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Anti-inflammatory Effects of Oct4/Sox2-overexpressing Human Adipose Tissue-derived Mesenchymal Stem Cells

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Abstract. *Background/Aim: The transcription factors Oct4 and Sox2 enhance the proliferation and pluripotency of human adipose tissue-derived mesenchymal stem cells (hAT-MSCs); however, the anti-inflammatory effects of Oct4- and Sox2-overexpressing hAT-MSCs (Oct4/Sox2-hAT-MSCs) are unclear. Here, we evaluated the anti-inflammatory effects of Oct4/Sox2-hAT-MSCs in vitro and in vivo. Materials and Methods: Supernatants from green-fluorescent protein (GFP)- and Oct4/Sox2-hAT-MSCs were used to treat lipopolysaccharide (LPS)-stimulated RAW264.7 cells and inflammatory cytokine expression was determined. In LPS-induced mice, GFP- and Oct4/Sox2-hAT-MSCs were injected intraperitoneally and survival rates, as well as sickness scores of mice, were monitored. Results: Decreased expression of pro-inflammatory cytokines was observed in Oct4/Sox2-hAT-MSC supernatant-exposed RAW264.7 cells compared to that in GFP-hAT-MSC supernatant-exposed RAW264.7 cells. The sickness score was reduced to 34.9% and the survival rate was increased by 11.1% in Oct4/Sox2-hAT-MSC-injected mice compared to that in GFP-hAT-MSC-injected mice. Conclusion: Our findings provide important insights into the development of therapies utilizing Oct4/Sox2-hAT-MSCs in inflammatory diseases.*

Inflammation is part of the complex biological response of vascular tissues to harmful stimuli (1). Macrophages, which are at the frontline of the innate immune response, are distributed throughout the circulation system, subsequently migrating to most tissues of the body and activating synthesis and release of pro-inflammatory mediators, such as interleukin (IL)-1β, IL-6 and tumor necrosis factor-α (TNF-α) (2). In macrophages activated by endotoxin, the rapid activation of nuclear factor (NF)-κB signaling *via* Toll-like receptor (TLR) 4 results in the transcription of immunity effectors (3). Although the inflammatory response is a universal and essential biological response, excessive production of pro-inflammatory cytokines may result in severe inflammation and, even, tissue necrosis.

Although the pathogenesis of systemic inflammation is not fully understood, numerous cytokines and factors involved in inflammation have been studied as therapeutic targets (4-7). However, formerly accepted treatments, such as anti-TNF-α antibodies (8), IL-1 receptor antagonists (9) and platelet-activating factor antagonists (10), have failed to demonstrate therapeutic effects or improve survival rates in systemic inflammation models. Recent studies on the effectiveness of cell-based therapy using mesenchymal stem cells (MSCs) have shown that bone marrow-derived MSCs may be effective for decreasing both systemic and local inflammatory responses (11-13).

The pluripotent transcription factors Oct4 and Sox2, which are involved in self-renewal and pluripotency, are expressed by MSCs at early passages. In previous studies, Oct4/Sox2 overexpression has been shown to enhance proliferation and differentiation ability in human adipose tissue-derived mesenchymal stem cells (hAT-MSCs) (14, 15). Based on these findings, we hypothesized that Oct4/Sox2 expression may enhance the anti-inflammatory effects of hAT-MSCs.

Accordingly, in this study, the anti-inflammatory effects of lentivirus-transduced Oct4/Sox2-hAT-MSCs were investigated by determining the expression of inflammation-related cytokines in macrophage cell lines treated with conditioned medium *in vitro*. In addition, sickness scores (diarrhea, eye condition, activity and fur condition) and survival rates were used to evaluate the anti-inflammatory effects of the engineered hAT-MSCs in a mouse model.

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Key Words: Inflammation, mesenchymal stem cell, Oct4, Sox2, lipopolysaccharide.

Materials and Methods

Cell culture. Human adipose tissues were harvested by suction of abdominal subcutaneous tissue from a volunteer, after obtaining informed consent according to the guidelines of the Institutional Review Board (IRB) of K-STEMCELL (Seoul, Korea). The cell medium was added to keratinocyte serum-free medium (SFM; Invitrogen, Carlsbad, CA, USA) containing 0.2 mM ascorbic acid, 0.09 mM calcium, 5 ng/ml recombinant epidermal growth factor (rEGF; Prospec, East Brunswick, NJ, USA) and 5% fetal bovine serum (FBS; PAN Biotech, Aidenbach, Germany). The cells were maintained for 5 days until confluent (passage 0). When the cells reached 90% confluency, they were subculture-expanded in keratinocyte SFM containing 0.2 mM ascorbic acid, 0.09 mM calcium, 5 ng/ml rEGF and 5% FBS. Isolated hAT-MSCs were used at passages 3-6 for the experiments. The medium was replaced every 2-3 days with fresh complete medium until cells reached 80-90% confluence. Cells were cultured at 37˚C in a humidified 5% CO_2 incubator.

RAW264.7 cells were purchased from the Korean Cell Line Bank (Seoul, Korea) and cultured in RPMI-1640 medium (PAN Biotech) supplemented with 10% heat-inactivated FBS (PAN Biotech), 200 IU/ml penicillin, and 200 mg/ml streptomycin at 37˚C in a humidified 5% CO_2 incubator.

hAT-MSC differentiation. To assess the ability of human hAT-MSCs to differentiate into adipocytes and osteocytes, 2×10^4 cells were seeded in 24-well cell culture plates. After 24 h of culture, the medium was replaced with fresh medium containing a StemPro adipogenesis and osteogenesis differentiation kit (Gibco, Carlsbad, CA, USA). The medium was replaced with fresh differentiation medium every 3 days for 2 weeks. Adipocytes and osteocytes were detected using Oil Red O and Alizarin Red S (both from Sigma, St. Louis, MO, USA) staining, respectively. For differentiation into chondrocytes, 1.6×10^7 hAT-MSCs were centrifuged and suspended in 1 ml normal saline. Micro-mass cultures were generated by seeding 5-μl droplets of cell solution into the center of the 24-well plate. The medium was replaced with fresh medium every 2 days for 3 weeks. After fixation, cells were stained with 1% Alcian Blue solution (Sigma) for 30 min for detection of chondrogenesis.

Oct4/Sox2 lentivirus vector construction and transduction into hAT-MSCs. The Oct4/Sox2 lentiviral plasmid (pSIN4-EF2-O2S) was purchased from Addgene (Cambridge, MA, USA). In order to prepare lentivirus particles, the Oct4/Sox2 lentiviral plasmids were transfected into 293FT cells using packaging mix (Invitrogen) and Lipofectamine 2000 transfection reagent (Invitrogen) at the appropriate volume, according to the manufacturer's instructions. In order to improve expression efficiency, hAT-MSCs were doubly transduced with lentivirus particles at a multiplicity of infection 20. Green-fluorescent protein (GFP) was inserted into the lentivirus vector, which was generated using the same methods and used as a transduction control.

Gene expression analysis. In order to detect the expression of *Oct4* and *Sox2* genes in the hAT-MSCs, total RNA was isolated from the cells using an Easy-Blue RNA Extraction Kit (iNtRON, Sungnam, Korea), according to the manufacturer's protocol. cDNA was synthesized using a reverse transcription kit (M-MLV cDNA synthesis kit, Cosmo Gentech, Seoul, Korea) with 1 μg of total RNA, according to the manufacturer's instructions (Enzynomics, Seoul, Korea). The samples were analyzed using 10 μl of AMPIGENE qPCR Green Mix Hi-ROX with SYBR Green dye (Enzo Life Sciences, Farmingdale, NY, USA) and 400 nM forward and reverse primers (Cosmo Genetech). Expression levels of the target genes were normalized to that of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). Primer sequences used in this study are listed in Table I.

Treatment of RAW264.7 cells with hAT-MSC conditioned medium. RAW264.7 cells (5×10^5 cells/well) were seeded, in duplicate, in 6-well plates and used for subsequent experiments. RAW264.7 cells were cultured in GFP- and Oct4/Sox2-hAT-MSC supernatants that had been harvested after 48 h of hAT-MSC culture. After 12 h of incubation, RAW264.7 cells were stimulated with 200 ng/ml lipopolysaccharide (LPS) for 6 h. After stimulation, total RNA from all groups of RAW264.7 cells was isolated using Easy-BLUE (iNtRON), following the manufacturer's protocol. Real-time polymerase chain reaction (PCR) analysis was performed to analyze the relative expression ratio using SYBR Green reagent (Enzynomics). PCR was performed using specific primers listed in Table I.

LPS-induced systemic inflammation mouse model and administration of Oct4/Sox2-hAT-MSCs. Male 7-week-old mice Balb/c mice (20±2 g) were purchased from NARABIO (Seoul, Korea) and housed (five per cage) under a 12-h light/dark cycle for 1 week for acclimation. All experimental procedures were approved by the Seoul National University Institutional Animal Care and Use Committee (IACUC, SNU-150102-2) and the animal study protocol was performed in accordance with the approved guidelines. The mice were separated randomly into three groups: control, GFP and Oct4/Sox2. Mice in each group were injected intraperitoneally with 10 mg/kg *Escherichia coli* LPS (055:B5; Sigma) suspended in 0.2 ml sterile phosphate-buffered saline (PBS). Three hours after injection of LPS, sterile PBS was administered by intraperitoneal injection to mice in the control group. The other two groups were treated intraperitoneally with injection of 5×10^6 GFP- and Oct4/Sox2-hAT-MSCs. After 20 h, the survival rates and abnormal behaviors were evaluated. Mouse behavior was evaluated using a sickness score parameter (Table II).

Statistical analysis. The results are presented as means±standard deviations. The data were analyzed by one-way analysis of variance. Results with *p*-values of less than 0.05 were considered statistically significant.

Results

hAT-MSC morphology and differentiation into the mesenchymal lineage. During cell culture, undifferentiated hAT-MSCs showed a fibroblast-like morphology. hAT-MSCs showed the capacity to differentiate into all three target phenotypes when cultured in differentiation medium, as shown in Figure 1. hAT-MSCs were capable of undergoing adipogenic differentiation, as indicated by Oil Red O staining of lipids. In addition, osteogenic differentiation was observed *via* Alizarin Red S staining of calcium deposits. Chondrogenesis was identified by Alcian Blue staining of proteoglycans.

Table I. *Primer sequences for RT-PCR amplification of human and mouse target genes.*

Genes	Sequences of primers	Genes	Sequences of primers
Human *OCT4*	F: 5'-ATCAAAGCTCTGCAGAAAGAACT-3' R: 5'-GCTTACACATGTTCTTGAAGCTAA-3'	Mouse *Il-6*	F: 5'-CTTTTCTACCCCAATTTCCA-3' R 5'-CGCACTAGGTTTGCCGAGTA-3'
Human *SOX2*	F: 5'-AGTACAACTCCATGACCAGCTC-3' R: 5'-GGAGTGGGAGGAAGAGGTAAC-3'	Mouse *Il-1β*	F: 5'-GTCTTTCCCGTGGACCTTC-3' R: 5'-TGTTCATCT-3'CGGAGCTGT-3'
Human *GAPDH*	F: 5'-TCAACGGATTTGGTCGTATT-3' R 5'-GATGGGATTTCCATTGATGA-3'	Mouse *Tnf-α*	F: 5'-CCCTCACACTCAGATCATCTTCT-3' R: 5'-GCTACGACGTGGGCTACAG-3'
Mouse *Gapdh*	F: 5'-CAAAATGGTGAAGGTCGGTG-3' R: 5'-CGTTGATGGCAACAATCTCC-3'	Mouse *Il-10*	F: 5'-CTTATCGGAAATGATCCAGTTTTAC-3' R: 5'-ACACCTTGGTCTTGGAGCTTATTA-3'

RT-PCR, Real-time polymerase chain reaction; F, forward; R; reverse.

Table II. *Parameters of the sickness score for assessing the individual sickness behavior after induction of the lipopolysaccharide challenge (34).*

Parameter (Sick Score)	Point						
	0	1	2	3	4	5	6
Condition of fur	Normal fur	Ruffled in the neck	Head and neck ruffled	Back completely ruffled	Head, neck and half stomach ruffled	Head, neck and stomach ruffled	Whole fur extremely ruffled
Diarrhea	No diarrhea	Small amount of stool at the anus	Area around anus smeared with stool	Area around anus strongly smeared with stool			
Activity/Apathy	Normal activity (exploration, grooming, eating)	Slightly reduced activity	Reduced activity with short intervals without activity	Extended intervals without activity	Strongly reduced exploration huddling	Huddling, no exploration no food intake, but reaction to environmental stimuli	No reaction to environmental stimuli
Condition of eyes	No encrustation, no swelling	Slightly swollen	Swollen	Strongly swollen	Swollen and slightly encrusted	Strongly swollen and encrusted	Completely encrusted

Oct4 and Sox2 overexpression in lentivirus-transduced hAT-MSCs. In order to detect Oct4 and Sox2 expression in hAT-MSCs transduced with GFP or Oct4/Sox2 lentivirus particles, PCR and immunostaining were performed. The mRNA levels of *Oct4* and *Sox2* genes were higher in Oct4/Sox2-hAT-MSCs than in GFP-hAT-MSCs (Figure 2A). Real-time PCR analysis indicated that the expression level of *Oct4* increased 1.7-fold, while that of *Sox2* increased 13.4-fold in Oct4/Sox2-hAT-MSCs compared to that in GFP-hAT-MSCs (Figure 2B). Concurrent immunofluorescent staining results revealed that the number of hAT-MSCs expressing Oct4 was higher in the Oct4/Sox2 group than in the GFP group. These findings indicate that Oct4/Sox2-hAT-MSCs were successfully generated using lentiviral gene engineering.

Effects of treatment with GFP- and Oct4/Sox2-hAT-MSC conditioned media on RAW264.7 cells. Conditioned media from GFP- and Oct4/Sox2-hAT-MSCs cultured for 48 h was collected and used to treat RAW264.7 cells. Decreases in the levels of *Tnf-α, Il-1β* and *Il-6* were observed in both hAT-MSC conditioned medium-treated groups; however, levels in the Oct4/Sox2 group exhibited stronger inhibition ability (Figure 3). In contrast, both GFP and Oct4/Sox2 groups expressed *Il-10*, although real-time PCR analysis indicated that Oct4/Sox2 overexpression significantly increased *Il-10* mRNA levels.

Evaluation of mouse sickness scores and survival rates. After LPS stimulation and administration of hAT-MSCs, clinical symptoms in mice were evaluated by assigning a sickness score and studying survival rates. The GFP and Oct4/Sox2 groups both exhibited significantly improved activity and eye conditions (Figure 4); however, the degree of improvement in the Oct4/Sox2 group was higher than that in the GFP group. The administration of hAT-MSCs, therefore, prolonged survival, with rates reaching 50%, 55.6% and

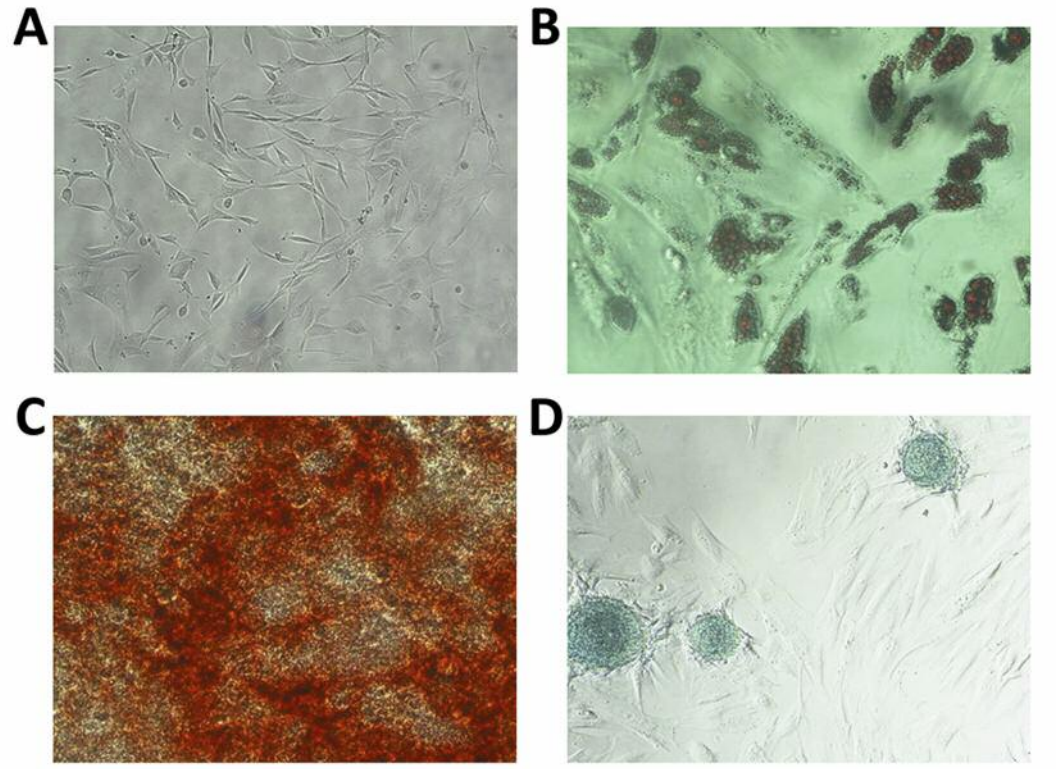

Figure 1. *Tri-lineage differentiation of hAT-MSCs. (A) hAT-MSCs exhibit a spindle-shaped fibroblastic morphology (100×). (B) Adipocyte staining as seen by Oil Red O (200×). (C) Differentiation into bone, as shown by Alizarin Red S staining (200×). (D) Differentiation into cartilage as shown by Alcian Blue staining (200×).*

66.7% for the PBS, GFP and Oct4/Sox2 groups, respectively (Figure 5). The survival of mice in the Oct4/Sox2 group was higher than that in the GFP group as a result of the amelioration of clinical signs of inflammation.

Discussion

Inflammation is a biological response that occurs in response to tissue injury (16). The inflammatory state is involved in the progression of diseases, such as arthritis, Crohn's disease, ulcerative colitis, ankylosing spondylitis, juvenile arthritis, lung or brain injury and myocardial infarction (17-19). As inflammation progresses, organ integrity is impaired and the levels of numerous small molecules, such as cytokines, are up- or down-regulated (19, 20).

MSCs were initially used as feeder layers. However, after exploration of the putative roles of these cells in replacing damaged cells, scientists found that MSCs limit tissue damage through numerous mechanisms, such as up-regulation of genes that modulate excessive inflammatory and immune reactions, the provision of a niche to enhance proliferation and differentiation of tissue-endogenous stem cells and transfer of vesicular components containing mitochondria and microRNAs (21). In our previous studies, we demonstrated that the increase in stemness of MSCs following the introduction of *Oct4* and *Sox2* promoted proliferation and differentiation into hepatogenic cells (14, 15). Based on these results, we hypothesized that increased stemness promotes the immune-modulating effects of hAT-MSCs. Using lentiviral vectors, transduction with *Oct4* and *Sox2* genes was performed to successfully generate Oct4/Sox2-hAT-MSCs. The production of pro-inflammatory cytokines by these cells was found to be down-regulated, whereas that of the anti-inflammatory cytokine IL-10 was up-regulated relative to control hAT-MSCs *in vitro*.

The IL-1 pathway plays a central role in sterile inflammation (22). Activated MSCs secrete an IL-1 receptor antagonist, suggesting that these cells are active during the initiation phase (21, 23). Its effects are similar to those of TNF-α, which is secreted during infectious inflammation (24, 25). A previous study showed that pro-inflammatory cytokine production by macrophages was reduced (26), consistent with the findings in the current study. Additionally, hAT-MSCs improved macrophage production of IL-10, an anti-inflammatory cytokine, upon LPS stimulation of RAW264.7

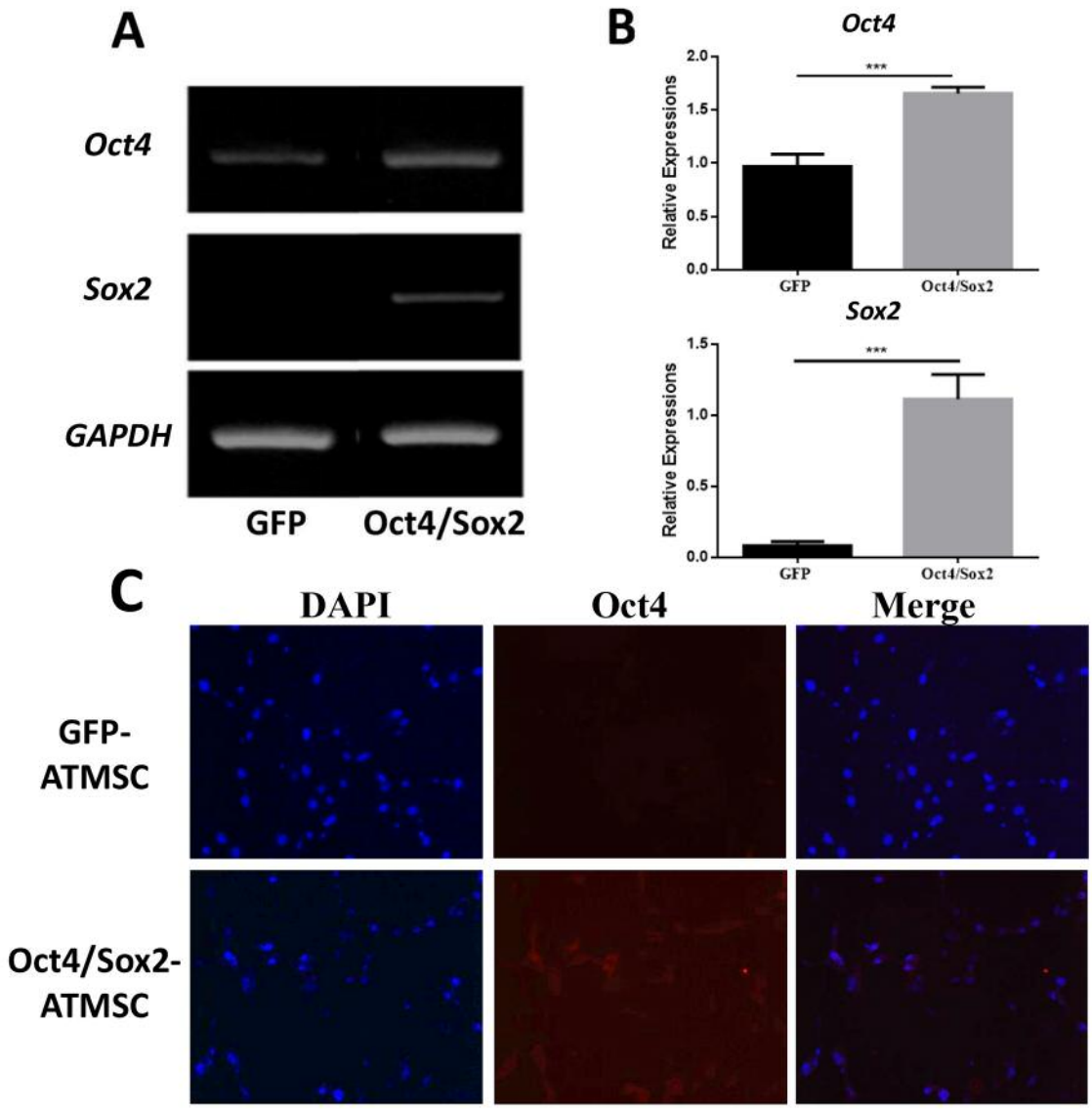

Figure 2. *Expression analysis of Oct4 and Sox2 in Oct4/Sox2-hAT-MSCs. (A) The mRNA expression levels of Oct4 and Sox2 were analyzed by PCR followed by agarose gel electrophoresis. (B) The results were evaluated by real-time PCR. (C) Oct4 protein expression was confirmed by fluorescence microscopy using immunostaining of Oct4/Sox2-hAT-MSCs. The results are representative of three independent experiments (**p<0.01, ***p<0.001).*

cells. Moreover, the increase in stemness of hAT-MSCs overexpressing Oct4 and Sox2 was accompanied by down-regulation of pro-inflammatory cytokine secretion and up-regulation of anti-inflammatory cytokine (IL-10) secretion relative to that of control hAT-MSCs *in vitro*.

Németh *et al*. (27) suggested that MSCs are capable of reprogramming macrophages into reducing type-one (M1) macrophages, characterized by the markers IL-6, IL-8, IL-1β and inducible nitric oxide synthases (iNOS), and increasing type two (M2) macrophages, characterized by the markers IL-10, IL-4 and CD206 (28). These findings were reproduced in our study, which showed a reduction in pro-inflammatory cytokines produced by macrophages (26). However, our findings were, to some extent, inconsistent because MSCs enhance the immune response during early inflammation but suppress the immune response during late-stage inflammation (29). Further studies are required to elucidate the effects of Oct4/Sox2-hAT-MSCs on cytokine production during early inflammation. Although numerous studies of the anti-inflammatory effects of MSCs have been performed, the mechanisms underlying the effects of these cells remain a subject of investigation (30-32).

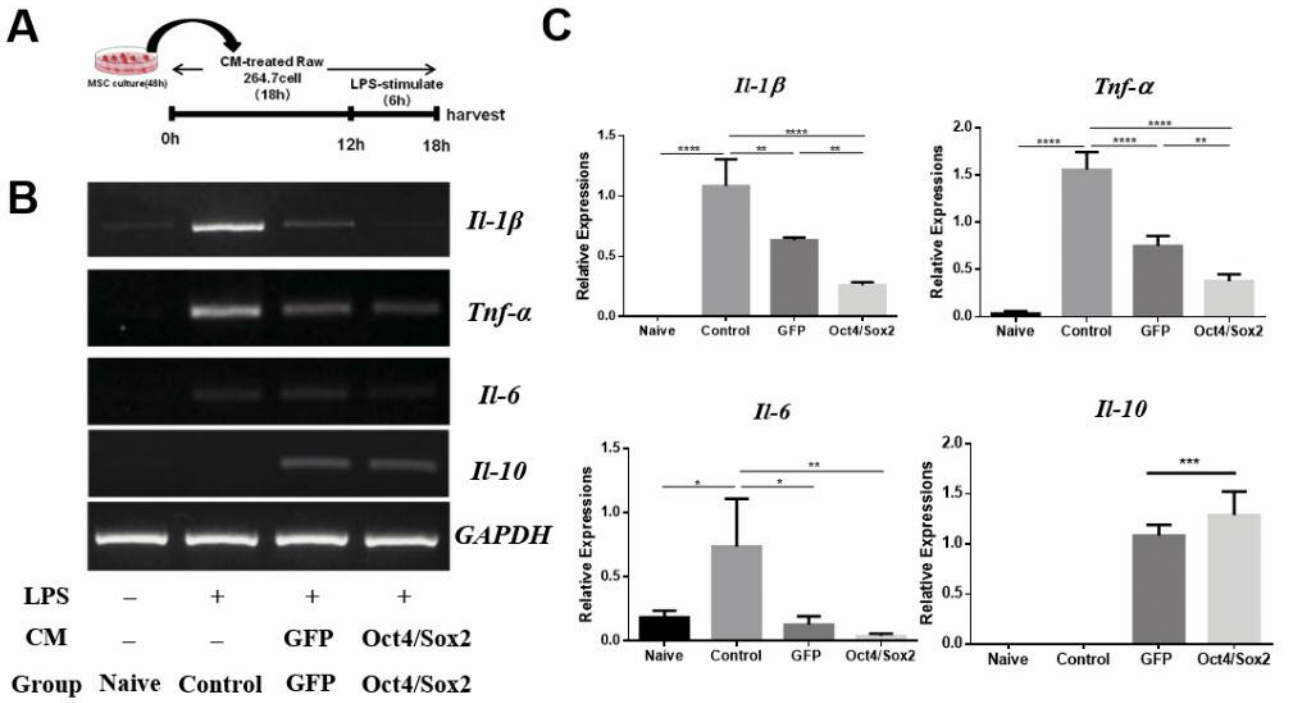

Figure 3. *Conditioned medium-treated RAW264.7 cells. (A) Conditioned medium-treated RAW264.7 cells were incubated for 12 h and stimulated with LPS (200 ng/ml) for 6 h. Total RNA from RAW264.7 cells was isolated. (B) mRNA expression levels of Il-1β, Tnf-α, Il-6 and Il-10 were evaluated by RT-PCR. (C) mRNA expression levels of Il-1β, Tnf-α, Il-6 and Il-10 were evaluated by real-time PCR. The results are representative of three independent experiments (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).*

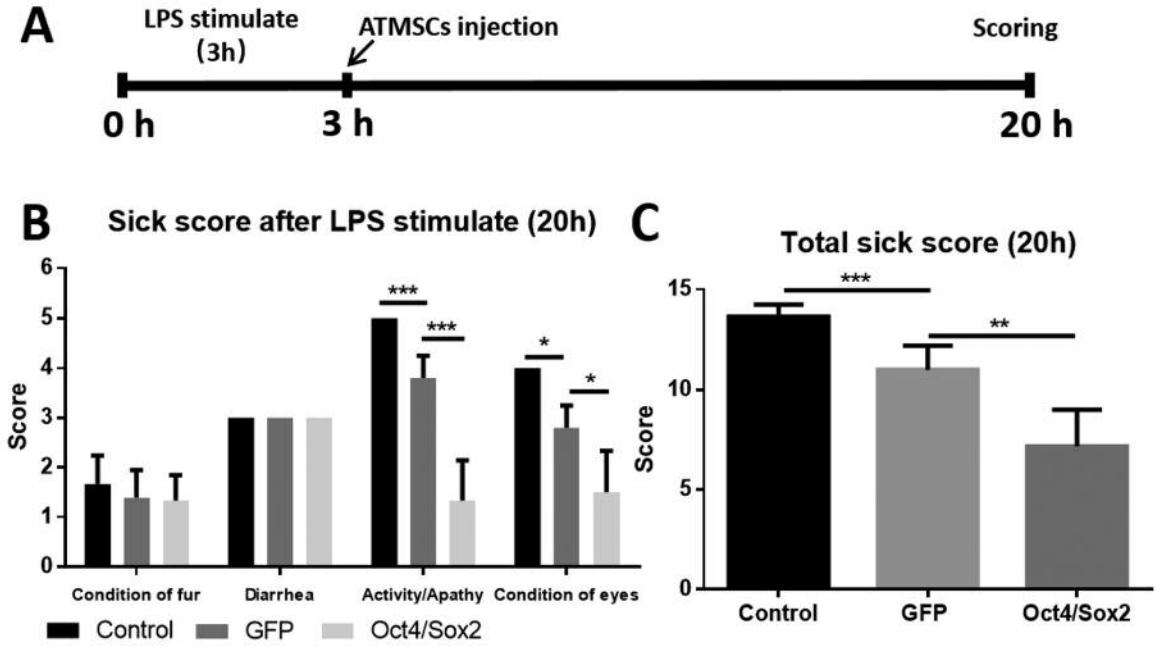

Figure 4. *Sickness scores of mice 20 h after LPS stimulation. (A) Mice were stimulated with a 20 mg/kg dose of LPS; after 3 h, PBS, GFP-hAT-MSCs and Oct4/Sox2-hAT-MSCs were administered to each group. The sickness scores and survival rates were evaluated after 20 h. (B) After 20 h of LPS stimulation, behavioral and health parameters were evaluated. (C) The average of the total value was compared for total sickness scores. (*p<0.05, **p<0.01, ***p<0.001).*

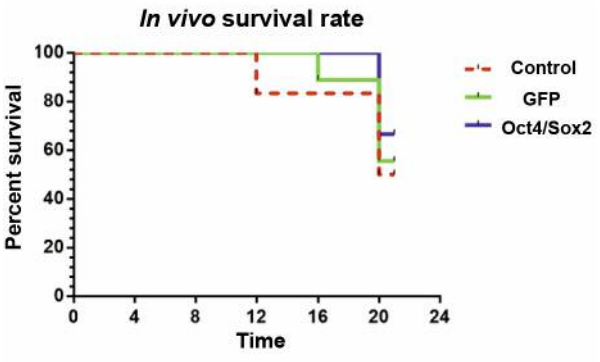

Figure 5. *In vivo survival rates following stimulation with LPS. Mice were treated with an intraperitoneal injection of LPS (10 mg/kg) and the survival rate for each group was recorded every 4 h. The survival rates are expressed as the percentage of surviving mice over total mice.*

This study has certain limitations. First, we only studied changes in cytokine production *in vitro* and *in vivo*. The inflammatory response is highly complex; accordingly, the effects of Oct4/Sox2-hAT-MSCs cells on the adaptive immune reaction (*e.g.*, activity of $CD4^+$ T helper cells, $CD8^+$ cytotoxic T cells and regulatory T cells) were not studied. Second, the anti-inflammatory effects were studied in only one animal model. Animal models are important for translating *in vitro* immunoregulatory properties of MSCs into therapeutic applications and for understanding the mechanisms underlying their efficacy. Mice injected with LPS *via* the intraperitoneal route represent a well-known animal model of systemic inflammation (33, 34). Disease-specific inflammatory animal models are selected according to the purpose of the study. In the present study, a general animal disease model was chosen to study the systemic effects of Oct4/Sox2-hAT-MSCs. In order to investigate the disease-specific anti-inflammatory effects of these cells, further studies are required using relevant animal models. Despite these limitations, however, our findings represent the first successful attempt to increase the immunomodulatory effects of MSCs by *Oct4* and *Sox2* gene engineering. Our findings may contribute to application of Oct4/Sox2-hAT-MSCs for the treatment of inflammatory disease.

Conclusion

hAT-MSCs were successfully transduced with the *Oct4*/*Sox2* genes *via* a lentivirus vector system. Oct4/Sox2-hAT-MSCs reduced the secretion of inflammatory mediators from macrophages and ameliorated clinical symptoms in an LPS-stimulated inflammatory mouse model.

Conflicts of Interest

There are no conflicts of interest to declare.

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