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Effect of Deltex-1 on proliferation and differentiation of bone marrow mesenchymal stem cells into smooth muscle cells

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Abstract. – OBJECTIVE: To investigate the effect of Deltex-1 on proliferation and differentiation of bone marrow mesenchymal stem cells (bMSCs) into smooth muscle cells (SMCs).

MATERIALS AND METHODS: bMSCs of rat were isolated from bone marrow, cultured and identified. The effect of Deltex-1 on the proliferation of bMSCs infected with adenovirus vector pAd/Deltex-1 was detected by cell count kit-8 (CCK-8). The expression of smooth muscle myosin heavy chain (SM-MHC), that is one of the markers of SMCs in bMSCs without treatment, with Deltex-1 virus infection or empty virus infection and co-cultured with SMCs, were detected by immunofluorescence cytochemistry staining, RT-PCR and Western blotting. The same detection of bMSCs and SMCs without treatment was used as normal control, respectively.

RESULTS: bMSCs of rat were isolated from bone marrow, cultured and identified. Compared with the control, the results of CCK-8 showed that the growth of bMSCs infected by Deltex-1 virus was slower, and began to appear more significant especially at 48 ($p<0.05$, $p<0.01$). The results of immunofluorescence cytochemistry, Real-time PCR and Western blot showed that bMSCs with Deltex-1 virus infection and co-cultured with SMCs significantly expressed SM-MHC, and weakly expressed Notch-1.

CONCLUSIONS: The proliferation of bMSCs with Deltex-1 over-expression could be inhibited and its differentiation into smooth muscle cells could be promoted.

Key Words

Deltex-1, Bone marrow mesenchymal stem cells, Smooth muscle cells, Cell differentiation.

Introduction

Stress urinary incontinence (SUI), a common disease in women, is affecting the life quality of females severely, with a prevalence increasing with the life span[1]. It is believed that the support dysfunction bladder neck and intrinsic sphincter deficiency (ISD) are the key pathogeneses, and the latter is contributed by the lesion or apoptosis of smooth muscle cells (SMCs) surrounding the urinary tract[2]. Currently, despite various treatment strategies for SUI, none has overcome the difficulties in restoring the function of sphincter in urinary tract. Adult stem cell transplantation has also become one of the major methods in regeneration medicine, providing evidence for the treatment of SUI[3].

Bone marrow mesenchymal stem cells (bMSCs), as the important adult stem cells, have been attracting the attention in terms of the genetic therapy for convenience in material preparation, rapid proliferation, autograft, and stable expression of exogenous target genes after modification[4].

With the potentials of differentiation into the osteoblasts, myocardial cells and SMCs, bMSCs are regulated by random inactivation, activation, microenvironment induction and signaling transduction pathways[5]. Therein, Notch pathway plays an important role in regulation[6]. Deltex-1 is involved in the neurogenesis and development of B cells, which is also correlated with the cellular differentiation[7]. Deltex-1 can regulate the expression of Notch signal through intracellular association between Domain I and the Notch signal. Thus, Deltex-1 is a critical regulator in Notch pathway[8]. Nevertheless, the correlation between Deltex-1 and bMSCs, and its role in differentiation of bMSCs into other cells, especially SMCs, are poorly understood at present.

Through isolation, culture and identification of rat bMSCs, we used the previously established pAd/Deltex-1 adenoviral vector[9] to infect the bMSCs. Using cell count kit-8 (CCK-8), proliferation was determined in infected bMSCs and the proliferation curve was prepared. After co-cul-

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ture of infected bMSCs and SMCs, we tested the changes in the expressions of smooth muscle myosin heavy chain (SM-MHC), the marker of SMCs, in bMSCs through immunocytochemistry, Real-time polymerase chain reaction and Western blot. Furthermore, we investigated the role of Deltex-1 in the differentiation of bMSCs into SMCs and provided basis for the treatment of female SUI by using the modified bMSCs.

Materials and Methods

Materials

Fifteen specific-pathogen free (SPF) Sprague Dawley (SD) rats weighed between 170 and 190 g [SCXK (Yu) 2012-0003] and purchased from the Experiment Animal Center of Third Military Medical University, were used for bMSCs isolation, culture and identification. pAd/Deltex-1 adenoviral vector[9]; fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA); Dulbecco's Modified Eagle Medium (DMEM) and 0.25% trypsin (HyClone, South Logan, NJ, USA); CCK-8 kit (DOjindo, Tokyo, Japan); SM-MHC monoclonal antibody (Abcam, Cambridge, MA, USA); horseradish-peroxidase (HRP) labeled goat anti-mouse IgG secondary antibodies, cy3-labeled goat anti-mouse immunofluorescent secondary antibodies, DAPI and mounting agent (Beyotime, China); primer design and synthesis (Generay, Shanghai, China); Marker DL200 and RT-PCR kits (TaKaRa, Otsu, Shiga, Japan); agarose gel and TAE (Tiangen, China); PVDF membrane (Bio-Rad, USA); superSignal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Waltham, MA, USA); SMCs of rat (ATCC).

Methods

Isolation, Culture and Identification of bMSCs

Isolation and culture of bMSCs in rat were carried out according the previously described methods[9], and identification was carried out with flow cytometer. Briefly, cell suspension was prepared using bMSCs in the 3rd generation at density of 1×10^5 in each group. In cell samples, CD44, CD45 and CD90 antibodies were added for incubation at 37°C in the dark for about 1.5 h, with cell samples without treatment of antibodies as control. Resuspension of cell was again prepared using PBS for detecting the cell samples with different signature of antibodies using flow cytometer. This procedure was carried out in triplicate.

Detection of Cell Proliferation Using CCK-8 Method

bMSCs in 3rd generation were inoculated in a 96-well plate (3000/well), followed by infection of pAd-Deltex-1 with multiplicity of infection of 100. Non-infected or empty virus-infected bMSCs served as control, while the cells treated with culture medium as blank control. At 24, 48, 72 and 96 h after infection, 96-well plate was taken out to replace the culture medium, where CCK-8 detection reagent was added (12 μL/well). Following 3.5 h of culture, plate was mixed gently for measuring the optical density (OD) at wavelength of 450 nm. With OD value (Y) and culture time (X), the proliferation curve of bMSCs was prepared. This procedure was repeated in triplicate.

Detection of the Expression of SM-MHC in bMSCs Through Immunocytochemistry

bMSCs in 3rd generation infected by adenovirus containing Deltex-1 for 24 h, and SMCs in regular culture, were co-cultured in 6-well plate, in which direct contact was maximally guaranteed. Non-infected, empty-virus-infected and SMCs-co-cultured bMSCs served as positive control, bMSCs infected by Deltex-1 and co-cultured with SMCs as negative control, and bMSCs and SMCs in separated culture as normal control. After 48 h of culture, cells were collected from immunocytochemistry to detect the expression of SM-MHC, a specific marker of SMCs that is not expressed in non-induced bMSCs; its positive expression suggested the differentiation of infected bMSCs into SMCs. Results were observed and photographed by laser scanning confocal microscope.

Detection of mRNA Expressions of SM-MHC Through RT-PCR

Primers of SM-MHC (BC166736.1) and β-actin (NM_031144.3) were designed as follows: SM-MHC, upstream, 5'-AGGGCGATGAGGTGGTTGTA-3', downstream 5'-CCGAGTAGGTGTAGATGAGGC-3', 195 bp; β-actin, upstream, 5'-TTGTCCCTGTATGCCTCTG-3', downstream, 5'-AGGAAGGAAGGCTGGAAG-3', 383 bp. Cells in groups above were collected to extract total RNA for quantification and reverse transcription with the corresponding kit to prepare cDNA. A cycle of temperature was set as follows: 30°C, 10 min; 42°C, 30 min; 99°C for 5 min; 5°C for 5 min. PCR detection was carried out according to

the instruction of manufacturer, and each reaction system contained 1.5 μL cDNA under the following condition: 94°C for 1 min, 94°C for 30 s, 85°C for 5 s, 58.6°C for 1 min, 72°C for 5 min, followed by 28 cycles. At the end of the reaction, 10 μL reaction liquid were collected for gel electrophoresis to verify the product of PCR. This procedure was conducted in triplicate at least.

Detection of SM-MHC and Notch-1 Protein Expressions Through Western blot

Cells in all groups were collected to extract the total proteins for quantification, followed by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) to isolate proteins. Proteins transferred on the membrane from the gel were incubated with monoclonal anti-mouse SM-MHC antibody (1:600) and polyclonal anti-rabbit Notch-1 antibody (1:200) diluted by skimmed milk (5%) overnight at 4°C. Then, they were probed with the horseradish-peroxidase (HRP) labeled goat anti-mouse IgG (1:1200) and goat anti-rabbit IgG (1:700) at room temperature for 1.5 h. Enhanced chemiluminescence reagent was used for band development with β-actin as internal reference. This procedure was repeated in triplicate.

Statistical Analysis

Measurement data were presented as mean ± standard deviation. Comparison between two groups was carried out with two-sample *t*-test, while those among groups with one-way analysis of variance. Tukey's HSD (honestly significant difference) test was used in conjunction with an ANOVA to find means that are significantly different from each other. $p<0.05$ suggested that the difference had statistical significance.

Results

Isolation, Culture and Identification of bMSCs

Under the inverted microscope, bMSCs in primary culture for 1 to 7 days were in sporadic growth, and cells were in circular or irregular shape, most of which were in fusiform or elongated spindle. After 5 to 8 days of culture, monoclonal cells in 1st generation in well growth were chosen for culture; thereafter, bMSCs in 2nd generation were passaged when 80-90% of the surface was covered by cells. In the 3rd generation, bMSCs were in unified elongated spindle and evenly distributed (Figure 1). The proliferation rate was more rapid than the primary bMSCs. Passaging was carried out after 4 to 6 days of culture.

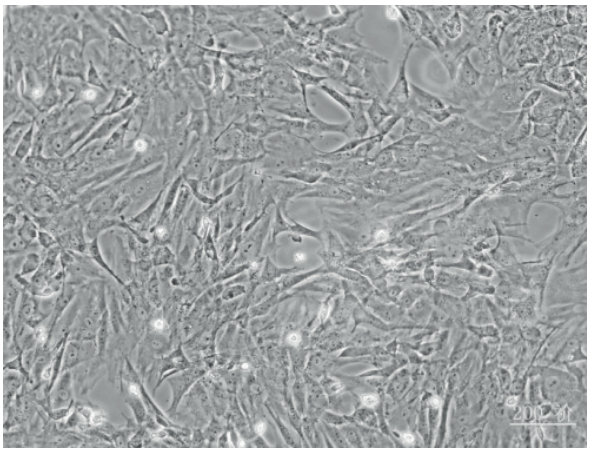

Figure 1. bMSCs in the 3rd generation under the inverted microscope (bar = 200 μm).

FCM results showed the stable expressions of CD44 and CD90 in the 2nd or 3rd generations with positive expression rates of 98.4% and 82.9%, and no or little expressions of CD45 with a positive expression rate of 0.943% (Figure 2).

Effect of Deltex-1 on Proliferation of bMSCs

CCK-8 detection revealed that bMSCs infected by Deltex-1 manifested a slower proliferation rate than that of bMSCs without infection or infected by empty vectors; more evident increase emerged at 48 h ($p<0.05$ or 0.01; Figure 3).

Deltex-1-Induced Differentiation of bMSCs into SMCs through Immunocytochemistry

As a functional indicator of SMCs, SM-MHC is not expressed in bMSCs that are not induced. Positive expression showed the differentiation of infected bMSCs into the SMCs. bMSCs, after 24 hours of adenoviral infection carrying Deltex-1 gene, were co-cultured with SMCs for 48 h, followed by immunocytochemistry detection. Observation using laser scanning confocal microscope showed that SM-MHC was not expressed in single culture of bMSCs (Figure 4A-D) and Deltex-1-infected bMSCs (Figure 4E-H), lowly expressed in co-culture of bMSCs not infected or infected by empty vector and SMCs, and highly expressed in Deltex-1-infected bMSCs that were co-cultured with SMCs (Figure 4Q-T) and single culture of SMCs (Figure 4U-X). These results suggested Deltex-1 can promote the differentiation of bMSCs into the SMCs through protein level.

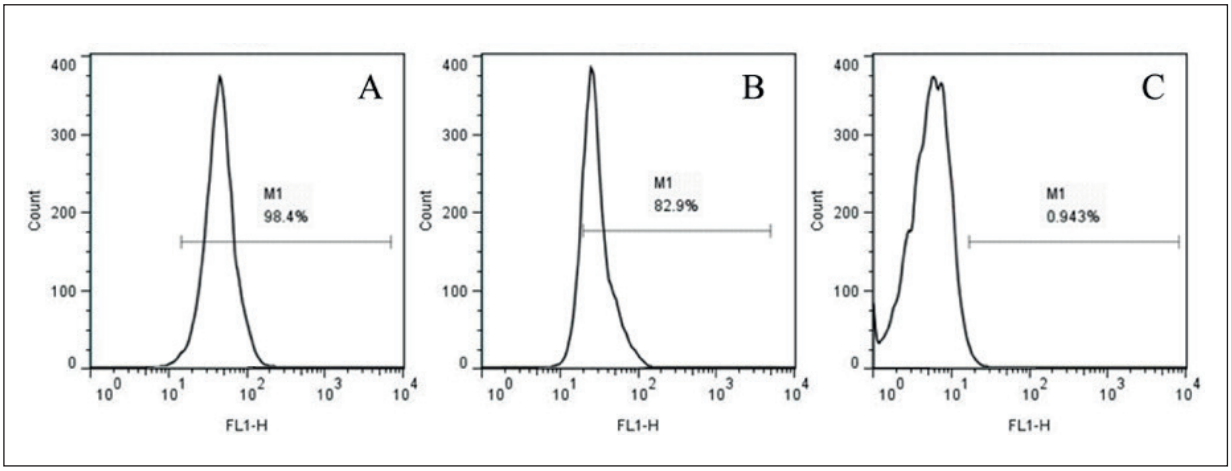

Figure 2. Positive expression rates of CD44, CD90 and CD45 in bMSCs through FCM.

RT-PCR Detection of the Promoting Effect of Deltex-1 on Differentiation of bMSCs into SMCs

RT-PCR results showed that SM-MHC was not expressed in single culture of bMSCs and Deltex-1-infected bMSCs, lowly expressed in co-culture of bMSCs not infected or infected by empty vector and SMCs, and highly expressed in Deltex-1-infected bMSCs that were co-cultured with SMCs and single culture of SMCs (Figure 5). These results suggested Deltex-1 can promote the differentiation of bMSCs into the SMCs through mRNA level.

Detection of the Promoting Effect of Deltex-1 on the Differentiation of bMSCs into SMCs through Western blot

Western blot assay showed that at the lane of 22.7×10^4 (RMW), SM-MHC was not expressed in single culture of bMSCs and Deltex-1-infected bMSCs, lowly expressed in co-culture of bMSCs not infected or infected by empty vector and SMCs, and highly expressed in Deltex-1-infected bMSCs that were co-cultured with SMCs and single culture of SMCs (Figure 6). At the lane of 4.0×10^4, Notch-1 was highly expressed in bMSCs that were not infected, lowely expressed in co-culture of bMSCs not infected and SMCs and

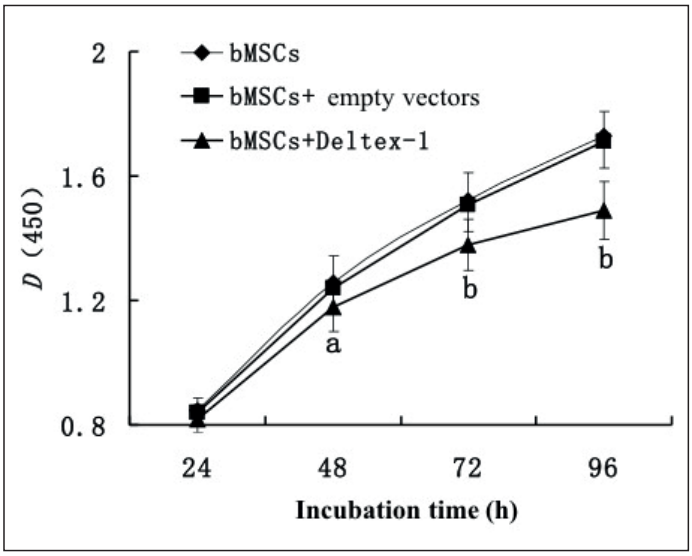

Figure 3. Effect of Deltex-1-infection on proliferation of bMSCs by CCK-8 kit. a: $p<0.05$; b: $p<0.01$ vs. bMSCs infected by empty virus or non-infected bMSCs.

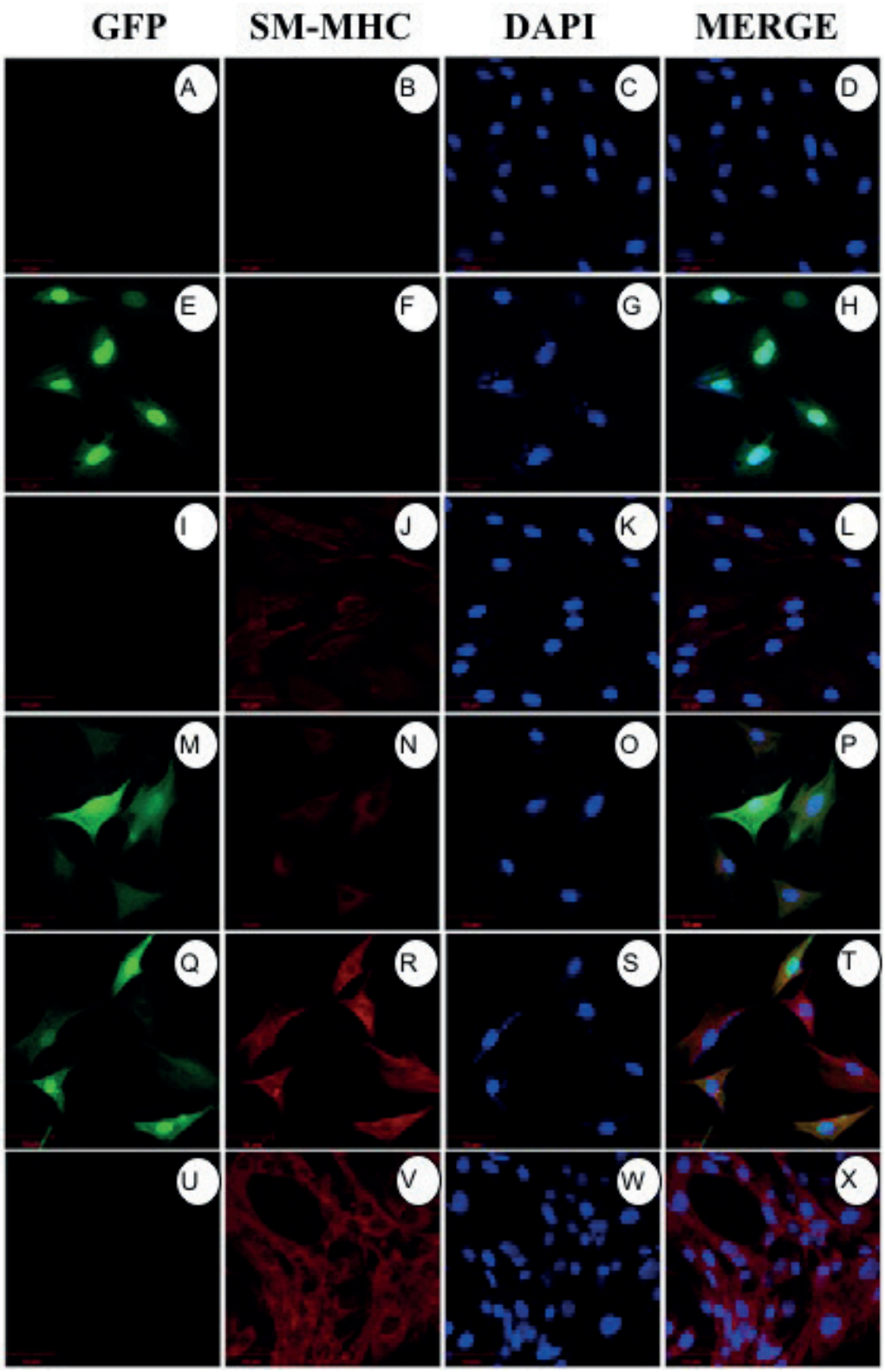

Figure 4. Immunofluorescence detection of SM-MHC expressions in bMSCs. **A**, **B**, **C** and **D**: Non-infected bMSCs; **E**, **F**, **G** and **H**: Deltex-1-infected bMSCs; **I**, **J**, **K** and **L**: Non-infected bMSCs that were co-cultured with SMCs; **M**, **N**, **O** and **P**: Empty-virus-infected bMSCs that were co-cultured with SMCs; **Q**, **R**, **S** and **T**: Deltex-1-infected bMSCs that were co-cultured with SMCs; **U**, **V** and **W**: Single-cultured SMCs.

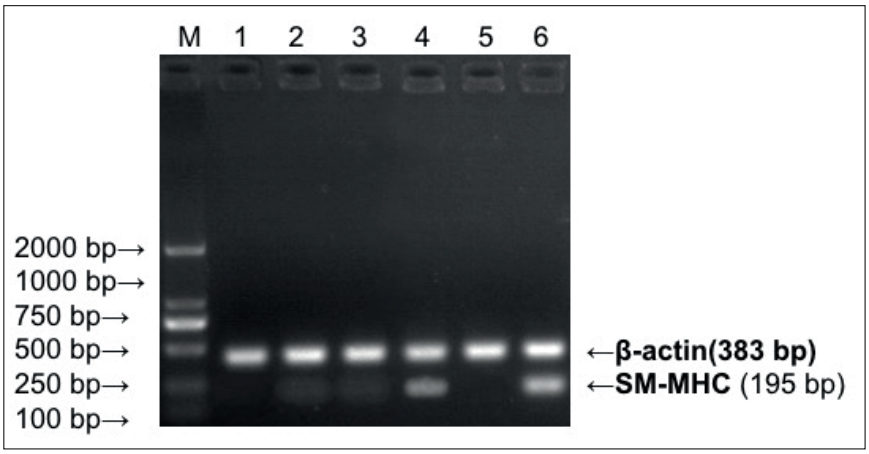

Figure 5. Detection of mRNA expression of SM-MHC in bMSCs through RT-PCR. M: DNA Marker (DL 2000); 1: Non-infected bMSCs; 2: Deltex-1-infected bMSCs; 3: Non-infected bMSCs that were co-cultured with SMCs; 4: Empty-virus-infected bMSCs that were co-cultured with SMCs; 5: Deltex-1-infected bMSCs that were co-cultured with SMCs; 6: Single-cultured SMCs.

Deltex-1-infected bMSCs, slightly or not expressed in co-culture of bMSCs infected by Deltex-1 virus and SMCs (Figure 7). These results suggested Deltex-1 can promote the differentiation of bMSCs into the SMCs through protein level, and this may be achieved through suppressing the proliferation of bMSCs by blocking the Notch signal pathway.

Discussion

Sphincter dysfunction is the major cause of SUI. Existing treatment methods fail to restore the function of sphincter in essence. Focus has been shifted to the stem cell transplantation for its application in repairing the muscle impairment and enhancing the contractibility of muscle[10,11]. However, it has not gained promising efficacy so far, which mainly owes to the tremendous critical problems in research on the *in-vivo* induction and differentiation of stem cells. Among these issues, the most important one is that the mechanisms of proliferation and differentiation of stem cells in post-transplantation microenvironment remain unclear.

bMSCs, with potential of self-renewal and diversified differentiation, can differentiate into the cells necessary for environment in different microenvironment, and research into the potential of proliferation and differentiation of bMSCs into SMCs has a great clinical significance for treatment of a variety of diseases, including SUI[12]. Differentiation-related potential is regulated by the random inhibition or activation of transcript factors, microenvironment induction and signal transduction pathway[5], among which Notch signal pathway is one of the major pathways regulating the stem cell activities, including the proliferation and differentiation of bMSCs. It can express the ligands that can interact with the signals released by adjacent cells, so as to determine the destiny of a variety of cells[13]. Combination between Notch and the corresponding ligands can result in the lysis of extracellular domain of Notch by ADAM through activating the γ-secretase, while the intracellular domain of Notch is transferred into the nuclei and binds to the CSI to form the transcription activator to activate the expression of

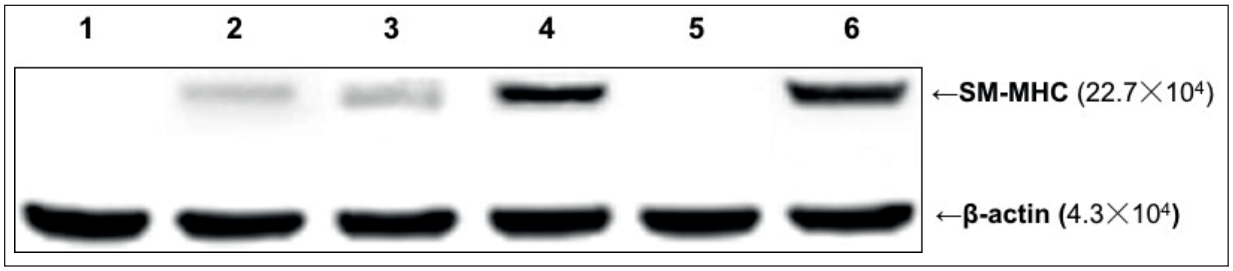

Figure 6. Detection of the protein expression of SM-MHC in bMSCs through Western blot. 1: Non-infected bMSCs; 2: Deltex-1-infected bMSCs; 3: Non-infected bMSCs that were co-cultured with SMCs; 4: Empty-virus-infected bMSCs that were co-cultured with SMCs; 5: Deltex-1-infected bMSCs that were co-cultured with SMCs; 6: Single-cultured SMCs.

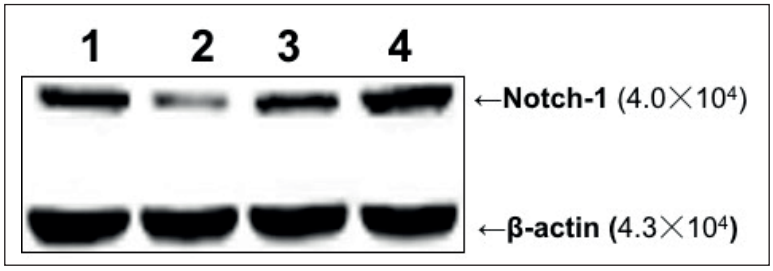

Figure 7. Detection of the protein expression of Notch-1 in bMSCs through Western blot. 1: Non-infected bMSCs that were co-cultured with SMCs; 2: Deltex-1-infected bMSCs that were co-cultured with SMCs; 3: Deltex-1-infected bMSCs; 4: Non-infected bMSCs.

HES, thus affecting the proliferation, differentiation and apoptosis of cells. Deltex family including Deltex-1, Deltex-2, Deltex-3 and Deltex-4, as the ligands of Notch, is closely correlated with the development, growth and differentiation of cells. Deltex-1 is the downstream molecule of Notch signal pathway and highly conservative in protein structure containing Domain I, Domain II and Domain III. Domain I, a necessary constituent of Deltex-1, can bind to the ANK repeated structure in the intracellular domain of Notch to regulate the expression of Notch signal[14] and also the critical regulator in Notch signal pathway[8]. Existing studies[15] have revealed that co-culture of SMCs and bMSCs can induce the differentiation of the latter into SMCs. Moreover, it has been confirmed that Notch signal pathway is involved in this process[16]. However, none has elucidated the role of Deltex-1 in Notch signal pathway. To explore the effect of Deltex-1 on the proliferation and differentiation of stem cells into SMCs, we isolated and cultured the bMSCs in rat for identification, and found that acquired bMSCs had the morphological features and surface markers of stem cells, indicating that they could be used in following experiments. Then, bMSCs were infected by virus containing Deltex-1 gene to detect the effect of Deltex-1 on bMSCs proliferation through CCK-8. Results showed that bMSCs with overexpression of Deltex-1 had a decreased proliferation rate. In further experiment, bMSCs with overexpression of Deltex-1 were co-cultured with SMCs, and immunocytochemistry, RT-PCR and Western blot assay were adopted to detect the effect of Deltex-1 on differentiation of bMSCS towards SMCs. Although there were some divergences between the RT-PCR results and Western blot results, mRNA expressions generally vary from the protein expressions. Meanwhile, the longer electrophoresis time resulted in the slighter expression of proteins in the 2nd and 3rd lanes. Taken together, these results suggested that SM-MHC was not expressed in single culture of bMSCs and Deltex-1-infected bMSCs, lowly expressed in co-culture of bMSCs not infected or infected by empty vector and SMCs, and highly expressed in Deltex-1-infected bMSCs that were co-cultured with SMCs and single culture of SMCs, suggesting that differentiation of bMSCs toward SMCs can only be initiated in co-culture with SMCs, consistently with previous investigations[16]. Moreover, the overexpression of Deltex-1 can accelerate this process.

Researches have reported that Notch pathway can facilitate the maintenance and proliferation of a variety of stem cells[17], and is involved in regulating the diverse differentiation-related potential[18]. Hsiao et al[14] have revealed that Deltex-1 can induce the ubiquitination and degradation of Notch receptor through binding to the intracellular domain of Notch, thereby inhibiting the Notch signal pathway. In addition, we found that in co-culture of bMSCs with Deltex-1 overexpression and SMCs, expression of Notch-1 was downregulated significantly. This suggested that Deltex-1 may facilitate the differentiation potential of bMSCs through blocking Notch signal pathway to inhibit the proliferation of bMSCs, and in culture with SMCs, variations in microenvironment can induce the differentiation of bMSCs towards SMCs.

Conclusions

We showed the facilitating effect of Deltex-1 on the differentiation of bMSCs into SMCs, which provides experimental basis for treatment of SUI. However, to figure out the specific molecular mechanism, requires further investigations.

Acknowledgements

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Conflict of interest

The authors report no conflicts of interest in this work.

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