A THESIS FOR THE DEGREE OF MASTER

Effect of ectopic OCT4 on canine adipose tissue-derived mesenchymal stem cells

개 지방 조직 유래 중간엽 줄기세포에서 OCT4의 효과에 관한 연구

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수의학과 수의내과학 전공

한 상 훈

Abstract

Sang Hun Han

Department of Veterinary Internal Medicine

The graduate School

College of Veterinary Medicine, Seoul National University

Gene modification to enhance the proliferative capacity of mesenchymal stem cells (MSCs) is critical to increase the therapeutic potentials of regenerative medicine and cell therapy for treating a wide range of diseases. To maximize the availability of MSCs for clinical application, their proliferative capacity is essential. It was hypothesized that the effect of lentivirus-mediated overexpression of canine OCT4 (cOCT4) may have an influence on the proliferation of canine adipose tissue-derived MSCs (cATMSCs).

cATMSCs were successfully transduced with an cOCT4-lentiviral vector and increased expression levels of cOCT4 were confirmed by RT-PCR and immunoblotting. To identify stem cell markers for phenotypic characterization of cOCT4- and Mock-transduced cATMSCs, cell surface antigens were evaluated by flow cytometry. Both of cOCT4- and Mock-cATMSCs showed high expression of CD29, CD44, CD73, CD90 and CD105, and the absence of CD31 and CD45 surface markers. Also, it was found that expression of CD44, CD73, CD90 and CD105 was increased relatively in cOCT4-cATMSCs compared to Mock-cATMSCs. In addition, the proliferative capacity was evaluated by a WST-1 cell proliferation assay and trypan blue exclusion. cOCT4-cATMSC showed a higher

proliferative ability than that of Mock-cATMSCs. The results of cell cycle analysis showed that overexpression of cOCT4 in cATMSCs caused an increase in the proportion of cells in S and G2/M phases. Increased cyclin D1 expression in cOCT4-cATMSCs was confirmed by western blot. Moreover, the expression of hepatocyte growth factor (HGF) at mRNA and protein levels was upregulated in cOCT4-cATMSCs.

The results of the current study show that lentivirus-mediated overexpression of cOCT4 increased the proliferative ability of cATMSCs and expression of HGF. This improvement using OCT4 expression in ATMSCs may be useful method to expand the population without loss of stemness.

Keywords : canine OCT4, adipose tissue derived mesenchymal stem cells, cell proliferation, hepatocyte growth factor, cell cycle analysis

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1. Introduction

Mesenchymal stem cells (MSCs) are found in several adult tissues, are readily available, and have therapeutic potentials in regenerative medicine and cell therapy for treating a wide range of diseases [1]. MSCs possess robust self-renewal, and are able to differentiate into numerous specialized cell types in vitro [2, 3]. Although clinical interest in cultured MSCs initially focused on their stem cell properties for tissue regeneration and repair, recent studies show that MSCs secrete a broad spectrum of bioactive macromolecules that influence regenerative microenvironments in tissue injury [4]. Thus, discovery of the paracrine properties of MSCs markedly increases their range of therapeutic applications for which they are currently studied. For these reasons, MSCs are currently thought to be excellent candidates for use in cell therapy.

One of the problems that still needs to be resolved is expansion of MSCs to yield high enough numbers of cells in vitro without an increase in chromosomal aberrations while maintaining self-renewal and multipotency. Rapid proliferation of desired cell phenotypes is a critical issue in stem cell therapy and tissue engineering approaches to repair damaged tissues with stem cells [5]. To obtain sufficient cell numbers for tissue repair, it is necessary to expand MSCs in culture. For example, ~4×10^6 MSCs are needed for a single injection aimed at infarcted heart repair [6]. In addition, Mastri et al. [7] evaluated the relationship between injected cell doses (2–40×10^6 cells/kg animal body weight) and therapeutic benefits, and found that effective cardiac repair was achieved with the highest cell dose. However, after several passages, MSCs enter senescence, characterized by

enlarged and irregular cell shapes and cessation of cell division, which causes MSCs to gradually lose their stem cell properties. To maximize the availability of MSCs for cell therapy, their proliferative capacity is essential. Enhanced proliferation of stem cells plays an important role in obtaining large numbers of stem cells, which may provide insights into improving the efficiency of stem cell transplantation [8, 9]. For this reason, proliferation is thought to be an important aspect to obtain an adequate number of stem cells with stemness properties for cell therapies, which makes them an excellent tool for regenerative medicine. Therefore, improvement of MSC proliferation is necessary for the clinical application of MSC-based tissue engineering [10].

Many studies have reported enhanced MSC proliferation by improving the culture system such as the medium condition, cell density and culture flasks [11] or applying mitogenic growth factors [12, 13]. The role of growth factors in enhancing MSC proliferation has been extensively studied over the past few years [12]. Fibroblast growth factor (FGF) and other growth factors, such as platelet-derived growth factor and epidermal growth factor, are often added to expansion medium [14]. It is known that these growth factors are pleiotropic, induce multiple biological effects as well as changes in cell motility, proliferation, morphogenesis and survival. Some studies have aimed to find growth factors that do not affect differentiation, whereas other reports have opted to study growth factors that induce a differentiation preference towards a specific lineage [12]. Basic FGF may decrease the doubling time of MSCs [15], although as the cells reach senescence, their growth factor receptors are downregulated and signals are highly attenuated, resulting in resistance to growth factor stimuli.

OCT4, encoded by POU5F1 belonging to the family of POU domain transcription factors, is a key transcription factor essential for self-renewal and survival of MSCs [16, 17], and has a unique role in development and determination of pluripotency. OCT4 is one of the four transcription factors, including SOX2, c-Myc and KLF4, used for induced pluripotent stem cell generation. Kim et al. [18] reported that OCT4 alone is sufficient to reprogram directly to pluripotent stem cells. When applied to MSCs, OCT4-expressing MSCs display a high proliferative capacity [19], whereas inhibition of OCT4 expression in MSCs results in inhibition of cell proliferation [20]. Moreover, OCT4 co-regulates certain genes encoding components of signaling pathways that control stem cell proliferation [17]. However, OCT4 is usually expressed at very low levels in early passage MSCs and disappears in MSCs at late passages [16]. Therefore, the use of MSCs alone can be hampered largely because of their limitations including low proliferation and gradual loss of their stem cell properties during in vitro expansion. For these reasons, OCT4 may be an attractive candidate gene for genetic modification of MSCs to increase cell proliferation.

The aim of this study is to investigate the effect of lentivirus-mediated overexpression of canine OCT4 on the proliferation of canine adipose tissue-derived MSCs (cATMSCs).

2. Materials and Methods

2.1. Cell isolation and culture

cATMSCs with defined characterization were provided by RNL Bio (Seoul, Korea). cATMSCs were obtained from canine subcutaneous fat tissue, and cultured in Dulbecco's low glucose modified medium (Thermo Scientific Inc, Utah, USA) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. After 24 hours, non-adherent cells were discarded, and the adherent cells were thoroughly washed with PBS twice. Fresh complete medium was added and replaced every 2–3 days until the cells reached 80–90% confluency. cATMSCs were subcultured once before use. cATMSCs were used at passages 2–5. All cells were cultured at 37℃ in a 5% CO2 incubator.

2.2. Engineering of cOCT4-overexpressing cATMSCs using a lentiviral vector system

Total RNA was extracted from canine ovarian tissue using an Easy-Blue Total RNA extraction kit (iNtRON Bio., Seoul, Korea). Following the manufacturer's method, 1 ml RNA extraction solution was added to 200 μl chloroform, followed by centrifugation at 13000 rpm and 4°C. Isopropanol (400 μl) was added to the tube and the upper solution was discarded to obtain the RNA pellet. The concentration of isolated total RNA dissolved in diethylpyrocarbonate-treated water was measured with a GeneQueant II RNA/DNA Calculator (Pharmacia

Biotech, Cambridge, UK). cDNA was synthesized using 3 μg RNA, Moloney murine leukemia virus reverse transcriptase (M-Mulv RT), and an oligo (dT)-18 primer according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). The following primers were used for PCR: cOCT4 (617 bp), forward 5'-ACTAGTGATGGCGGGACACCTGGCTT-3'; and reverse 5'-CTCGAGTCAATTTGAATGCATGGGAG-3'. The PCR volume of 50 μl consisted of 5 μl 10× buffer, 4 μl dNTPs, 1 μl forward and reverse primer each, 0.5 μl pfu Taq polymerase (Bio-online) and 1 μl cDNA template. PCR conditions were initial denaturation at 94℃ for 5 minutes, 30 cycles of amplification at 94℃ for 30 seconds, annealing at 57℃ for 30 seconds and extension at 72℃ for 30 seconds, followed by a final extension at 72℃ for 10 minutes. PCR products were electrophoresed on 1.5% agarose gels, stained with ethidium bromide for 20 minutes, visualized under ultraviolet illumination and photographed with a Gel-Doc system (BioRad, Hercules, CA, USA). The 617 bp DNA band was extracted and purified by a Qiaquick Gel Extraction kit (Qiagen, Hilden, Germany).

To construct the lentiviral vector containing the cOCT4 gene, a pLenti6/v5/D-TOPO plasmid was used with a pLenti6/V5 Directional TOPO® Cloning Kit (Invitrogen). The amplified DNA was cloned into the pLenti6/v5/D-TOPO® vector with 16 ng PCR products, 1 μl vector and up to 6 μl water added. E.coli JM109 cells were transformed with the recombinant vector, which were placed on Luria Bertani (LB) agar plates with ampicillin and incubated overnight at 37℃. Several colonies containing plasmids were picked up from LB agar plates and incubated overnight in LB broth with ampicillin (100 μg/ml) at 37°C. Plasmid DNA was isolated from cultured cells by a PureLink™ HiPure Plasmid Midiprep kit

(Invitrogen) and treated with SpeI and XhoI restriction enzymes (Fermentas, Vilnius, Lithuania) to identify that the cOCT4 gene was successfully inserted into the plasmid DNA. The plasmid was sequenced using the cytomegalovirus forward primer and the V-5 (C-term) reverse primer. Mock vector without an insert was used as a control (Mock-lentiviral expression vector).

2.3. Transfection of cATMSCs using the cOCT4-lentivirus

cATMSCs (5 × 104 cells) were grown in 100-mm culture plates. The culture medium was replaced with 200 μl viral supernatant mixed with 8 ml medium and 6 μg polybrene. After incubation for 24 hours at 37°C, the medium was replaced with OPTI-MEM containing 10% FBS, 1% penicillin/streptomycin and 5 μg/ml blasticidin to select transduced cells. Selection was continued until all of the cells were killed in the mock wells. Finally, the cells were washed with PBS and cultured in Dulbecco's high glucose modified medium (Thermo Scientific Inc, Utah, USA) containing 10% FBS and 1% penicillin/streptomycin.

2.4. RT-PCR analysis

Total RNA was isolated from cOCT4-and Mock-transduced cATMSCs using Easy-Blue RNA extraction reagents following the manufacturer's protocol. Briefly, samples were transferred to a tube containing 1 ml RNA extraction solution. The homogenate was then chloroform extracted, isopropanol precipitated, ethanol

washed and resuspended in 30 μl distilled water. RNA concentration and purity were determined by absorbances at 260 and 280 nm. Samples exhibited an absorbance ratio (260/280) of ≥ 1.8. First strand cDNA was obtained by reverse transcription using 3 μg total RNA, M-MuLV RT and an oligo (dT)-18 primer according to the manufacturer's instructions (Invitrogen). PCR volumes were 20 μl consisting of 2.5 μl 10× buffer, 2 μl dNTPs, 0.25 μl rTaq polymerase (TaKaRa, Otsu, Shiga), 1 μl cDNA template and 1 μl cOCT4, cHGF or canine glyceraldehyde-3-phosphate dehydrogenase (cGAPDH) forward and reverse primers each (Table 1). PCR products were electrophoresed on 1.5% agarose gels to verify DNA fragment sizes.

Table 1 - Primer sets used for RT-PCR amplification of target genes

Gene	Genbank Accession number	Sequences of primer	Product size(bp)
cOCT4	XM_538830	F : ACTAGTGATGGCGGGACACCTGGCTT R : CTCGAGTCAATTTGAATGCATGGGAG	1096
cHGF	AB090353.1	F : GCAGCAGCTCCTCCTGCACC R : GTGCCGGTGCGGTGTCTGAT	694
cGAPDH	AB038240	F : GGTCACCAGGGCTGCTTT R : ATTTGATGTTGGCGGGAT	209

2.5. Western blot analysis

OCT4, cyclin D1, HGF and β-actin expression levels were examined in Mock- and cOCT4-cATMSC by western blot. Whole cell lysates were prepared in

Proprep™ protein extraction solution (iNtRON bio). The protein concentration was determined with Bradford reagent (BioRad). Total protein (20 μg for β-actin and OCT4 analysis and 120 μg for cyclin D1 analysis) was separated on 10% polyacrylamide gels, and then transferred onto a polyvinylidene fluoride (PVDF) membrane (Whatman, Maidstone, UK). Total protein (80 μg) for cHGF analysis was separated on 8% polyacrylamide gels, and then transferred onto a PVDF membrane. The membranes were blocked with 5% skim milk powder in 0.1% Tween 20, 10 mM Tris, and 150 mM NaCl (TBST) for 2 hours at room temperature. After a brief wash with TBST, each membrane was incubated with antibodies against OCT4 (1:500), cyclin D1 (1:200), HGF (1:200) and β-actin (1:1000) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). The membranes were washed again with TBST and incubated overnight with each secondary antibody at 4℃. Proteins were detected with an enhanced ECL plus kit (Gendepot, TX, USA).

2.6. Flow cytometric analysis of cell surface marker expression in cATMSCs

To analyze cell surface marker expression in Mock- and cOCT4-cATMSCs, flow cytometry was used. Analysis was performed by a FACSCalibur (BD Biosciences, Bedford, MA, USA) and CellQuest Pro software (BD Biosciences). By passage 4, a homogenous population of rapidly dividing cells with fibroblastoid morphology was obtained. cATMSCs were fixed with 70% ethanol at 4℃ and stained for 30 min on ice with primary antibodies that recognized various surface molecules.

cATMSCs were incubated with the following primary antibodies: Anti-Rat CD29 (BD Pharmingen, USA), Anti-Canine CD44 (Serotec, USA), Anti-Mouse CD73 (BD Pharmingen), Anti-Canine CD90 (thy.1) (Serotec), Anti-Mouse CD105 (BD Pharmingen), Anti-Human CD31 (BD Pharmingen) and Anti-Human CD45 (BD Pharmingen). Cells were incubated with a Mouse Anti-Rat IgG FITC secondary antibody (BD Pharmingen) only as a control.

2.7. Cell proliferation assay

Cell proliferation and viability were evaluated by a WST-1 cell proliferation assay and trypan blue exclusion. For the WST-1 cell proliferation assay, cells were seeded in 96-well plates at 5 × 103 cells/well. Each cell was assayed in triplicate, including Mock-cATMSC wells. Cells were then incubated for 6 (day 0), 24 (day 1), 48 (day 2) and 72 hours (day 3), then 10 µl WST-1 reagent was added to each well. The reaction proceeded for 2 hours at 37℃ with 5% CO2. The absorbance of the samples at 450 nm was measured by a microplate reader. For trypan blue exclusion, cATMSCs were seeded into 6-well culture plates at 2 × 104 cells/well in duplicate and then incubated at 37℃ with 5% CO2 for 5 days. Cells were then collected and stained with a 0.4% trypan blue solution. Enumeration of viable cells was carried out under a phase contrast microscope with a hemocytometer at day 5 after seeding. Cell viability and proliferation were separately examined by phase contrast microscopy.

2.8. Cell cycle analysis by flow cytometry

Mock- and cOCT4-cATMSCs (4 × 105 cells) were cultured for cell cycle analysis by flow cytometry. Mock- and cOCT4-cATMSCs were harvested and washed twice with 1 mM EDTA-PBS. Cells were fixed with 70% ethanol for at least 1 day. Then, cells were washed with EDTA-PBS, treated with 10 μg ribonuclease A (Sigma-Aldrich) at 37°C for 1 hour, and then combined with 10 μg propidium iodide (Sigma-Aldrich) for flow cytometric analysis using a FACSCalibur. A total of 2 × 103 cells were analyzed.

2.9. Statistical analysis

All statistical differences were analyzed using Excel 2010 and GraphPad software (2003). Differences were considered significant at *P < 0.05 and **P < 0.01 compared with the corresponding control values as determined by one-way ANOVA with the Tukey test or Student's t-test.

3. Results

3.1. Identification of packaging plasmids that can produce lentiviral vectors containing the canine OCT4

The open reading frame of canine OCT4 (cOCT4) was amplified by blunt-end PCR and cloned in a V5-D-TOPO vector using porcine cytomegalovirus(pCMV) promotor. Additionally, the pLenti6/V5-D-TOPO containing cOCT4 was digested with *Spe*I and *Xho*I, restricted enzymes, and a band fo the insert gene at 1096-bp in sizes was detected in (Fig 1.)

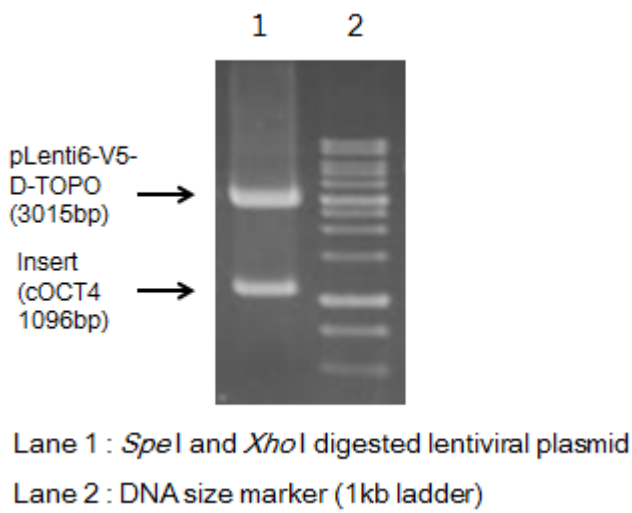


Figure 1. Construction of lentiviral expression vector carrying c*OCT4* gene. Lentiviral-cOCT4 plasmid was digested with *Spe*I and *Xho*I and the inserted gene was confirmed at 1.5% agarose gel.

After the lentiviral vector containing the c*OCT4* gene was transfected to the 293FT cells, a comparative reverse transcriptase-polymerase chain reaction (RT-

PCR) was performed on the remained 293FT cells and with untransfected 293FT cells. Expression of cOCT4 mRNA was significantly increased in the transfected 293FT cells compared with non-transfected cells used as a negative control in (Fig 2.)

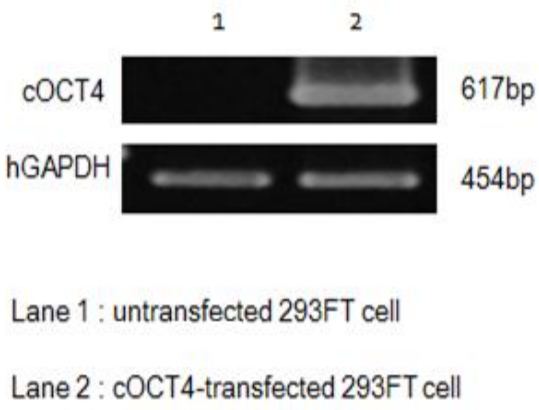


Figure 2. RT-PCR analysis of cOCT4 mRNA expression in cOCT4 transfected 293FT cell compared with untransfected 293FT Cell. Expression of human GAPDH (h-GAPDH) mRNA was analyzed from transfected 293FT cells and untransfected 293FT cells. Expression of cOCT4 mRNA was significantly increased in cOCT4 transfected 293FT cells compared with in untransfected cells.

3.2. Lentivirus-mediated transduction of cATMSCs with cOCT4

The cOCT4 gene was transduced into cATMSCs using a lentiviral expression vector. At the third passage, cATMSCs were transduced overnight in OPTI-MEM without serum and in the presence of polybrene. Cells were incubated in complete

growth medium containing blasticidin (5 μg/ml) to select successfully transduced cATMSCs. After selection, semi-quantitative RT-PCR and western blot analyses of selected cOCT4- and Mock-cATMSCs were performed. Up-regulation of cOCT4 mRNA and protein expression was confirmed in cOCT4-cATMSCs (Fig. 3a, b). These results showed that cATMSCs were successfully transduced with the cOCT4-lentiviral vector.

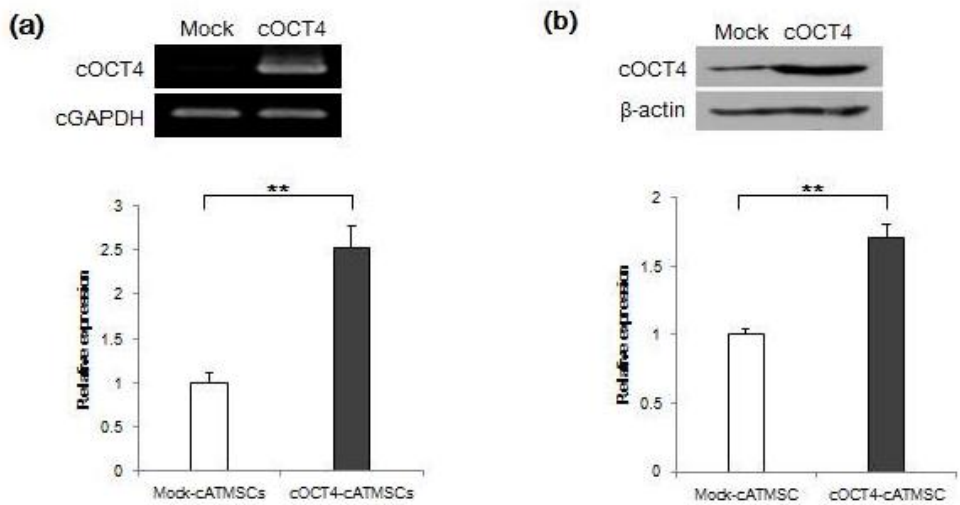


Figure 3. Expressional analysis of cOCT4 in cATMSCs transduced with lentiviral-mediated cOCT4 gene. cOCT4 expression was analyzed from cOCT4-cATMSCs cultured for 3 days by RT-PCR (a) and western blot (b). The results of both RT-PCR and western blot showed significant increase of OCT4 expression in cOCT4-cATMSCs compared with Mock-cATMSCs. Band densities in each lane of RT-PCR and western blot were evaluated semi-quantitatively by scanning densitometry. Data are representative of three independent experiments with similar results. Result is ratio of OCT4 expression normalized to GAPDH mRNA (a) or β-actin protein (b) expressed as means +SD. *P < 0.05 and **P < 0.01

compared with the control (Mock-cATMSCs) value as determined one way ANOVA with Tukey test.

3.3. Expression of stem cell markers in cOCT4-cATMSCs

To identify markers for phenotypic characterization of cOCT4- and Mock-cATMSCs, cell surface antigens were evaluated by flow cytometry (Fig. 4). The presence of surface molecules was analyzed using specific monoclonal antibodies against CD29, CD44, CD73, CD90, CD105, CD31 and CD45. Flow cytometric analysis confirmed high expression of CD29 (99.86% for Mock; and 99.91% for cOCT4), CD44 (63.81% for Mock; and 81.71% for cOCT4), CD73 (61.70% for Mock; and 74.46% for cOCT4), CD90 (60.08% for Mock; and 78.19% for cOCT4) and CD105 (65.92% for Mock; and 80.47% for cOCT4), while endothelial cell marker CD31 (1.17% for Mock; and 1.19% for cOCT4) and myeloid/hematopoietic marker CD45 (1.77% for Mock; and 2.17% for cOCT4) were rarely detected. CD44, CD73, CD90 and CD105 were highly expressed in cOCT4-cATMSCs compared with those in Mock-cATMSCs. Phenotypic characterization confirmed that these cells were MSCs, although expression differences in CD markers were found. In addition, these data indicate that cOCT4 could enhance stem cell marker expression of MSCs in vitro.

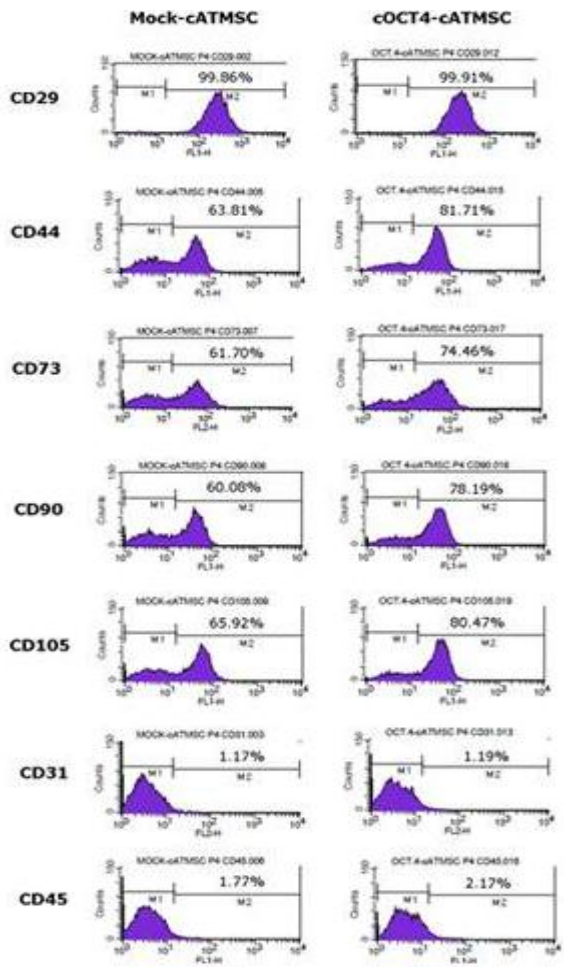
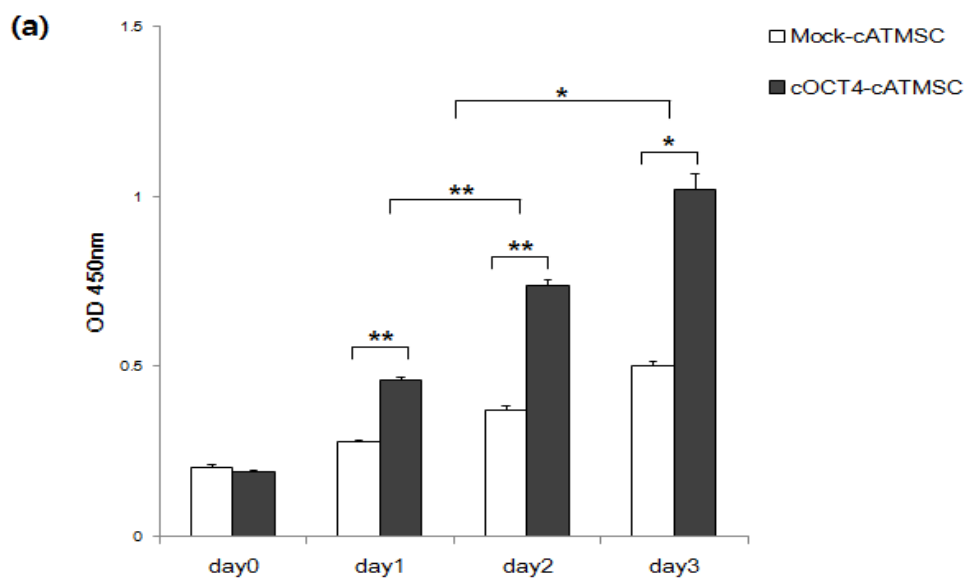


Figure 4. Immunophenotyping of cOCT4-cATMSCs by flow cytometric analysis. Mock- and cOCT4-cATMSCs at passage 3 (or 4) were immunophenotyped for CD29, CD31, CD45, CD44, CD73, CD90 and CD105 by flow cytometric analysis. Both Mock- and cOCT4-cATMSCs showed high expression of CD29, CD44, CD73, CD90 and CD105, and the absence of CD31 and CD45 surface markers. In addition, these results revealed that expression of CD44, CD73, CD90 and CD105 was increased relatively in cOCT4-cATMSCs compared to Mock- cATMSCs. Data are representative of three independent experiments with similar results.

3.4. Enhanced proliferative ability of cOCT4-cATMSCs

To evaluate the proliferative ability of cOCT4-cATMSCs, a WST-1 cell proliferation assay was performed, which measures cell viability relative to the metabolic activity, after culturing the cells for 24 h followed by an additional 6, 24, 48 and 72 h of incubation. Figure 3a shows the effect of OCT4 overexpression on the proliferative ability of cATMSCs. OCT4 overexpression in cATMSCs resulted in a time-dependent increase in proliferation as indicated by the WST-1 cell proliferation assay. It was also confirmed that proliferation of cOCT4-cATMSCs was enhanced, compared with that of Mock-cATMSCs, by counting cells using trypan blue exclusion. Compared with Mock-cATMSCs, cOCT4 overexpression increased the proliferation rate of MSCs by an average of 1.95-fold (Fig. 5c). During passaging, it was also observed that cells displayed markedly enhanced cell population doubling (Fig. 5b). These results suggest that cOCT4 may have an important role in the proliferative ability of cATMSCs.



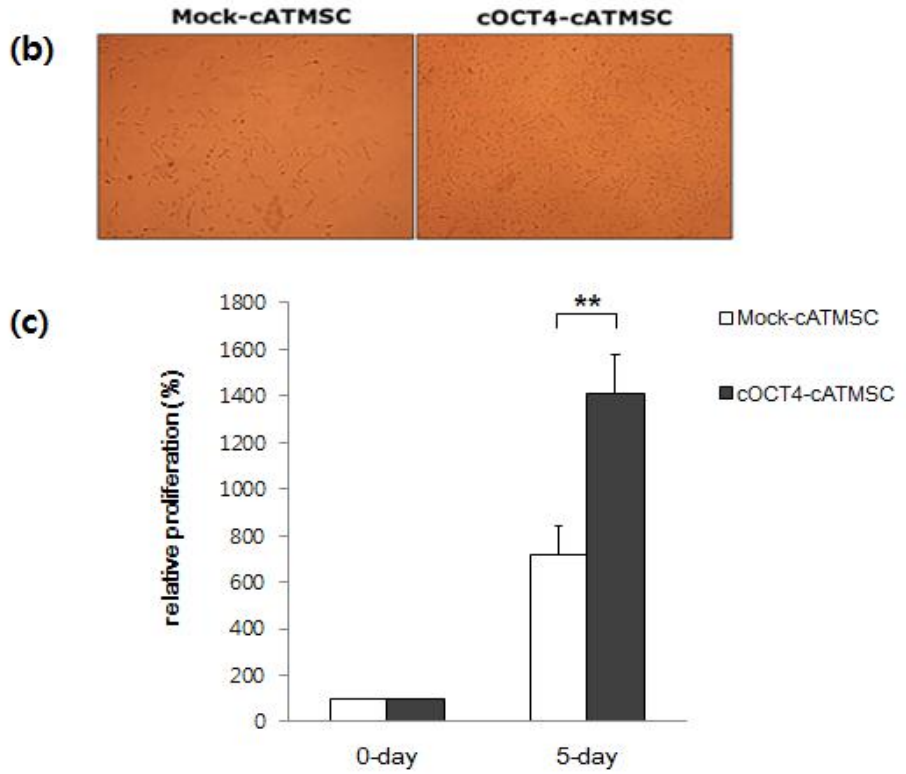
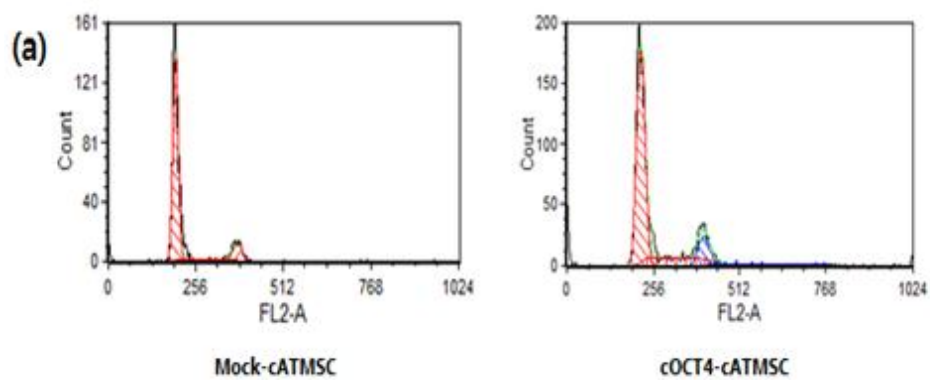


Figure 5. Proliferation assay of cOCT4-cATMSCs. (a) Canine MSCs were incubated for 6 (day 0), 24 (day 1), 48 (day 2) and 72 hours (day 3), and then cell viability was measured by a WST-1 reagent. Significant differences in the proliferation rate were observed in cOCT4-cATMSCs compared with Mock-cATMSCs. Also, cOCT4-cATMSCs were incubated at 100-mm dish for 5 days and then the cells were photographed (b), and counted by trypan blue exclusion (c). Results of trypan blue exclusion assay showed increased cell viability and proliferation ability (2-fold) in cOCT4-cATMSCs compared with Mock-cATMSCs. Data are representative of three independent experiments with similar results. Data are expressed as the mean + SD. *P < 0.05 and **P < 0.01 compared with the corresponding control value as determined by one-way ANOVA with the Tukey test.

3.5. Increase of S and G2/M phase composition in cOCT4-cATMSCs by cell cycle analysis

To assess the proliferative ability of cOCT4-cATMSCs, cell cycle analysis was performed after culturing the cells for 24 h. Mock- and cOCT4-cATMSCs (4 × 105 cells) were harvested for cell cycle analysis by flow cytometry. Cell cycle analysis showed that 82.30 ± 0.89%, 2.00 ± 0.10% and 15.33 ± 0.93% of Mock-cATMSCs were in G0/G1, S and G2/M phases, respectively. For cOCT4-cATMSCs, G0/G1, S and G2/M phases represented 76.26 ± 1.71%, 3.56 ± 0.83% and 20.02 ± 1.24% of the cell population, respectively (Fig. 6b). Cell cycle analysis showed that overexpression of cOCT4 in cATMSCs caused an increase in the proportion of cells in S and G2/M phases. Progression of proliferation during the cell cycle is closely regulated by cyclin D1, a protein that activates cyclin-dependent kinases (CDKs). Result from western blot analysis showed a marked increase in the expression of genes known to be involved in S phase and mitosis, such as cyclin D1 (Fig. 6c), when cOCT4 was overexpressed in cATMSCs. These data showed that cOCT4 overexpression improved cATMSC proliferation.



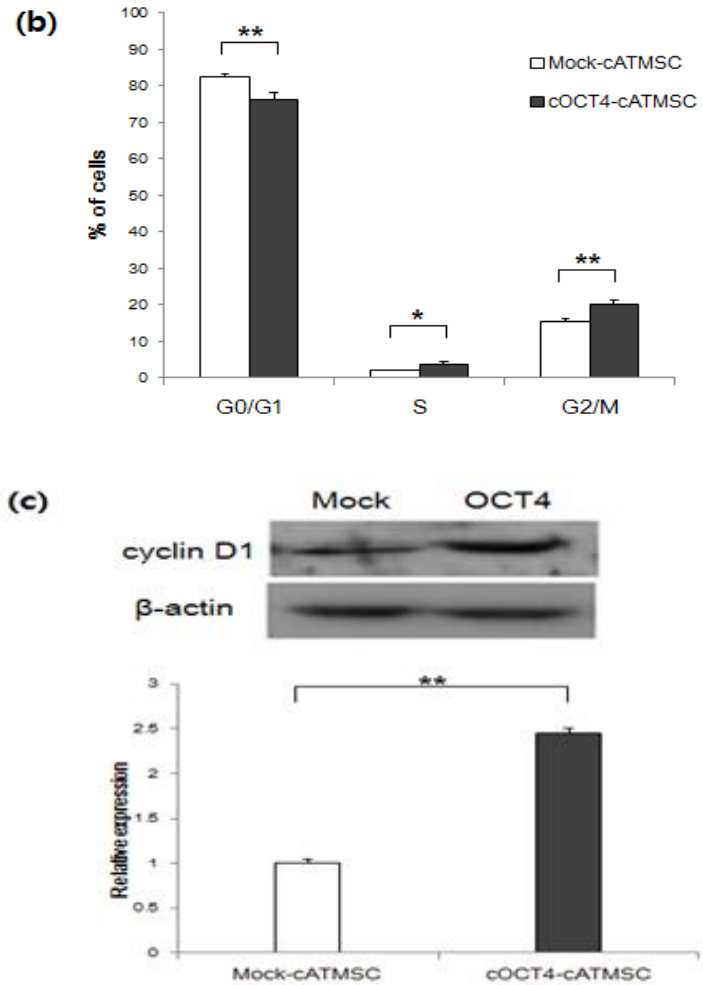


Figure 6. Cell cycle analysis by flow cytometry, and western blot analysis of cyclin D1 in cOCT4-cATMSCs. cOCT4-cATMSCs were incubated in DMEM media containing 10% FBS in triplicate and harvested at 3 days for flow cytometric analysis (a, b). Results of cell cycle analysis showed that cOCT4-cATMSC decreased G0/G1 phase at 3 days whereas increased S and G2/M phase compare to control (Mock-cATMSCs). In addition, cyclin D1 expression of cOCT4-cATMSCs was assessed by western blot (c). The results revealed increase of cyclin D1 expression in cOCT4-cATMSCs. Data are representative of three independent

experiments with similar results. Data are expressed as the mean + SD. *P < 0.05 and **P < 0.01 compared with the corresponding control value as determined by the Student's t-test.

3.6. Upregulation of hepatocyte growth factor in cOCT4-cATMSCs

To quantify hepatocyte growth factor (HGF) mRNA expression levels in cOCT4-cATMSCs, quantitative RT-PCR was performed (Fig. 7a). cOCT4-cATMSCs exhibited a higher HGF mRNA level than that in Mock-cATMSCs. Moreover, cOCT4-cATMSCs exhibited an increased HGF protein level as shown by western blot (Fig. 7b). OCT4 mRNA and protein levels were increased by 1.91-fold and 1.72-fold in cOCT4-cATMSCs, respectively, compared with those in Mock-cATMSCs. These results showed that HGF mRNA and protein levels were upregulated in cOCT4-cATMSCs compared with those in Mock-cATMSCs.

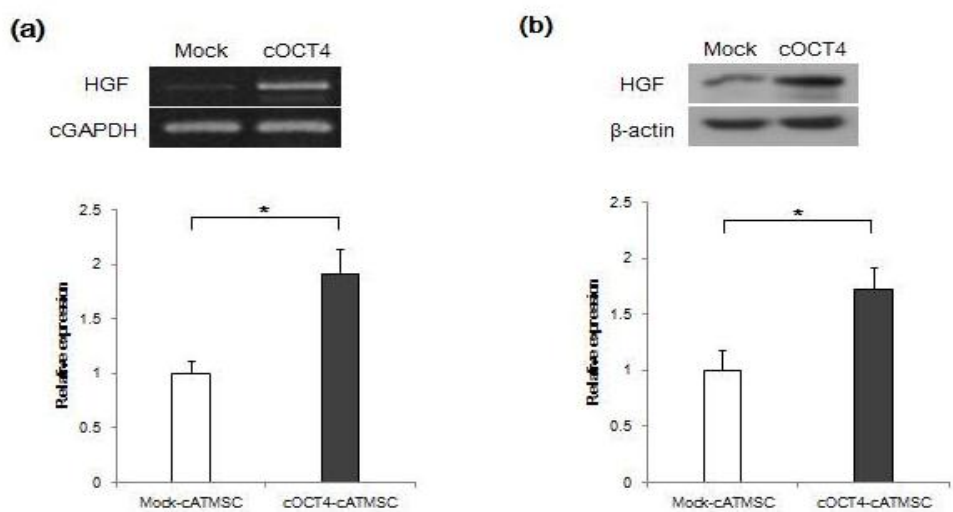


Figure 7. Upregulation of HGF in cOCT4-cATMSCs. cOCT4-cATMSCs were incubated in DMEM media containing 10% FBS in triplicate and harvested at 3 days for semi-quantitative RT-PCR (a) and western blot of HGF (b). Expression of HGF-mRNA and protein was significantly increased in cOCT4-cATMSCs compared with Mock-cATMSCs. Band densities in each lane of RT-PCR and western blot were evaluated semi-quantitatively by scanning densitometry. Data are representative of three independent experiments with similar results. Result is ratio of HGF expression normalized to GAPDH mRNA (a) or β-actin protein (b) expressed as means +SD. *$P < 0.05$ compared with the control value as determined one way ANOVA with Tukey test.

4. Discussion

In this study, cATMSCs was successfully engineered to overexpress cOCT4 using a lentiviral expression vector that has the advantage of integration into its host cell genome, thereby allowing long-term expression of the exogenous gene. Lentiviral vector-mediated genetic modification of MSCs with genes encoding for specific transcription factors can efficiently induce and maintain a proliferative potential [16, 21, 22]. In addition, the frequency of lentiviral transduction of target cells is far better than that using non-viral vectors [23], as shown by a previous study in which the frequency of transduction was over 90% [24]. The results demonstrated high efficiency of exogenous cOCT4 transduction and up-regulation of expression in transduced cATMSCs, indicating the effectiveness of the lentiviral expression vector system (Fig. 1a, b).

OCT4, a POU domain-containing transcription factor encoded by Pou5f1, is expressed in pluripotent embryonic stem cells and germ cells, and has a unique role in development and determination of pluripotency. OCT4 has potent self-renewal as well as proliferative effects [16]. It has been reported that OCT4-expressing MSCs display a high proliferative capacity [19], whereas OCT4 knockdown significantly lowers the growth rates of MSCs [25]. A study by Hochedlinger et al. [26] showed that only progenitor cells respond to exogenous expression of OCT4 with increased proliferation. Conversely, exogenous expression of OCT4 in differentiated fibroblasts has adverse effects on cell proliferation and results in a 2-fold reduction in cell proliferation of fibroblasts, indicating that OCT4 overexpression has converse effects on the proliferation of progenitors and

differentiated cells [16]. In agreement with the findings that OCT4 increases the proliferation of progenitor/stem cells rather than that of differentiated cells, these findings demonstrated that OCT4 overexpression promoted the proliferation of MSCs. cOCT4-overexpressing cells showed significantly different population doubling as measured by the WST-1 cell proliferation assay and trypan blue exclusion (Fig. 3a–c). cOCT4-overexpressing cells had significantly higher viability and proliferation rates than those of control cells. The enhanced growth activity was further confirmed by cell cycle analysis and western blot of cyclin D1. The cell cycle is a critical process for determining cell proliferation and senescence [8]. G0/G1 phase is a non-proliferative or resting phase, S phase is DNA synthesis, and G2/M phase is mitosis. The overexpression of cOCT4 in cATMSCs caused an increase in the proportion of cells in S and G2/M phases compared with that of Mock-cATMSCs. These results indicate that transduction of the cOCT4 gene increases the DNA content of S phase by inducing the transition of MSCs from G0/G1 to S phase, thus promoting the proliferation of MSCs. In addition, delaying G0/G1 to S phase transition slows stem cell self-renewal and possibly prepares stem cells for differentiation [27]. Cell cycle progression is tightly regulated by cyclin D1 [28], which is a critical regulator of transition from G1 to S phase, and is expressed to transfer the DNA replication signals [29]. Its expression influences cell cycle progression and consequently stem cell proliferation.

Characterization of cOCT4-overexpressing cATMSCs was performed by flow cytometry (Fig. 2). CD marker expression is a typical standard for MSC identification, and cell surface marker characteristics provided convincing evidence that the transduced cATMSCs were phenotypically MSCs [30]. It was shown that

both cOCT4- and Mock-cATMSCs were negative for hematopoietic markers CD31 and CD45, and consistently positive for MSC markers CD29, CD44, CD73, CD90 and CD105, although there was a difference caused by species-specific or cross-reacting monoclonal antibodies [31]. Data from the current study were consistent with those of other studies that reported MSCs exhibit high levels of CD29, CD44, CD73, CD90 and CD105 expression [32-34]. In addition, CD44, CD73, CD90 and CD105 were highly expressed in cOCT4-cATMSCs compared with those in Mock-cATMSCs. The results suggest that OCT4 overexpression may promote expression of mesenchymal stem cell markers such as CD44, CD73, CD90 and CD105.

Interestingly, It was found that cOCT4 overexpression in MSCs induced upregulation of HGF (Fig. 5a,b). HGF, a pleiotropic cytokine of mesenchymal origin which promotes migration, proliferation and survival in a wide spectrum of cells, can also modulate various biological responses in stem cells [35]. All biological effects of HGF are mediated by a single tyrosine kinase receptor, c-Met [36, 37], and the role of the HGF/c-Met pathway in this process is still unclear [38]. c-MET activation by its ligand HGF induces c-MET kinase catalytic activity that triggers phosphorylation of the tyrosine kinase c-Met receptor [39]. Signaling initiated by the receptor promotes progression of cell growth through autocrine mechanisms activated by expression of the c-Met ligand, HGF [39]. It has been reported that increased HGF supports tissue regeneration by promoting the viability of stem cells, but also by enhancing cell growth in stem cell-based tissue engineering [35, 40]. In this study, it was assumed that upregulation of HGF expression was caused by cOCT4, which improved the proliferative ability of cATMSCs. Although there are some studies that show OCT4 increases the

proliferation of MSCs, the relevance between HGF/c-Met signaling and OCT4 has not been previously reported. However, the results suggest that upregulation of HGF expression might have influenced the improvement of MSC proliferation as a result of OCT4 overexpression. Therefore, further study should be focused on whether OCT4 can directly stimulate HGF/c-Met signaling.

A current limitation of MSC application for regeneration includes obtaining sufficient numbers of these stem cells in a timely manner from the challenging in vivo milieu [12]. In this context, MSCs transduced with OCT4 may be of particular interest for clinical applications to shorten the MSC culture duration and obtain sufficient cell numbers. A high growth-promoting ability resulting from OCT4 overexpression in MSCs has been reported by several studies [16, 17, 20, 41], and these experiments confirmed that cOCT4-overexpressing cATMSCs exhibit a higher proliferative capacity while maintaining their stem cell properties. Therefore, the effect of lentivirus-mediated overexpression of cOCT4 on the proliferation of cATMSCs is critical to elucidate clinical applications of MSC-based tissue engineering.

Conclusions

These data demonstrate that overexpression of cOCT4 promotes the proliferation of cATMSCs, suggesting that cOCT4 plays an important role in the proliferation of cATMSCs. The results suggest that OCT4 gene modification may be an efficient method to improve MSC proliferation.

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국 문 초 록

개 지방 조직 유래 중간엽 줄기세포에서 OCT4의 효과에 관한 연구

지도교수 : 윤화영

한 상 훈

서울대학교 대학원

수의학과 수의내과학 전공

중간엽 줄기세포의 증식 능력을 향상시키기 위한 유전자 조작 방법은 다양한 질병의 치료를 위한 재생 의학과 세포 치료의 잠재력을 높이는데 중요하다. 임상 적용에 대한 중간엽 줄기세포의 이용을 최대화하기 위하여 증식 능력은 필수적이다. 렌티바이러스 발현 벡터 시스템을 이용한 OCT4의 과발현이 개 지방 조직 유래 중간엽 줄기세포의 증식 능력에 영향을 미칠 것이라고 가정하였다.

RT-PCR 과 Western blot분석을 통하여 OCT4가 형질 도입된 개 지방 조직 유래 중간엽 줄기세포가 성공적으로 제작되었음을 확인하였다. OCT4가 형질 도입된 개 지방 조직 유래 중간엽 줄기세포가 중간엽 줄기세포의 특성을 가지는지를 확인하기 위하여 flow cytometry방법을 이용하여 줄기세포 표지 단백질을 분석하였다. OCT4가 형질 도입된 개 지방 조직 유래 중간엽 줄기세포와 대조군에서 양성 표지 단백질인 CD29, CD44, CD73, CD90, CD105가 각각 높은 비율을 나타냈으며, 음성 표지 단백질인 CD31 과 CD45는 거의 나타나지 않는 것을 확인할 수 있었다. 또한 OCT4가 형질 도입된 개 지방 조직 유래 중간엽 줄기세포에서 대조군에 비해 CD44, CD73, CD90, CD105가 상대적으로 높은 비율을 나타내었다. 추가적으로 WST-1 cell proliferation assay 와 trypan blue exclusion 방법을 이용하여 증식 능력을 평가하였다. 그 결과 OCT4가 형질 도입된 개 지방 조직 유래 중간엽 줄기세포에서 대조군에 비해서 높은 증식 능력을 보이는 것을 확인할 수 있었다. 세포 주기 분석 결과는 OCT4가 과발현된 개 지방 조직 유래 중간엽 줄기세포에서 DNA 합성 단계인 S기와 유사분열 단계인 G2/M기에서 세포의 비율이 증가하였음을 보여준다. Western blot 분석을 통해서

OCT4가 형질 도입된 개 지방 조직 유래 중간엽 줄기세포에서 G1에서 S기로의 진행의 조절과 DNA 복제 신호에 매우 중요한 역할을 하는 cyclin D1의 발현량이 유의적으로 증가하였음을 확인하였다. 게다가, OCT4가 형질 도입된 개 지방 조직 유래 중간엽 줄기세포에서 HGF(Hepatocyte growth factor)가 mRNA와 단백질 수준에서 과발현 되었음을 확인하였다.

본 연구의 결과는 렌티바이러스 발현 벡터 시스템을 이용한 OCT4의 과발현이 개 지방 조직 유래 중간엽 줄기세포의 증식 능력과 HGF의 발현을 촉진시켰음을 보여준다. 이와 같이 지방 조직 유래 중간엽 줄기세포에서의 OCT4 과발현을 이용한 방법은 stmeness를 잃지 않고 세포의 증식을 촉진시킬 수 있는 유용한 방법일 것이다.

주요어 : OCT4, 지방 조직 유래 중간엽 줄기세포, 세포 증식, HGF, 세포 주기 분석

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