction group and DMSO group. These suggested that COL-2A1 appeared after chondrogenic induction.

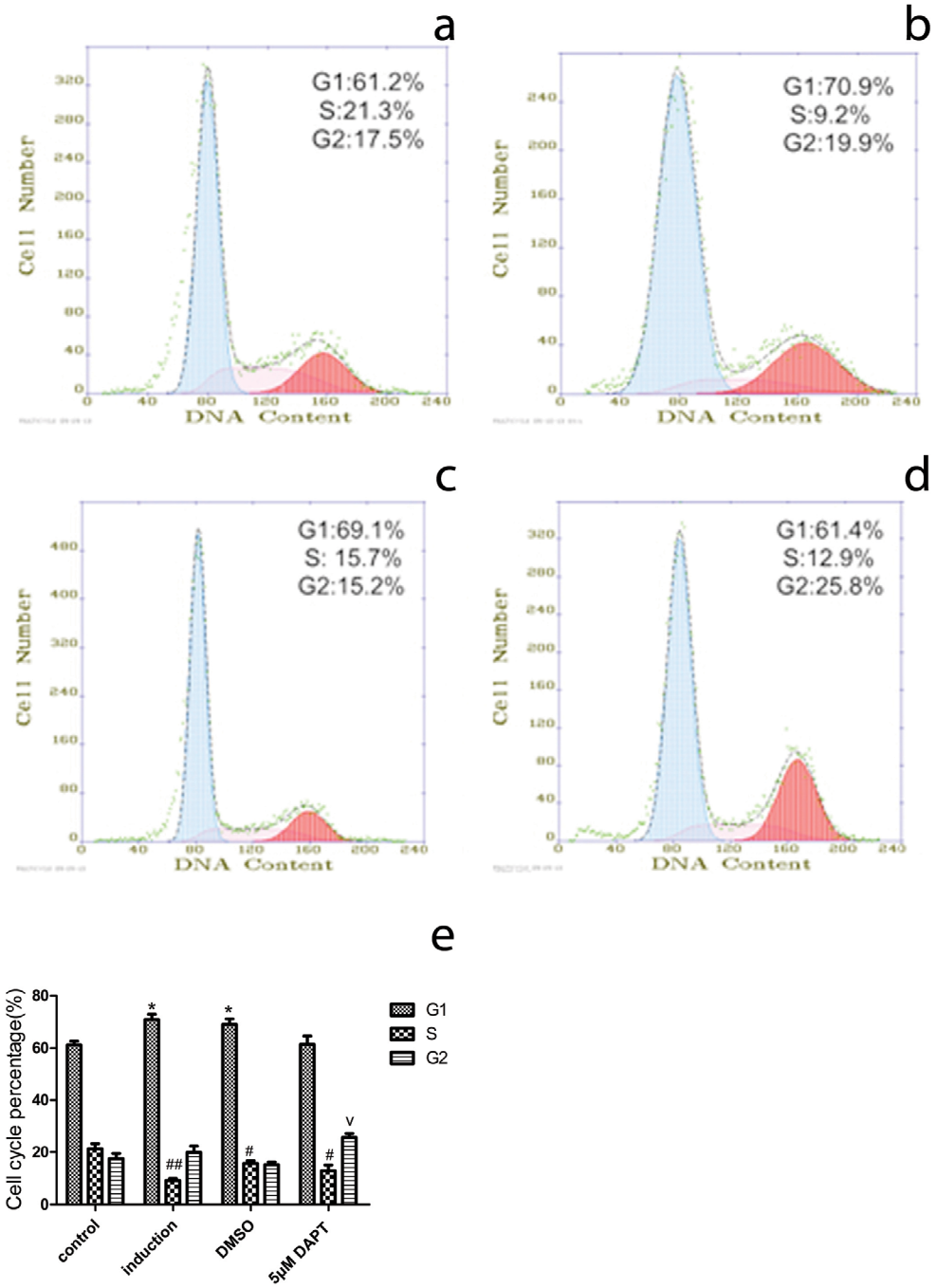

Figure 6: Cell cycle of UC-MSCs: a. control group, b. induction group, c. DMSO group, d. 5 μM DAPT group, e. Cell cycle percentage. Error bars indicate mean ± SD of 3 independent experiments. *, #, v represent $p < 0.05$, vs. corresponding control group, there were significant differences, ## represent $p < 0.01$, vs. the control group of S period, there were very significant differences.

3.8 Gene expression of Notch-1 and Hes-1 after addition of DAPT

Form Fig. 8, compared with the induction group, the gene expression of Notch-1 and Hes-1 decreased significantly at all time points with 5 μM DAPT. These results suggested that DAPT blocked the Notch signaling effectively, and the Notch signaling strength declined obviously, but the gene expression of Notch-1 and Hes-1 between the induction group and DMSO group had no significant differences at all time points ($p > 0.05$).

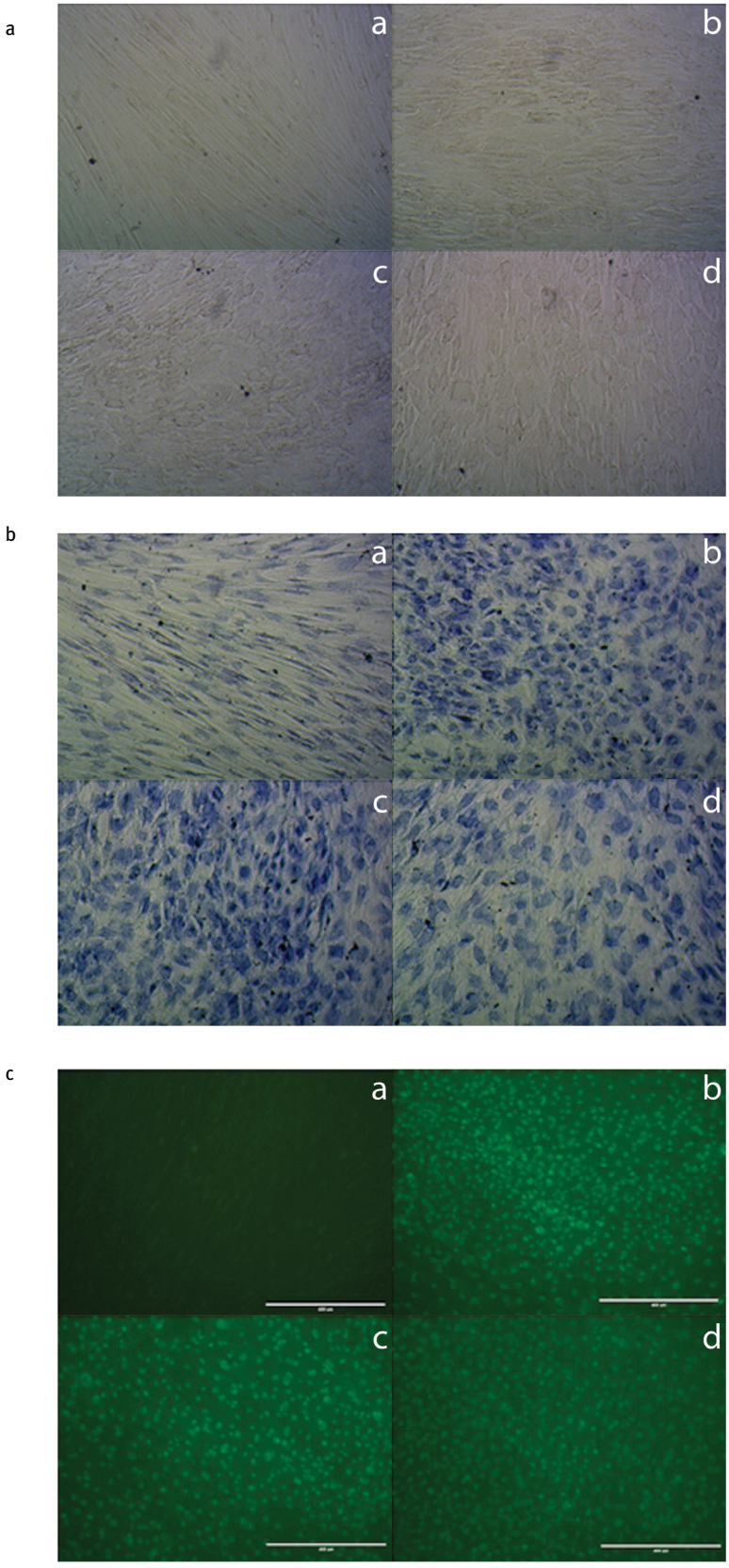

Figure 7: a. Morphology of UC-MSCs (×100): a-a: control group, a-b. induction group, a-c. DMSO group, a-d. 5 μM DAPT group; b. Toluidine Blue staining of UC-MSCs (×100): b-a. control group, b-b. induction group, b-c. DMSO group, b-d. 5 μM DAPT group; c. immunofluorescence staining of UC-MSCs (bar 400 μm): c-a. control group, c-b. induction group, c-c. DMSO group, c-d. 5 μM DAPT group.

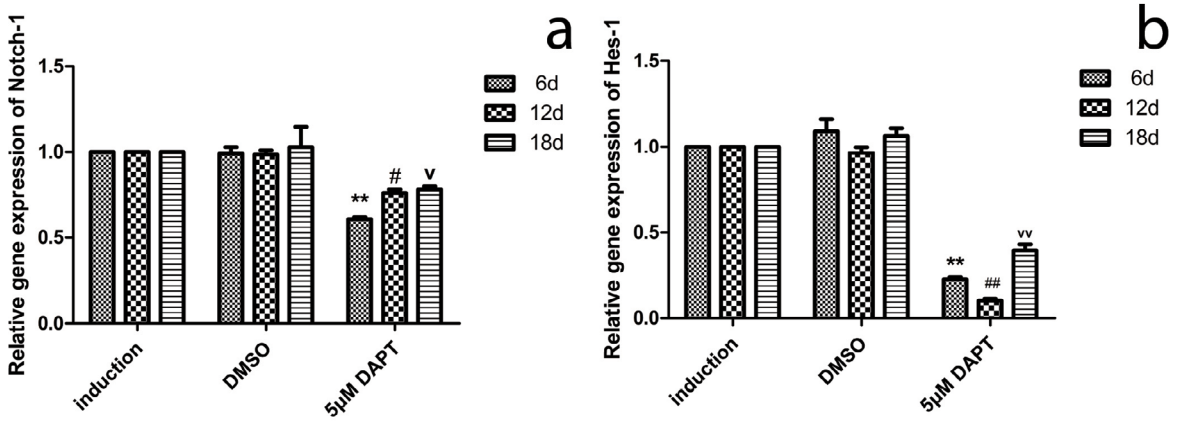

Figure 8: Gene expression of Notch-1 and Hes-1. Error bars indicate mean ± SD of 3 independent experiments. a. Gene expression of Notch-1, b. Gene expression of Hes-1. #, v represent $p < 0.05$, vs. corresponding induction group, there were significant differences. **, ##, vv represent $p < 0.01$, vs. corresponding induction group, there were very significant differences.

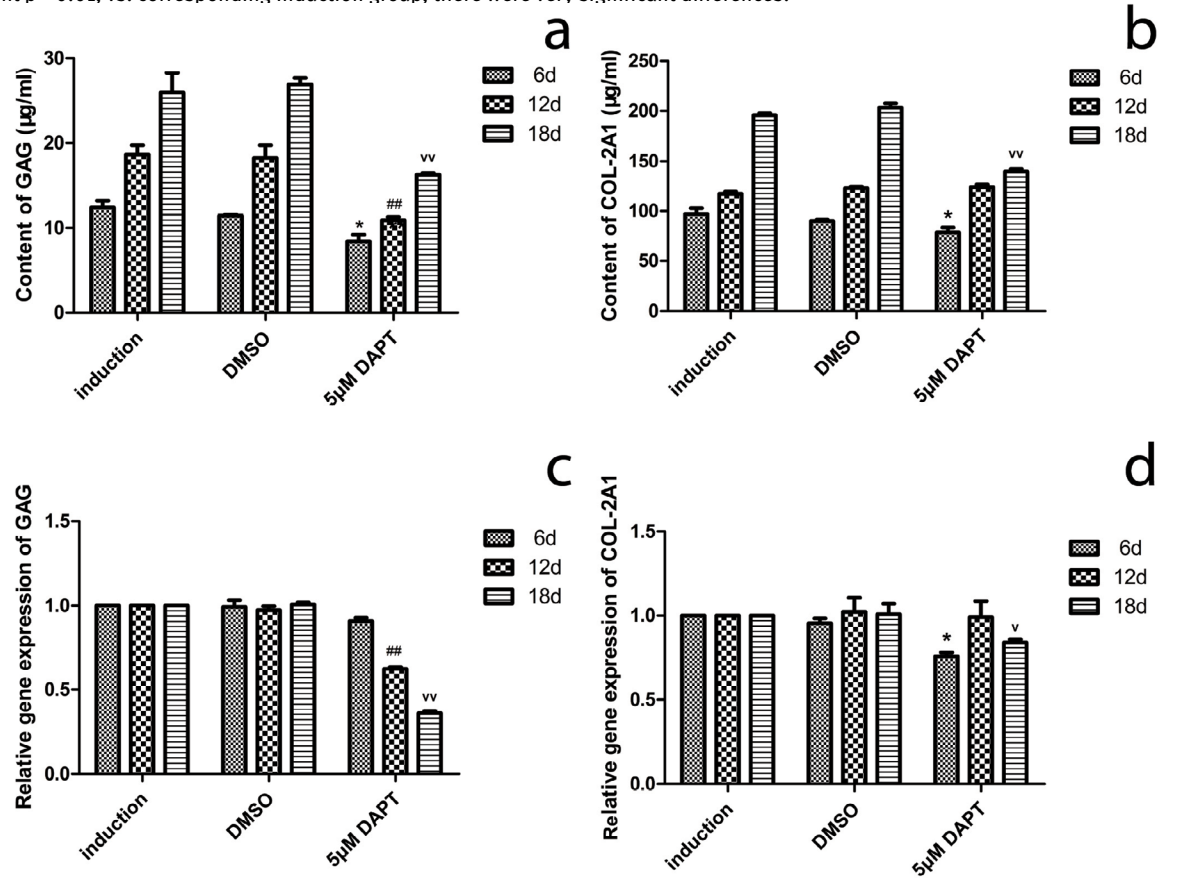

Figure 9: a. Content of GAG, b. Content of COL-2A1, c. Gene expression of GAG, d. Gene expression of COL-2A1. Error bars indicate mean ± SD of 3 independent experiments. * represent $p < 0.05$, vs. corresponding induction group, there were significant differences. ##, vv represent $p < 0.01$, vs. corresponding induction group, there were very significant differences.

3.9 Content and gene expression of GAG and COL-2A1 after addition of DAPT

As showed in Fig. 9 a and b, the content of GAG in the DAPT group was lower than the induction group at all time points ($p < 0.05$). On the sixth day, the content of COL-2A1 in the DAPT group was lower than the induction group ($p < 0.05$) and was much lower ($p < 0.01$) on the eighteenth day, but the content on the twelfth day had no obvious difference ($p > 0.05$). These results suggested that the expression of GAG and COL-2A1 was restrained by DAPT, and this effect was more obvious with time increasing. As showed in Fig. 9 c and d, compared with the induction group, the gene expression of GAG in the DAPT

group had no significant difference ($p > 0.05$) on the sixth day, but was much lower on the twelfth and eighteenth day ($p < 0.01$). The gene expression of COL-2A1 in the DAPT group was lower than the induction group on the sixth and eighteenth day ($p < 0.05$), but on the twelfth day, they had no significant difference ($p > 0.05$). These results showed that DAPT inhibited the gene expression of GAG obviously, and this effect became more significant with the time increasing, but this suppressive effect of DAPT for the gene expression of COL-2A1 was less significant. In addition, the content and gene expression of GAG and COL-2A1 had no significant differences between the induction group and DMSO group at all time points ($p > 0.05$).

4 Discussion

Due to the lack of nerves and vessels, articular cartilage has little ability for self-repair, and the limited source of chondrocytes has become the major obstacle for healing [30]. In recent years, cartilage tissue engineering has been considered one of the methods that may solve this medical issue. Besides, UC-MSCs have become the ideal seed cells because of their abundant source, low immunogenicity and no ethical debate [16,31]. In this study, UC-MSCs were separated from human umbilical cord by 0.2% collagenase II successfully, they were adherent and spindle (Fig. 1). UC-MSCs not only meet the standard of ectomesenchymal of international cell therapy and ectomesenchymal stem cell established by Tissue Stem Cells commission, but also express some original stem cell marker [15,32,33]. At the same time, UC-MSCs inhibits the proliferation of lymphocytes [34]. In this study, we use the methods of novel myeloid precursor cell line PC-MDS characteristics [35]. The flow cytometry analysis showed that the cells highly expressed mesenchymal cell makers CD13, CD44, CD90 and CD105, and lowly expressed hepatoeitic cell makers CD11b, CD34, CD45 and human leucocyte antigen HLA-DR (Fig. 2). In addition, they had the ability to differentiate into osteogenic, chondrogenic and adipogenic cells (Fig. 3), these all satisfied the characteristics of MSCs [17,31].

To date, numerous researches on cartilage tissue engineering concentrate on the optimization of induced conditions rather than the molecular mechanism during chondrogenesis, which becomes an obstacle for the development of cartilage tissue engineering [36]. Notch signaling is a highly conservative pathway during hemogenesis and organic evolution, it plays a role in the rat intestinal adaptation [37], the osteogenic differentiation of human BM-MSCs [38], the differentiation of myocardial cells and cardiac regeneration [13,39], the proliferation and differentiation of human neural stem cell [40], the maintaining of stemmas characteristics of neural precursor cells [41]and the adipogenic differentiation of human adipose mesenchymal stem cells [42]. Besides, previous studies have suggested that Notch signaling participates in the dedifferentiation of mouse articular chondrocytes and avian cartilage development [43].

In this study, the gene expression of Notch-1 and Hes-1 declined significantly after chondrogenic induction (Fig. 4), which demonstrated that Notch signaling exists in UC-MSCs, and Notch signaling may block the differentiation of hUC-MSCs and remain their proliferative activity, which was analogous with Rebecca Williams' conclusion [43] and Karlsson's findings [44]. The content and gene expression of GAG and COL-2A1 all increased with time increasing after chondrogenic induction (Fig. 5). Besides, the morphology became polygon, which was closer to chondrocytes, and the results of Toluidine Blue staining and immunofluorescence staining (Fig. 6) were all positive, which suggested that GAG and COL-2A1 were expressed. These all revealed that UC-MSCs were induced to differentiate into chondrocytes successfully. The cell cycle showed that the percentage of interkinesis increased after chondrogenic induction, but the percentage in the DAPT group was nearly equal to the control group (Fig. 7). These results demonstrated that the decline of Notch signaling strength could weaken the chondrogenic differentiation of UC-MSCs and may remain their proliferative ability. Previous study also proved that Notch signaling was closely related with the formation of osteoblasts and chondrocytes in perichondrium by controlling both the differentiation and proliferation [21]. The gene expression of Notch-1 and Hes-1 all declined after the addition of DAPT, which suggested that the Notch signaling strength with chondrogenic differentiation was weakened effectively by DAPT (Fig. 8). The content and gene expression of GAG and COL-2A1 all decreased with DAPT, (Fig. 9), which suggested that DAPT inhibited the expression of GAG and COL-2A1, and the effect on GAG was more obvious. These findings illustrated that the Notch signaling strength with chondrogenic induction may be suitable for the expression of GAG and COL-2A1, further blocking the Notch signaling with DAPT would prevent the chondrogenesis. This may be relative with the regulation of SOX9 [28,45] and was similar with Oldershaw's result [25]. It's worth mentioning that in our preliminary trials, 5 μM DAPT had the least effect on the growth of UC-MSCs (data was not shown here). Besides both the gene expression of Notch-1, Hes-1, GAG, COL-2A1 and the content of GAG and COL-2A1 had no significant

difference between the induction group and DMSO group, which suggested that the low concentration of DMSO had little effect on the chondrogenesis of UC-MSCs.

5 Conclusion

Our study demonstrated that Notch signaling exists in UC-MSCs and it maintains the proliferative state of UC-MSCs. Once UC-MSCs are induced to differentiate into chondrocytes, Notch signaling strength will decline greatly, and DAPT inhibits the chondrogenesis of UC-MSCs. Because there are many other signaling pathways during chondrogenesis [46,47], it's uncertain whether any other signaling pathway interacts with Notch signaling. Therefore, a further investigation is needed in the future.

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References

[1] Wang X.B., Rackwitz L., Noth U., Tuan R.S., Cartilage Development, Physiology, Pathologies, and Regeneration, In: M. Santin (Ed.), Strategies in Regenerative Medicine, Springer Berlin, 2009, 367-393

[2] Coates E.E., Fisher J.P., Engineering superficial zone chondrocytes from mesenchymal stem cells, Tissue Eng. Part C Methods, 2014, 20 (8), 630-40

[3] Steward A.J., Liu Y.X., Wagner D.R., Engineering Cell Attachments to Scaffolds in Cartilage Tissue Engineering, JOM, 2011, 63 (4), 74-82

[4] Marlovits S., Zeller P., Singer P., Resinger C., Vécsei V., Cartilage repair: Generations of autologous chondrocyte transplantation, Eur. J. Radiol., 2006, 57 (1), 24-31

[5] Pelttari K., Steck E., Richter W., The use of mesenchymal stem cells for Chondrogenesis, Injury, 2008, 39 (S1), S58-S65

[6] Peeters C.M., Leijs M.J., Reijman M., van O.G., Bos P.K., Safety of intra-articular cell-therapy with culture-expanded stem cells in humans: a systematic literature review, Osteoarthritis Cartilage, 2013, 21 (10), 1465-73

[7] Obradovic B., Radisic M., Novakovic G.V., Tissue engineering of cartilage and myocardium, App. Cell Immobilisation Biotechnol., 2005, 8B, 99-133

[8] Keeney M., Lai J.H., Yang F., Recent progress in cartilage tissue engineering, Curr. Opin. Biotechnol., 2011, 22 (5), 734-40

[9] Panseri S., Russo A., Cunha C., Bondi A., Di M.A., Patella S., et al., Osteochondral tissue engineering approaches for articular cartilage and subchondral bone regeneration, Knee Surg. Sports Traumatol. Arthrosc., 2012, 20 (6), 1182-91

[10] Aerts F., Wagemaker G., Mesenchymal stem cell engineering and transplantation, Gen. Eng. Mesenchymal Stem Cells, 2006, 1-44

[11] Abdallah B.M., Kassem M., Human mesenchymal stem cells from basic biology to clinical applications, Gene Ther., 2008, 15 (2), 109-16

[12] Chen P.M, Yen M.L, Liu K.J, Sytwu H.K, Yen B.L., Immunomodulatory properties of human adult and fetal multipotent mesenchymal stem cells, J. Biomed. Sci., 2011, 18 (1), 49

[13] Li H.W., Yu B., Zhang Y., Pan Z.W., Xu W., Li H., Jagged1 protein enhances the differentiation of mesenchymal stem cells into cardiomyocytes, Biochem. Biophys. Res. Commun., 2006, 341 (2), 320-5

[14] Fong C.Y., Subramanian A., Gauthaman K., Venugopal J., Biswas A., Ramakrishna S., et al., Human Umbilical Cord Wharton's Jelly Stem Cells Undergo Enhanced Chondrogenic Differentiation when Grown on Nanofibrous Scaffolds and in a Sequential Two-stage Culture Medium Environment, Stem Cell Rev., 2011, 8 (1), 195-209

[15] Weiss M.L., Medicetty S., Bledsoe A.R., Rachakatla R.S., Choi M., Merchav S., et al., Human Umbilical Cord Matrix Stem Cells: Preliminary Characterization and Effect of Transplantation in a Rodent Model of Parkinson's Disease, Stem Cells, 2006, 24 (3), 781-92

[16] Fan C.G., Zhang Q.J., Zhou J.R., Therapeutic Potentials of Mesenchymal Stem Cells Derived from Human Umbilical Cord, Stem Cell Rev. Res., 2008, 7 (1), 195-207

[17] Cooper K., Viswanathan C., Establishment of a mesenchymal stem cell bank, Stem Cells Int., 2011, 10, 4061-4068

[18] Guo J., Yang J., Cao G.F., Fan H.H, Guo C.Z., Ma Y.E., et al., Xenogeneic immunosuppression of human umbilical cord mesenchymal stem cells in a major histocompatibility complex-mismatched allogeneic acute graft-versus-host disease murine model, Eur. J. Haematol., 2011, 87(3), 235-43

[19] Tsakonas S.A., Rand M.D., Lake R.J., Notch Signaling: Cell Fate Control and Signal Integration in Development, Science, 1999, 284 (5415), 770-6

[20] Hayes A.J., Dowthwaite G.P., Webster S.V., Archer C.W., The distribution of Notch receptors and their ligands during articular cartilage development, J. Anat., 2003, 202 (6), 495-502

[21] Watanabe N., Tezuka Y., Matsuno K., Miyatani S., Morimura N., Yasuda M., et al., Suppression of differentiation and proliferation of early chondrogenic cells by Notch, J. Bone Miner. Metab., 2003, 21 (6), 344-352

[22] Colombo M., Thümmler K., Mirandola L., Garavelli S., Todoerti K., Apicella L., et al., Notch signaling drives multiple myeloma induced osteoclastogenesis, Oncotarget., 2014, 5 (21), 10393-406

[23] Chiba S., Concise Review: Notch Signaling in Stem Cell Systems, Stem Cells, 2006, 24 (11), 2437-47

[24] Zhang S.B., Loch A.J., Radtke F., Egan S.E., Xu K.L., Jagged1 is the Major Regulator of Notch-Dependent Cell Fate in Proximal Airways, Dev Dyn., 2013, 242 (6), 678-686

[25] Oldershaw R.A., Tew S.R., Russell A.M., Meade K., Hawkins R., McKay T., et al., Notch Signaling Through Jagged-1 Is Necessary to Initiate Chondrogenesis in Human Bone Marrow Stromal Cells but Must Be Switched off to Complete Chondrogenesis, Stem Cells, 2008, 26 (3), 666-74

[26] Jundt F., Probsting K.S., Anagnostopoulos I., Muehlinghaus G., Chatterjee M., Mathas S., et al., Jagged1-induced Notch signaling drives proliferation of multiple myeloma cells, Blood, 2004, 103 (9), 3511-15
[27] Mirandola L., Internati M.C., Montagna D., Locatelli F., Zecca M., Ranzani M., et al., Notch1 regulates chemotaxis and proliferation by controlling the CC-chemokine receptors 5 and 9 in T cell acute lymphoblastic leukaemia, J. Pathol., 2012, 226, 713–22
[28] Grogan S.P., Olee T., Hiraoka K., Lotz M., Repression of Chondrogenesis Through Binding of Notch Signaling Proteins HES-1 and HEY-1 to N-box Domains in the COL2A1 Enhancer Site, Arthritis Rheum., 2008, 58 (9), 2754-63
[29] Zhou J.X., Han J.B., Chen S.M., Xu Y., Kong Y.G., Xiao B.K., γ Inhibition Combined with Cisplatin Enhances Apoptosis of Nasopharyngeal Carcinoma Cells, Exp. Ther. Med., 2012, 3 (2), 357-361
[30] Peretti G.M., Pozzi A., Ballis R., Deponti D., and Pellacci F., Current Surgical Options for Articular Cartilage Repair, Acta Neurochir. Suppl., 2011, 108, 213-9
[31] Troyer D.L., Weiss M.L., Concise Review: Wharton's Jelly-Derived Cells Are a Primitive Stromal Cell Population, Stem Cells, 2008, 26 (3), 591-9
[32] Conconi M.T., Burra P., Liddo D.R., Calore C., Turetta M., Bellini S., Bo P., et al., CD105 (+) cells from Whartonps jelly show in vitro and in vivo myogenic differentiative potential, Int. J. Mol. Med., 2006, 18 (6), 1089-96
[33] Koh S.H., Kim K.S., Choi M.R., Jung K.H., Park K.S., Chai Y.G., et al., Implantation of human umbilical cord-derived mesenchymal stem cells as a neuroprotective therapy for ischemic stroke in rats, Brain Res., 2008, 1229, 233-248
[34] Uccelli A., Moretta L., Pistoia V., Immunoregulatory function of mesenchymal stem cells, Eur. J. Immunol., 2006, 36 (10), 2566-73
[35] Bogdanovic G., Jurisic V., Kurtovic N.K., Mrdjanovic J., Jakimov D., Krtolica K., et al., Characteristics of novel myeloid precursor cell line, C-MDS, established from a bone marrow of the patient with therapy-related myelodysplastic syndrome, Leuk. Res., 2007, 31 (8), 1097–1105
[36] Onyekwelu I., Goldring M.B., Hidaka C., Chondrogenesis, Joint Formation, and Articular Cartilage Regeneration, J. Cell Biochem., 2009, 107 (3), 383-92
[37] Chen G., Sun L., Yu M., Meng D., Wang W., Yang Y., Yang H., et al., The Jagged-1/Notch-1/Hes-1 Pathway Is Involved in Intestinal Adaptation in a Massive Small Bowel Resection Rat Model, Dig. Dis. Sci., 2013, 58 (9), 2478-86
[38] Lu Z.Z, Wu Z.Z., Z Q.W., H W., Jia X.X., Duan H.F., et al., Notch signaling stimulates osteogenic, differentiation of human bone marrow-derived mesenchymal stem cells, Chinese Sci. Bull., 2004, 49 (8), 815-818
[39] Sassolia C., Pinia A., Mazzanti B., Quercioli F., Nistri S., Saccardi R., et al., Mesenchymal stromal cells affect cardio-myocyte growth through juxtacrine Notch-1/Jagged-1 signaling and paracrine mechanisms: clues for cardiac regeneration, J. Mol. Cell Cardiol., 2011, 51 (3), 399-408
[40] Wang Y., Tu W., Lou Y., Xie A., Lai X., Guo F., et al., Mesenchymal stem cells regulate the proliferation and differentiation of neural stem cells through Notch signaling, Cell Biol. Int., 2009, 33 (11), 1173-9
[41] Woo S.M., Kim J., Han H.W., Chae J.I., Son M.Y., Cho S., et al., Notch signaling is required for maintaining stem-cell features of neuroprogenitor cells derived from human embryonic stem cells, BMC Neurosci., 2009, 10 (97) ,1-12
[42] Osathanon T., Subbalekha K., Sastravaha P., Pavasant P., Notch signalling inhibits the adipogenic differentiation of single-cell-derived mesenchymal stem cell clones isolated from human adipose tissue, Cell Biol. Int., 2012, 36(12), 1161-70
[43] Williams R., Nelson L., Dowthwaite G.P., Evans D.J., Archer C.W., Notch receptor and Notch ligand expression in developing avian cartilage, J. Anat., 2009, 215 (2), 159-69
[44] Karlsson C., Jonsson M., Asp J., Brantsing C., Kageyama R., Lindah A., Notch and HES5 are regulated during human cartilage differentiation, Cell Tissue Res., 2007, 327 (3), 539-51
[45] Hardingham T.E., Oldershaw R.A., Tew S.R., Cartilage, SOX9 and Notch signals in chondrogenesis, J. Anat., 2006, 209 (4), 469-80
[46] Kita K., Kimura T., Nakamura N., Yoshikawa H., Nakano T., PI3K/Akt signaling as a key regulatory pathway for chondrocyte terminal differentiation, Genes Cells, 2008, 13 (8), 839-50
[47] Bradley E.W., Drissi M.H., Wnt5b Regulates Mesenchymal Cell Aggregation and Chondrocyte Differentiation Through the Planar Cell Polarity Pathway, J. Cell Physiol., 2011, 226 (6), 1683-1693