Analysis of Bone Marrow-derived Mesenchymal Stem Cell Kinetics after Short-term Stimulation with Tumor Necrosis Factor-α (TNF-α)

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Running title: Effect of Short Term TNF-α Stimulation of BMSCs

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Abstract: Bone marrow-derived mesenchymal stem cells (BMSCs) have considerable potential for self-renewal and multi-differentiation. Tumor necrosis factor-α (TNF-α) is an inflammatory cytokine and is involved in tissue regeneration during wound healing. It was already reported that cultured human dental pulp cells acquire stem cell properties following short-term stimulation by TNF-α. However, it has not been clarified if BMSCs acquire stem cell properties after TNF-α treatment. The purpose of this study was to investigate the effect of short-term stimulation with TNF-α on BMSCs. Rat BMSCs were cultured up to 60% confluence and then incubated with 1–100 ng/ml of recombinant rat TNF-α (rTNF-α) for a further 2 days. After reaching subconfluence, cells were passaged once to remove rTNF-α completely before subsequent assays. Cells in the control group were passaged without stimulation. Expression levels of *Nanog* and *Oct4* stem cell markers were significantly increased in the rTNF-α 10 ng/ml stimulation group. rTNF-α stimulation did not affect cell proliferation compared with the control group. However, rTNF-α stimulation led to a delay in cell differentiation. This study suggests that short-term TNF-α stimulation of BMSCs significantly increased their stem cell phenotype, but delayed their osteogenic cell differentiation.

Key words: TNF-α, Bone marrow-derived mesenchymal stem cells, Short-term stimulation, Cell proliferation, Cell differentiation

Introduction

Current advances in regenerative medicine are founded on many previous studies in embryonic development, stem cell biology and tissue engineering technology[1-3]. The generation of stem cells from different sources, such as tissue-derived stem cells, embryonic stem (ES) cells and induced pluripotent stem (iPS) cells, is an attractive concept in regenerative medicine[4,5]. Many studies using stem cells have attempted to regenerate damaged tissues and organs that have structural and functional disorders[6,7]. The issue of immunological rejection of transplanted cells or tissues would ideally be prevented by autologous transplantation. Therefore, utilizing stem cells derived from patients is currently the first choice in regenerative medicine[8]. Bone marrow-derived mesenchymal stem cells (BMSCs), which can be collected from patients and easily cultured in the laboratory, have a considerable potential for self-renewal and multi-differentiation into various functional cells[9,10]. The use of BMSCs in applications such as the treatment of severe burn injury, bone regeneration and myocardial reproduction, has featured in human clinical trials[9,11,12]. Hence, BMSCs are widely expected to play an important part in the future of regenerative medicine.

When considering the use of stem cell transplantation and tissue engineering technology, a key factor is the number of mesenchymal stem cells as this greatly affects the success rate of tissue regeneration and refunctionalization[13,14]. There are very few cells with a stem cell phenotype from among the population of BMSCs collected from bone marrow[15]. It

has been reported that the number and functionality of the stem cell population in bone marrow decreases in an age-dependent manner[16]. Therefore, elderly patients, who may need regenerative medicine, are likely to have only a small number of stem cells and a reduced stem cell activity[17]. Furthermore, mesenchymal stem cells can easily decrease their stem cell functionality during *in vitro* cell culture[18]. It is therefore difficult to obtain a large amount of stem cells for transplantation and other applications[19]. A novel culture method for maintaining/improving stem cell properties *in vitro* could therefore make regenerative technology available for elderly patients.

One regenerative technique employs the application of growth factors and cytokines to *in vitro* stem/progenitor cell cultures that contributes to cellular activation resulting in cell growth and cell differentiation[20,21]. This technique is often adopted to differentiate undifferentiated stem cells, including mesenchymal stem cells or pluripotent stem (ES/iPS) cells[22]. Cell culture technology using various cytokines has also been developed to maintain the cellular properties and characteristics of stem cells[23,24]. However, there is no culture method whereby stem cell functionality is obtained from cells degenerated as a result of aging, disease and injury[25]. Tumor necrosis factor-α (TNF-α) induces a range of biological phenomena, such as bone resorption, joint destruction, swelling and edema, through the action of inflammatory responses[26,27]. However, it is suggested that TNF-α plays a role in tissue regeneration through the induction of other cytokines found in the wound healing process[28,29]. Although cultured

human dental pulp cells acquire stem cell properties following short-term stimulation with TNF-α[30], it is not known if BMSCs acquire stem cell properties after treatment with TNF-α.

In this study, we demonstrated that in rat BMSCs, TNF-α treatment affected the acquisition of stem cell properties. Short-term stimulation with TNF-α in cultured cells accelerated the upregulation of stem cell markers and increased the stem cell phenotype of BMSCs. This novel cell culture method may have the potential in future to aid in the rejuvenation of degraded stem cells derived from elderly patients.

Materials and Methods

Cell culture

BMSCs were collected from five to six-week-old Sprague Dawley rats. The femur and tibia were harvested, then the epiphysis of the bones were cut off, and the bone marrow cavity was flushed with culture medium, as defined below, using a 23 gauge needle with 5 ml syringe. The resulting whole bone marrow suspension was mixed well and filtered through a 70 μm cell strainer (BD Falcon, Franklin Lakes, NJ, USA) to obtain BMSCs. The cells were cultured with basal medium consisting of alpha-minimum essential medium (α-MEM) (Nacalai Tesque Inc., Kyoto Japan), 10% heat inactivated fetal bovine serum (FBS) (Thermo Fisher Scientific, MA, USA), 50 μg/ml L-ascorbic acid-2-phosphate (Sigma-Aldrich Co., Tokyo, Japan) and 2 mM ß-glycerophosphate (Nacalai Tesque Inc.), at 37°C under 5% CO_2 in air.

BMSCs were cultured to 60% confluence and incubated with 1–100 ng/ml of recombinant rat TNF-α (rTNF-α) (R&D Systems Inc., MN, USA) for 2 days. Before reaching confluence, cells were passaged once using accutase (Nacalai Tesque Inc.) to remove rTNF-α completely before subsequent assays (Fig. 1a). Cells were examined using light microscopy (Nikon Eclipse TS100, Tokyo, Japan). All of the animal procedures were approved by the Board for Animal Experiments at Tokushima University (Admission Number: T27-79).

Induction of differentiation

BMSCs were cultured up to subconfluence and induced to differentiate into osteoblasts, chondrocytes, neurons and adipocytes using a specific induction medium as detailed below.

To analyze the osteogenic differentiation potential, cells were cultured in osteogenic induction medium containing α-MEM, 10% FBS, 0.01 μM dexamethasone (Sigma-Aldrich Co.), ß-glycerophosphate (Nacalai Tesque Inc.), 0.1 mM L-ascorbic acid-2-phosphate and 1% penicillin-streptomycin solution (Nacalai Tesque Inc.).

For chondrogenic differentiation, cells were cultured in chondrocyte induction medium containing high-glucose DMEM (Nacalai Tesque Inc.), 10% FBS, 1% insulin-transferring selenium (Corning, NY, USA), 0.1 μM dexamethasone, 40 μg/ml L-proline (Nacalai Tesque Inc.), 50 μg/ml L-ascorbic acid-2-phosphate, 1 mM sodium pyruvate (Sigma-Aldrich Co.) and 1% penicillin-streptomycin solution.

To evaluate the neurogenic differentiation ability, cells were cultured with pre-induction medium consisting of DMEM low-glucose medium (Nacalai Tesque Inc.) supplemented with 10% FBS, 10 ng/ml epithelium growth factor (EGF) (PeproTech, London, UK), 20 ng/ml basic fibroblast growth factor (bFGF) (PeproTech) and 10 ng/ml brain derived neurotrophic factor (BDNF) (R&D Systems Inc.) for 3 days. After pre-induction, the cells were induced for 4 days in neurogenic medium consisting of DMEM low-glucose medium, 10% FBS, 120 μM indomethacin (Nacalai Tesque Inc.), 3 μg/ml insulin (Sigma-Aldrich Co.) and 300 μM isobutyl-methylxanthine (Nacalai Tesque Inc.).

To evaluate the adipogenic differentiation ability, cells were cultured in adipocyte induction medium. Medium for adipocyte differentiation contained α-MEM, 10% FBS, 0.5 mM 3-isobytyl-1-methylxanthine (Nacalai Tesque Inc.), 60 μM indomethacin (Nacalai Tesque Inc.), 1 μg/ml insulin, 2 mM L-glutamine (Thermo Fisher Scientific), 0.1 mM L-ascorbic acid-2-phosphate and 1% penicillin-streptomycin solution.

Quantitative real-time polymerase chain reaction (PCR)

BMSCs were cultured under each condition for 7 days and total RNA extracted using TRIzol (Life Technologies, CA, USA). The final quantity and concentration of total RNA was calculated with a spectrophotometer (NanoDrop® ND-1000, Nanodrop Technologies, Inc., DE, USA) at 260 nm. RNase-free DNase I (Invitrogen, Thermo Fisher Scientific, CA, USA) was

utilized to remove any DNA contaminants from the purified total RNA. The cDNA was synthesized from 1 µg of total RNA using a High Capacity RNA-to-cDNA Kit (Applied Biosystems, CA, USA). Quantitative real-time PCR was performed by Step One Plus (Applied Biosystems) using the primers for the following genes Octamer-binding transcription factor 4 *(Oct4), Nanog,* Osteocalcin *(Ocn),* Runt-related transcription factor 2 *(Runx2),* Collagen type II alpha 1 chain *(Col2a1), Aggrecan,* Glial fibrillary acidic protein *(Gfap),* Neuron-specific class III beta-tubulin *(Tuj1),* Adipocyte protein *2 (Ap2),* Peroxisome proliferator-activated receptor ɤ *(Ppar-ɤ)* genes, and *ß-actin* as shown (Table 1). The thermal cycle conditions consisted of an initial denaturation step at 94°C for 3 min, 40 cycles of amplification at 94°C for 30 s, 60°C for 30 s and 72°C for 1 min. The specificity of PCR products was calculated using melting curve analysis at the end of the PCR cycles. The reaction products were quantified by standard curves generated from serial dilutions of each sequence at a known concentration. The mRNA content of each target was normalized to the values of *ß-actin* mRNA in each sample.

Immunofluorescence staining

After 7 days of BMSC culture, the expression of NANOG and OCT4 proteins was detected using immunofluorescence. The cells were fixed in 4% paraformaldehyde (PFA) for 10 min, washed three times with phosphate buffered saline (PBS), permeabilized in PBS with 0.2% Triton X-100 for 45 min and blocked with 5% horse serum in PBS. After washing with

PBS, the cells were incubated with primary antibodies at 4°C overnight, and then incubated with secondary antibodies at room temperature for 45 min in the dark. The NANOG primary antibody (1:100, Abcam, Cambridge, MA, USA) was followed by an Alexa Fluor® 488 goat anti-mono IgG3 (γ3) (1:500, Invitrogen) secondary antibody, and the OCT4 primary antibody (1:100, Abcam) was followed by a goat polyclonal antibody to rabbit IgG Alexa Fluor® 488 (1:1000, Abcam). Cell nuclei were then stained with 4′6-diamidino-2-phenylindole (DAPI; NucBlue™ Fixed Cell Stain ReadyProbes, Invitrogen). Immunofluorescence staining was observed using a fluorescence microscope (Nikon Eclipse TE2000-U, Tokyo, Japan). The number of positive cells were counted per unit area, and compared between control group and rTNF-α (10 ng/ml) stimulation group.

Colony Forming Unit Fibroblast assay (CFU-F assay)

The CFU-F assay was performed to evaluate colony-forming ability. BMSCs were seeded in 25 cm^2 flasks (BD Falcon) at a density of 100 cells/flask and cultured for 2 weeks. Cells were fixed with 4% PFA and stained with toluidine blue. Colonies with more than 50 cells were counted and the average value calculated in 5 flasks per condition.

MTS assay

Cell proliferation was assessed at days 1, 3, 5, and 7 by the MTS (3- (4,5-

dimethylthiazol-2-yl) -5- (3-carboxymethoxyphenyl) -2- (4-sulfophenyl) -2H- tetrazolium) assay (CellTiter 96® Aqueous One Solution Cell Proliferation Assay, Promega, WI, USA). After aspirating the medium, cells were incubated in the mixed solution containing α-MEM medium and CellTiter 96® Aqueous One Solution Reagent in the ratio of 5:1 for 1 hour in a 5% CO_2 atmosphere at 37°C. Supernatant was transferred into a 96-well plate, and read by a plate reader at a wavelength of 492 nm.

Detection of apoptotic cells

Apoptotic cells were detected using the TdT-mediated dUTP nick end labeling method (Apoptosis in situ Detection Kit Wako, Wako, Osaka, Japan) in accordance with the manufacturer's protocol. Experiments were carried out using cells 2 days after rTNF-α stimulation and 3 days after cell passage. The percentage of apoptotic cells were calculated per unit area and then compared for each concentration.

Determination of alkaline phosphatase activity

Alkaline phosphatase activity was measured on days 3, 5, and 7 by p-Nitrophenyl Phosphate Substrate method (LabAssay™ ALP, Wako) in accordance with the manufacturer's protocol.

Alizalin red staining

Alizarin red staining was performed to determine calcification. Cells were washed with PBS and stained with 1% alizarin red solution (Muto Pure Chemicals Co., Tokyo, Japan) for 10 minutes. The culture plates were washed three times with distilled water. The calcification was observed using light microscopy.

Statistical analysis

Statistical differences between groups were determined by the Student's t-test or one-way analysis of variance (ANOVA) with a post hoc test (Bonferroni) using EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan). EZR is a modified version of R (The R Foundation for Statistical Computing, Vienna, Austria) designed to calculate statistical functions frequently used in biostatistics. The Student's t-test was used to analyze: the number of cells with positive immunofluorescence, the CFU-F assay, and quantitative real time PCR of *Ocn, Runx2, Col2a1, Aggrecan, Gfap, Tuj1, Ap2, Ppar-γ*. One way ANOVA was utilized for quantitative real time PCR of *Nanog* and *Oct4*, the MTS assay, the ALP assay and apoptosis analysis. P values of less than 0.05 were considered to be statistically significant.

Results

BMSCs acquire stem cell properties after rTNF-α treatment

Expression levels of *Nanog* and *Oct4* stem cell markers were examined by quantitative real-time RT-PCR and immunofluorescence staining. Quantitative real-time PCR showed that expression levels of *Nanog* and *Oct4* significantly increased in the rTNF-α 10 ng/ml stimulation group compared with the control group (Fig. 1b). Immunofluorescence staining showed that the ratio of NANOG and OCT4 positive cells increased in the rTNF-α 10 ng/ml stimulation group. There was also a significant increase in the number of positive cells per unit area in the rTNF-α 10 ng/ml stimulation group as compared with the control group (Fig. 1c, d). This suggests that BMSCs treated with rTNF-α acquired stem cell properties and that 10 ng/ml rTNF-α is the optimal concentration to stimulate BMSCs.

The effect of rTNF-α on the morphology and proliferative ability of BMSCs

BMSCs were observed by light microscopy. There was no difference in cell morphology between the control group and the rTNF-α 10 ng/ml stimulated group (Fig. 2a). Colony-forming ability was evaluated by the CFU-F assay. Stem cells were able to form colonies, and there was no difference in colony-forming ability between BMSCs from the control group and from the rTNF-α 10 ng/ml stimulation group (Fig. 2b). Cell proliferation was analyzed by the MTS assay on days 1, 3, 5, and 7. There was no significant difference in cell proliferation between the rTNF-α stimulated (1 ng/ml, 10 ng/ml) and the control groups on days 1, 3, 5, and 7. However, cell proliferation was significantly inhibited by higher concentrations

of rTNF-α stimulation, such as 50 ng/ml and 100 ng/ml, as compared with the other three groups on days 3, 5 and 7 (Fig. 2c). TNF-α treatment is reported to induce apoptosis. Apoptotic cells were detected by the TUNEL method and the ratio of apoptotic cells was examined. In BMSCs stimulated with rTNF-α, but before cell passage, the ratio of apoptotic cells was significantly increased in the rTNF-α 50 ng/ml and 100 ng/ml groups as compared with the other three groups. However, in BMSCs after three days of cell passage, there was no significant difference between groups (Fig. 2d). These findings suggest that stimulation of rTNF-α 10 ng/ml did not affect cell behavior in terms of morphology and proliferative ability.

Examination of BMSC multipotency after rTNF-α stimulation

Quantitative real-time PCR was performed to examine the multipotency of BMSCs following TNF-α stimulation. *Ocn* and *Runx2* were used as marker genes for osteogenic differentiation. Quantitative real-time PCR showed that expression levels of *Ocn* and *Runx2* were significantly decreased in the rTNF-α 10 ng/ml stimulated group compared with the control group (Fig. 3a). *Col2a1* and *Aggrecan* were used as markers of chondrocyte differentiation and their expression levels were significantly decreased in the rTNF-α 10 ng/ml stimulated group compared with the control group (Fig. 3b). Expression levels *Gfap* and *Tuj1*, markers of neural differentiation, were significantly decreased in the rTNF-α 10 ng/ml stimulated group compared with the control group (Fig. 3c). However, there was no significant

difference in the expression levels of adipocyte differentiation markers *Ap2* and *Ppar-ɤ* between the control groups and rTNF-α 10 ng/ml stimulated group (Fig. 3d).

rTNF-α stimulation delayed osteogenic differentiation

After short-term stimulation of BMSCs by rTNF-α, there was a decrease in the degree of differentiation. Therefore, we investigated the effect of long-term rTNF-α stimulation on differentiation of BMSCs. Osteogenic differentiation was investigated by analyzing ALP activity and alizarin red staining. Significantly higher levels of ALP activity were shown on days 5 and 7 in the control group compared with the stimulation group (Fig. 4a). Alizarin red staining showed that there was a decrease in the formation of calcified nodules in the stimulated group compared with the control group up until day 21. However, the formation of calcified nodules in the stimulated group had recovered to the same level as the control group by 28 days (Fig. 4b).

These findings suggest that short-term stimulation of rTNF-α may cause delayed differentiation of BMSCs.

Discussion

TNF-α, one of the inflammatory cytokines, plays an important role in tissue repair during the wound healing process[29]. TNF-α promotes bone regeneration through the mediation

of early bone remodeling at bone fracture sites[28]. Although the use of high concentrations of TNF-α inhibits bone healing, there is an optimal concentration of TNF-α stimulation for tissue repair[31,32]. In addition, an *in vitro* study demonstrated that short-term stimulation of TNF-α increased the stem cell population of dental pulp cells[30]. In this study, we found that short-term stimulation with TNF-α significantly promoted the stem cell phenotype of BMSCs by increasing the gene expression of stem cell markers and the number of positive cells. Our study showed that 10 ng/ml TNF-α stimulation was the optimal concentration for increasing the stem cell population of BMSCs. Furthermore, our culture method did not affect cell morphology, cell proliferation or survival. These findings indicate that short-term stimulation of BMSCs by TNF-α potentially leads to the acquisition of stem cell properties.

The same study on TNF-α-stimulated dental pulp cells reported that the cells increased their ability to differentiate into different cell types such as those in osteogenic and adipogenic pathways[30]. In this study, we showed that TNF-α stimulation significantly decreased the ability of TNF-α treated BMSCs to differentiate into osteogenic, cartilaginous and neural pathways, but not adipogenic ones. In long-term osteogenic induction, TNF-α treated BMSCs gradually produced mineralized nodules, and there were no differences in the mineralized nodule formation compared with non-treated BMSCs by the 28th day of osteogenic induction. Several studies have reported that TNF-α inhibited Type I collagen production and ALP expression based on the blockage of osteoblast differentiation in osteoblastic clonal cell lines[33-35].

Furthermore, Inoue *et al*. have shown that short-term stimulation by TNF-α delayed cell differentiation in osteoblast-like cells[36]. In this study of a heterogeneous BMSC population, it is not clear if there are different bone marrow subpopulations that acquire stem cell properties after TNF-α stimulation[37]. Further studies are needed to investigate the reactivity of each cell type within bone marrow when applying this culture method for use in regenerative medicine.

In the clinic patients may expect the provision of stem cell-based regenerative medicine for tissue repair and functional recovery[6]. However, these patients, especially elderly patients, may have a small number of stem cells with low stem cell functionality, and it may therefore difficult to prepare a sufficient amount of stem cells for regenerative medicine[17]. Recently, iPS cells have been proposed as a source of cells that can differentiate into target stem cells. However, there are many problems, such as risk of tumor formation and a complex culturing process, that may rule out their practical use[38,39]. The current cell culture technologies for maintaining cellular properties have established using various cytokines and matrix coating materials[23,24]. However, the culture method for acquiring stem cell properties from degenerated and senescent cells has not been yet developed[25]. This study developed a novel culture method using short-term TNF-α stimulation that resulted in the acquisition of stem cell properties *in vitro.* Our cell culture technology provides a simple and safe method for effective stem cell culture that could be useful for making regenerative medicine available for elderly patients.

In conclusion, this study shows that short-term TNF-α stimulation significantly

increases the stem cell phenotype of BMSCs, but that osteogenic cell differentiation may be delayed in TNF-α treated BMSCs.

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Conflict of Interest

The authors declare no conflicts of interest associated with this manuscript.

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Figure legend

Figure 1. Short-term treatment with rTNF-α enhances the stem cell properties of BMSCs.

(a) rTNF-α stimulation method. BMSCs were cultured up to 60% confluence, following which, they were exposed to 1–100 ng/ml of recombinant rat TNF-α for 2 days. BMSCs were then passaged once to remove the rTNF-α completely before subsequent assays. (b) Effects of rTNF-α pre-treatment on mRNA levels of stem cell markers *Oct4* and *Nanog*. Pretreatment of 10 ng/ml rTNF-α significantly increased gene expression of *Oct4* and *Nanog.* *P <0.05 (by ANOVA and post hoc test). (c and d) Immunofluorescence expression of stem cell markers NANOG and OCT4 upon rTNF-α treatment. Treatment with rTNF-α markedly increased the number of cells positive for each marker. Bar = 200 μm *P <0.05 (by Student's t-test).

Figure 2. Effect of rTNF-α stimulation on morphology and proliferative capacity of BMSCs.

(a) Observation of cell morphology. BMSCs pre-exposed to rTNF-α were passaged once and further cultured for 3 days. Bar = 50 μm (b) CFU-F assay. The graph on the right shows quantitative analysis of the total number of cell colonies containing more than 50 cells (by Student's t-test). (c) MTS assay showing cell proliferation over time after rTNF-α addition. Stimulation with 50 ng/ml and 100 ng/ml rTNF-α significantly suppressed BMSC proliferation. *P <0.05 (by ANOVA and post hoc test). (d) Detection of apoptotic cells. The graph on the left shows the percentage of apoptotic BMSCs immediately following rTNF-α stimulation at each concentration. The figure on the right shows the percentage of apoptotic BMSCs after 3 days of culture including 2 days of rTNF-α stimulation and passage. *P <0.05 (by ANOVA and post hoc test).

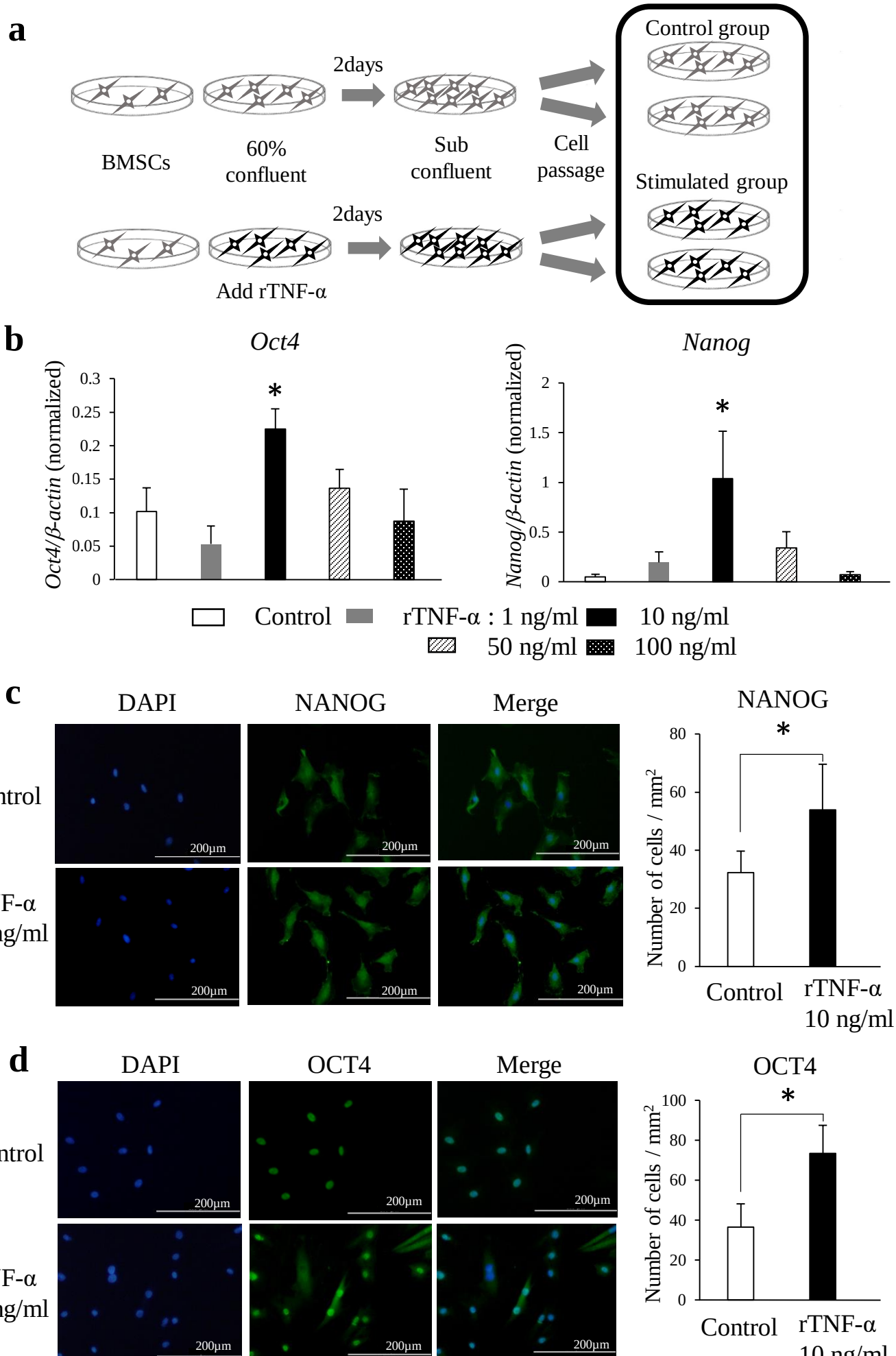
Figure 3. Effect of rTNF-α stimulation on the multipotency of BMSCs.

Analysis of mRNA expression of each differentiation gene assessed by quantitative real-time PCR. (a) Osteoblast markers *Ocn* and *Runx2*. (b) Chondrocyte markers *Col2a1* and *Aggrecan*. (c) Nerve cell markers *Gfap* and *Tuj1*. (d) Adipocyte markers *Ap2* and *Ppar-ɤ*. *P <0.05 (by Student's t-test).

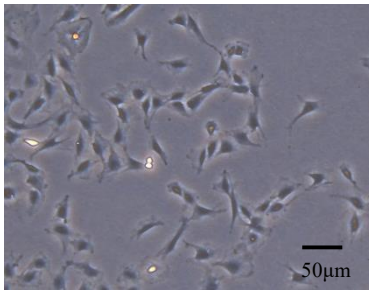
Figure 4. Analysis of osteogenic differentiation potential of BMSCs after rTNF-α stimulation. (a) ALP assay. ALP activity consistently increased on days 3, 5, and 7 in both groups. ALP activity was significantly lower in the rTNF-α-treated group compared with the control group on days 5 and 7; *P <0.05 (by Student's t-test). (b) Alizarin red staining. Alizarin red staining was suppressed in the rTNF-α group compared with the control group on days 14 and 21. On day 28, alizarin red staining was comparable between both the control and rTNF-α groups. Bar = 200 μm

Table 1. Quantitative real-time PCR primer

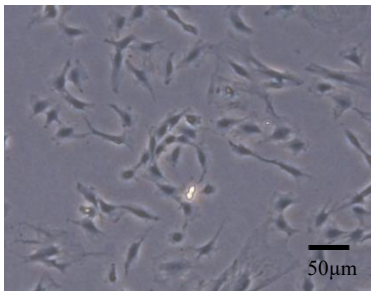
Primer name	Sequence(5`-3`)
Oct4	F: ctcctggagggccaggaatc
	R: atatacacaggccgatgtgg
Nanog	F: atgcctcacacggagactgt
	R: aagtgggttgtttgcctttg
Ocn	F: gaacagacaagtcccacac
	R: gagctcacacacctccctg
Runx2	F: tgccacctctgacttctgc
	R: gatgaaatgcctgggaactg
Col2a1	F: ctcaagtcgctgaacaacc
	R: ctatgtccacaccaaattcc
Aggrecan	F: aagtgctatgctggctggtt
	R: ggtctggttggggtagaggt
Gfap	F: agtggtatcggtccaagtttgc
	R: tggcggcgatagtcattagc
Tuj1	F: agatgtacgaagacgacgaggag
	R: gtatccccgaaaatataaacacaaa
Ap2	F: gatttccttcaaactgggcg
	R: tgacacattccaccaccagc
Ppar-γ	F: actgccggatccacaaaa
	R: tctccttctcggcctgtg
β-actin	F: cacccgcgagtacaaccttc
	R: cccatacccaccatcacacc


a
2days
BMSCs
60%
confluent
Sub
confluent
Cell
passage
Control group
Stimulated group
2days
Add rTNF-α
b
Oct4
Nanog
Oct4/β-actin (normalized)
Nanog/β-actin (normalized)
Control
rTNF-α : 1 ng/ml
10 ng/ml
50 ng/ml
100 ng/ml
c
DAPI
NANOG
Merge
Control
rTNF-α
10 ng/ml
200μm
NANOG
Number of cells / mm2
Control
rTNF-α
10 ng/ml
d
DAPI
OCT4
Merge
Control
rTNF-α
10 ng/ml
200μm
OCT4
Number of cells / mm2
Control
rTNF-α
10 ng/ml

a

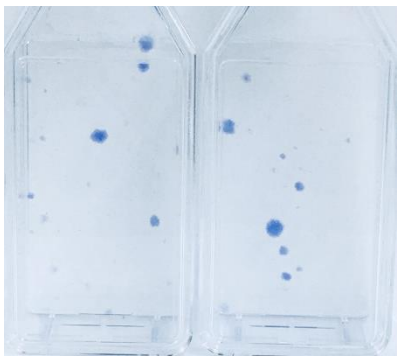


Control

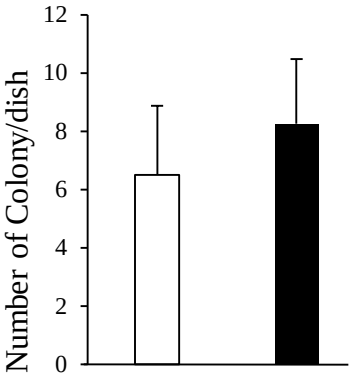


rTNF-α 10 ng/ml

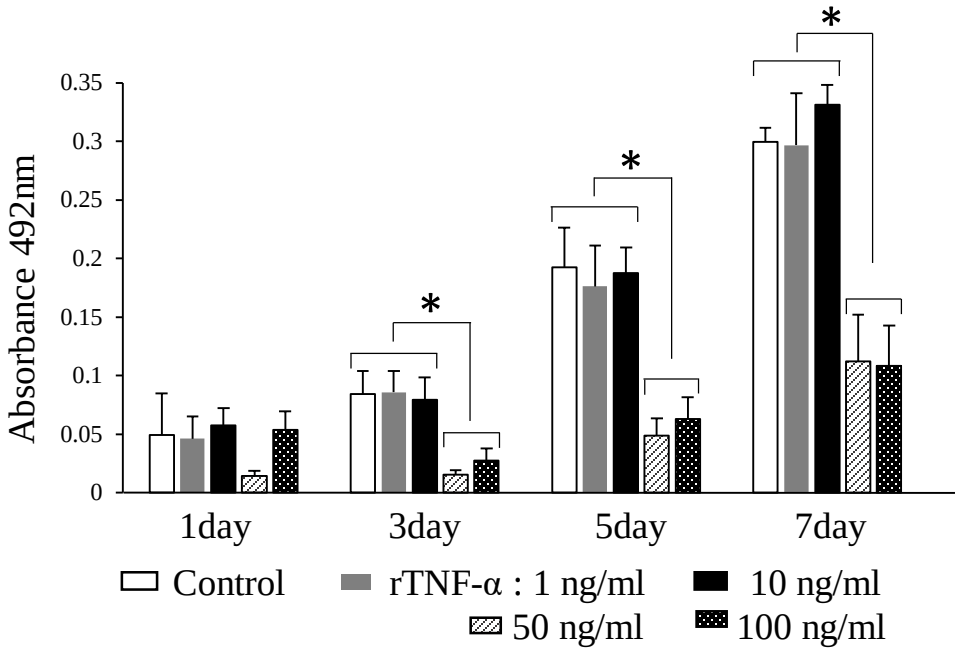
b



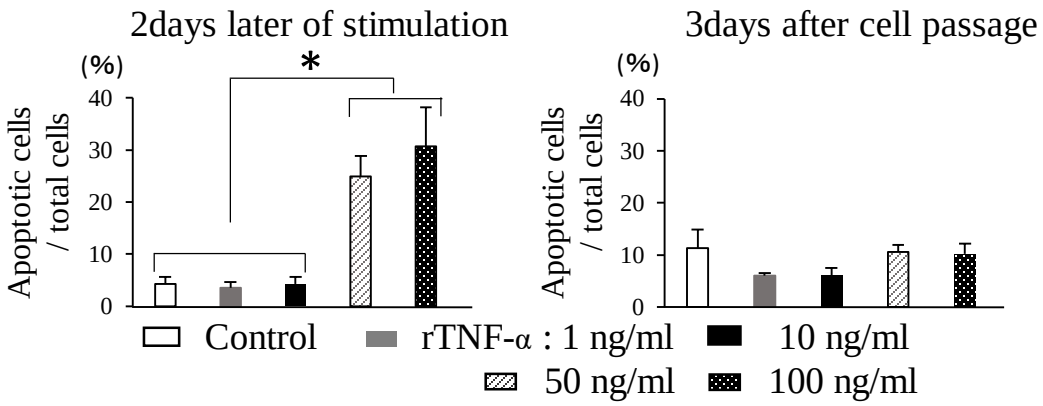
Control rTNF-α 10 ng/ml

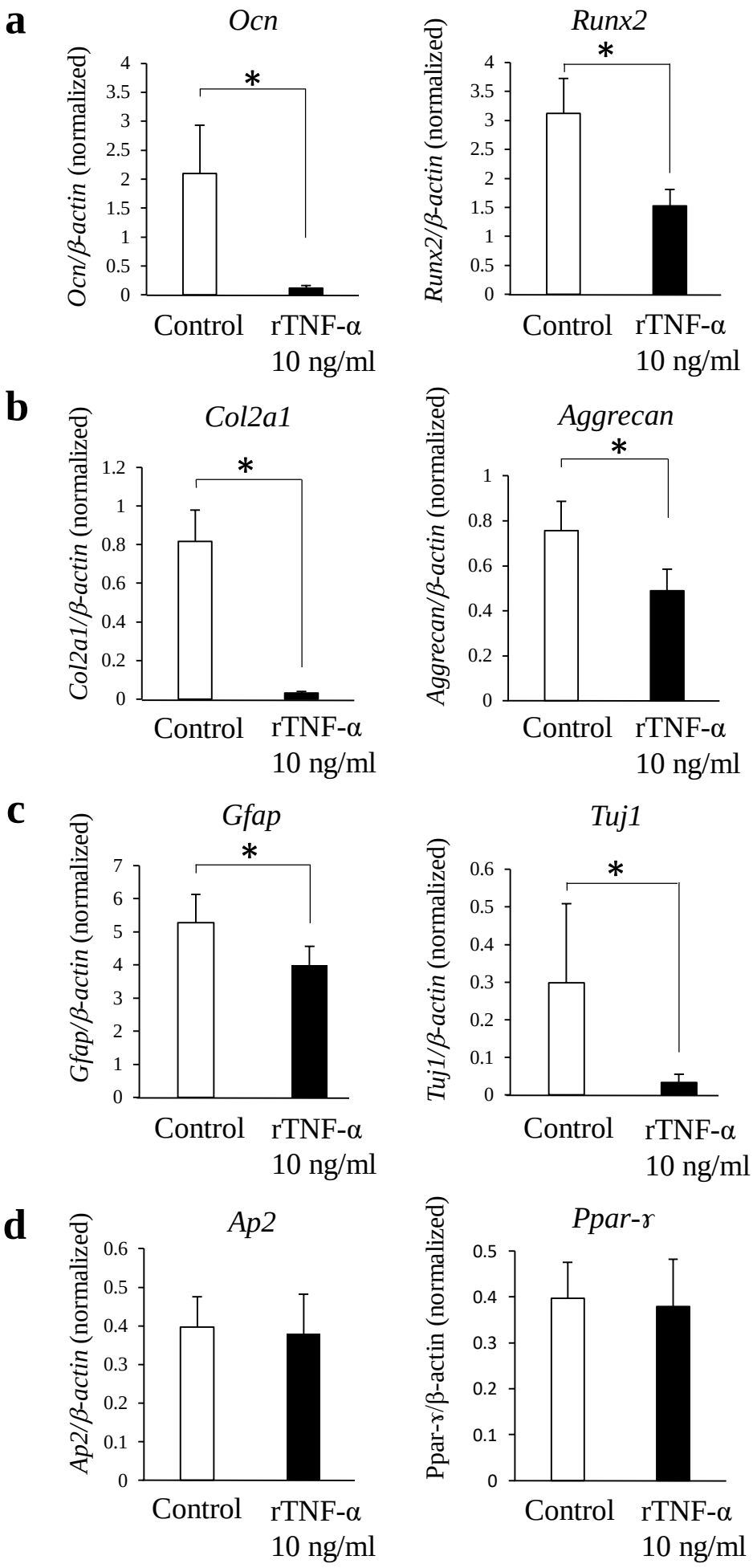


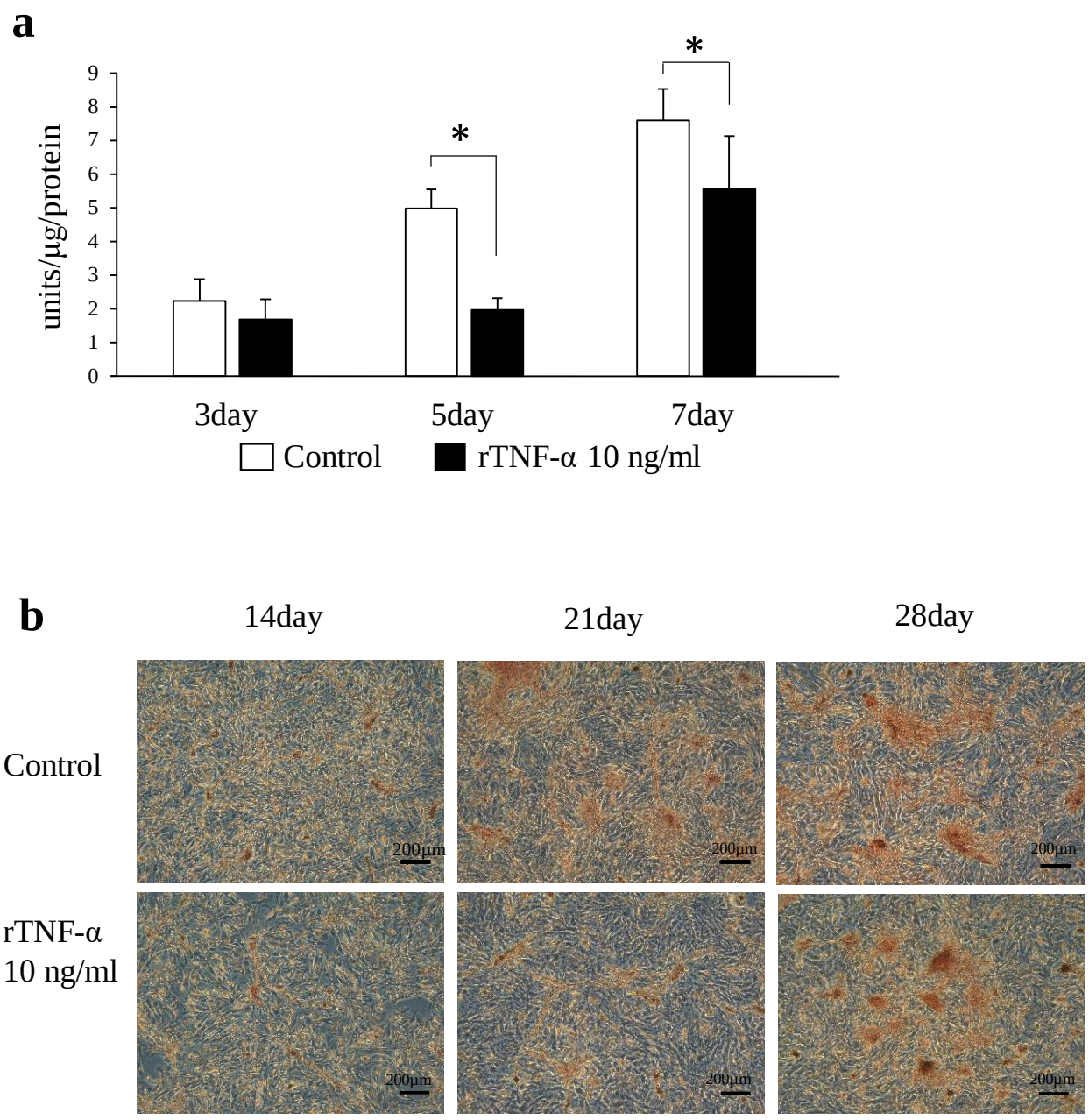
c



d




a
Ocn
Ocn/β-actin (normalized)
4
3.5
3
2.5
2
1.5
1
0.5
0
*
Control
rTNF-α
10 ng/ml
Runx2
Runx2/β-actin (normalized)
4
3.5
3
2.5
2
1.5
1
0.5
0
*
Control
rTNF-α
10 ng/ml
b
Col2a1
Col2a1/β-actin (normalized)
1.2
1
0.8
0.6
0.4
0.2
0
*
Control
rTNF-α
10 ng/ml
Aggrecan
Aggrecan/β-actin (normalized)
1
0.8
0.6
0.4
0.2
0
*
Control
rTNF-α
10 ng/ml
c
Gfap
Gfap/β-actin (normalized)
7
6
5
4
3
2
1
0
*
Control
rTNF-α
10 ng/ml
Tuj1
Tuj1/β-actin (normalized)
0.6
0.5
0.4
0.3
0.2
0.1
0
*
Control
rTNF-α
10 ng/ml
d
Ap2
Ap2/β-actin (normalized)
0.6
0.5
0.4
0.3
0.2
0.1
0
Control
rTNF-α
10 ng/ml
Ppar-ɤ
Ppar-ɤ/β-actin (normalized)
0.5
0.4
0.3
0.2
0.1
0
Control
rTNF-α
10 ng/ml


a
units/μg/protein
9
8
7
6
5
4
3
2
1
0
*
*
3day
5day
7day
Control
rTNF-α 10 ng/ml
b
14day
21day
28day
Control
rTNF-α
10 ng/ml
200μm
200μm
200μm
200μm
200μm
200μm