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Clinorotation prevents differentiation of rat myoblastic L6 cells in association with reduced NF-κB signaling

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Abstract

In this study, we examined effects of the three-dimensional (3D)-clinorotation, a simulated-model of microgravity, on proliferation/differentiation of rat myoblastic L6 cells. Differentiation of L6 cells into myotubes was significantly disturbed in the 3D-clinorotation culture system, although the 3D-clinorotation had no effect on the proliferation. The 3D-clinorotation also suppressed the expression of myogenesis marker proteins, such as myogenin and myosin heavy chain (MHC), at the mRNA level. In association with this reduced differentiation, we found that the 3D-clinorotation prevented accumulation of ubiquitinated proteins, compared with non-rotation control cells. Based on these findings, we focused on the ubiquitin-dependent degradation of IκB, a myogenesis inhibitory protein, to clarify the mechanism of this impaired differentiation. A decline in the amount of IκB protein in L6 cells was significantly prevented by the rotation, while the amount of the protein in the non-rotated cells decreased along with the differentiation. Furthermore, the 3D-clinorotation reduced the NF-κB-binding activity in L6 cells and prevented the ubiquitination of IκB proteins in the IκB- and ubiquitin-expressing Cos7 cells. Other myogenic regulatory factors, such as deubiquitinases, cyclin E and oxygen, were not associated with the differentiation impaired by the clinorotation. Our present results suggest that simulated microgravity such as the 3D-clinorotation may disturb skeletal muscle cell differentiation, at least in part, by inhibiting the NF-κB pathway.

Keywords: 3D-clinorotation; Rat myoblastic L6 cell; Ubiquitination; NF-κB signaling; IκB

1. Introduction

Microgravity and its simulated conditions preferentially disturb differentiation of skeletal muscle cells [1,2]. Reculturing of the space-flown L8 myoblastic cells after landing failed to stimulate them to fuse and differentiate into myotubes [1]. Mouse satellite cells cultured in a rotating wall vessel bioreactor proliferated normally, but their differentiation was significantly inhibited [2]. These inhibitory effects of microgravity have been suggested to contribute to the retarded recovery from microgravity-induced muscle atrophy, since differentiation of satellite cells and their fusion to myofibers are necessary for the recovery [3]. Therefore, an increased understanding of molecular mechanisms of this impaired muscle cell differentiation may

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; IKK, IκB kinase; MHC, myosin heavy chain; JAXA, Japan Aerospace Exploration Agency; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; RT-PCR, reverse transcription-polymerase chain reaction; 3D-clinorotation, three-dimensional clinorotation; β-TrCP, β-transducin repeat-containing protein

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lead to the development of effective therapies for the recovery from muscle atrophy.

Several signal transduction molecules, including myogenin and cyclin E, mediate differentiation of myoblasts into myotubes [4,5]. Recent investigations have shown that proteolytic systems, especially the ubiquitin-proteasome pathway, are involved in activation or inactivation of these myogenic regulatory factors [6–8]. For example, NF-κB activation by 26S proteasome is essential for up-regulating myogenin expression during myotube formation [6]. In contrast, we previously reported that space shuttle flight (STS-90) as well as tail-suspension specifically activated the ubiquitin-dependent proteolysis in rat skeletal muscles in vivo [9]. Based on these findings, we envisaged that the differentiation of skeletal muscle cells under microgravity is modified by the ubiquitin-dependent proteolytic system.

To address this issue, we examined the effects of three-dimensional (3D)-clinorotation on the differentiation and protein-ubiquitination of rat L6 myoblastic cells. The 3D-clinorotation is a microgravity-simulating model that has two independent axes of rotation to disperse the gravity vector [10]. Myotube formation of L6 cells cultured with a 3D-clinorotation apparatus was impaired, and the 3D-clinorotation prevented the expression of myosin heavy chain (MHC) and myogenin in L6 cells. Ubiquitinated proteins in L6 cells were accumulated during myotube formation, whereas the 3D-clinorotation significantly repressed the protein-ubiquitination in L6 cells. We also found that the 3D-clinorotation prevented the ubiquitin-dependent IκB degradation, leading to the reduction of NF-κB signaling in L6 cells. Our results suggest that the reduced NF-κB signaling contributes, at least in part, to skeletal muscle cell differentiation disturbed by the 3D-clinorotation.

2. Materials and methods

2.1. Cell culture

L6 cells were purchased from Dainippon Pharmaceutical Co. (Osaka, Japan). Cos7 cells were a kind gift from Dr. Taketani, The University of Tokushima School of Medicine, Tokushima, Japan. L6 or Cos7 cells were maintained and proliferated at 37 °C with 5% CO_2/95% air in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin.

2.2. Transfection

We constructed an expression vector for rat IκB using a reverse transcription-polymerase chain reaction (RT-PCR) and cloning techniques as described previously [9,11]. Total RNA was extracted from L6 cells with an acid guanidinium thiocyanate–phenol–chloroform mixture (Isogen™; Nippon Gene, Tokyo, Japan) according to the standard protocol [12]. First-strand cDNAs were reverse-transcribed at 37 °C for 50 min from 1 μg of the extracted total RNA with oligo-dT15 primer and SuperScript II™ reverse transcriptase (Invitrogen, Carlsbad, CA). After initial denaturation at 94 °C for 2 min, second-strand synthesis and DNA amplification with Pfx™ Taq polymerase (Invitrogen) and the IκB primer set (5′-CACCATGTTTCAGCCAGCTGGGCA-3′ and 5′-TAACGTCAGACGCTGGCCTCCAAAC-3′) [13] were accomplished through 30 cycles of the following incubations: 15 s at 94 °C, 30 s at 60 °C, 90 s at 68 °C by using a thermal cycler (MJ Research, Watertown, MA). The PCR products were sequenced and cloned into an expression vector pcDNA3.1/V5-His (Invitrogen). The plasmid containing FLAG-tagged ubiquitin cDNA was kindly provided by Dr. Ishidoh, Juntendo University School of Medicine, Tokyo, Japan. Cos7 cells were transfected with 1 μg/dish of the purified plasmid containing IκB or FLAG-tagged ubiquitin by using FuGene6 (Roche Diagnostics, Mannheim, Germany) according to the method of Hellgren et al. [14]. IκB- and ubiquitin-expressing Cos7 cells were subjected to the 3D-clinorotation 24 h after transfection as described below.

2.3. 3D-clinorotation

We subjected L6 cells or the transfected Cos7 cells to 3D-clinorotation in an apparatus (Mitsubishi Heavy Industries, Kobe, Japan) according to the method of Arase et al. [10]. Flasks containing L6 cells at about 30% and 75% confluence were filled with DMEM in the presence of 10% FCS (proliferation medium) and 2% horse serum (differentiation medium), respectively. They were rotated at 37 °C on the 3D-clinostat apparatus in a 5% CO_2 chamber. The rate and cycle of rotation were controlled by the computer to randomize the gravity vector both in magnitude and in direction, and then the dynamic stimulation of gravity to cells was cancelled in any direction. Reagents and media in the flasks were not changed during rotation (7 days). Control cells were incubated in parallel under the same conditions except for the rotation. Flasks containing the transfected Cos7 cells were rotated in the same manner after being filled with the proliferation medium.

2.4. Immunoblot analysis

Immunoblot analysis was performed as described previously [9]. The whole-cell extracts (40 μg protein/lane) from L6 or Cos7 cells were subjected to SDS-8%, 10% or 12% polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidene difluoride membrane at 35 mA for 6 h at 4 °C. The membrane was blocked with 3% skim milk and then incubated for 1 h at 25 °C in phosphate-buffered saline (PBS) with a 1:500 dilution of antiserum

against rabbit skeletal fast-type MHC, bovine ubiquitin, rabbit cyclin E (Sigma, St. Louis, MO), FLAG, human IκB (Upstate biotechnology, Lake Placid, NY), rat myogenin (PharMingen International, Tokyo, Japan) or chicken β-actin (Oncogene Research Products, San Diego, CA). The bound antibodies were detected by using the enhanced chemiluminescence system (Amersham, Little Chalfont, England, UK). Signals were quantitated by densitometric analysis. The 3D-clinorotation did not change the level of β-actin protein in 40-μg protein of cell extracts. Therefore, each protein level was standardized by that of β-actin protein.

2.5. Immunoprecipitation

The transfected Cos7 cells cultured with or without the 3D-clinorotation for the indicated times were washed in PBS three times and resuspended in 50 mM Tris-HCl buffer, pH 7.5, containing 150 mM NaCl, 1% Triton X-100, 1 tablet/25 ml protease inhibitor cocktails (Roche Diagnostics) and 10 μM epoxomicin. The cells were centrifuged in a microcentrifuge at 10,000×*g* for 15 min at 4 °C. Antibody against IκB was preadsorbed to protein G-Sepharose at 4 °C for 1 h and washed twice with the buffer before being incubated with the supernatant containing the cytoplasmic fraction overnight at 4 °C. The immunopellets obtained by the centrifugation at 5,000×*g* for 1 min at 4 °C were washed four times in the same buffer and subjected to SDS-PAGE.

2.6. Semi-quantitative RT-PCR

To measure the mRNA level of differentiation markers, a semi-quantitative RT-PCR was performed as described previously [9]. Following the synthesis of first-strand cDNAs from mRNAs, second-strand synthesis and amplification of target genes were performed as described above. In this case, PCR buffer contained two sets of primers to amplify a target gene cDNA and an internal standard β-actin cDNA simultaneously. The following oligonucleotide primers were used for amplification: 5′-GAAGGCCAAGAAGGCCATC-3′ and 5′-CTCGCCTCTCGTGTTTTCG-3′ for rat type 2A MHC cDNA [15]; 5′-GCAGTGCCATCCAGTACATTGAGC-3′ and 5′-GGAAGGTGACAGACATATCCTCCAC-3′ for rat myogenin cDNA [16]; 5′-AGTTCTCTGTCCAGAGGTTCC-3′ and 5′-GAACAAATAGGCGTCGCTGGT-3′ for cDNA of rat UBP 45/69 deubiquitinating enzyemes, which were derived from alternative splicing of the primary transcript [17]; 5′-ATGAATTCCAGATTGTCAGTAGT-3′ and 5′-TTATCTGGAGATGTAGGTGTATGT-3′ for rat β-transducin repeat-containing protein (β-TrCP); 5′-TCACCGAGGCCCCTCTGAACCCTA-3′ and 5′-GGCAGTAATCTCCTTCTGCATCCT-3′ for rat β-actin [18]. Since sequence data for rat β-TrCP has not been reported, we designed its primers from homology sequence between mouse and human β-TrCP cDNAs [19,20]. The amplification was terminated 15 min later at 72 °C when PCR products were linearly amplified. The PCR products were separated by electrophoresis in an 8% polyacrylamide gel and detected with a highly-sensitive nucleic acid staining reagent (TaKaRa, Tokyo, Japan). The intensities of the target bands and internal standard gene cDNAs were estimated with an image analyzer (FMBIO II, TaKaRa), and the intensity ratio of a target gene cDNA to the internal standard gene cDNA was calculated. We used β-actin as an internal standard gene, since the 3D-clinorotation did not change the level of β-actin mRNA.

2.7. Gel mobility shift assay

The cellular protein was prepared according to the method described previously [21]. Collected cells were homogenized on ice in 20 mM HEPES, pH 7.4, containing 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM dithiothreitol, 0.42 M NaCl, 20% (v/v) glycerol, 0.5 mM PMSF and 0.5 mM leupeptin. The homogenate was ultracentrifuged at 100,000×*g* for 30 min at 4 °C. The supernatants were dialyzed against 10 mM Tris–HCl buffer, pH 7.8, containing 50 mM NaCl, 1 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM PMSF, 0.5 mM leupeptin and 5% glycerol by using an oscillatory microdialysis system (Bio-Tech International, Bellevue, WA, USA). The dialyzed sample was stored at −80 °C until analysis. The synthetic oligonucleotide was radiolabeled with 5′-end labeling one strand by T4 polynucleotide kinase and [γ-^{32}P]ATP. The sequences of the used probes (NF-κB-binding element and its mutant) were 5′-AGTTGAGGGGACTTTCCCAGGC-3′ [22] and 5′-AGTTGAGCTCACTTTATCAGGC-3′, respectively. The whole-cell extract (7 μg protein) was mixed with 10 mM Tris–HCl buffer, pH 7.8, containing 50 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 5-μg bovine serum albumin, 2-μg poly(dI–dC)·poly(dI–dC) and 0.1-ng [^{32}P]-labeled probe (1–2×10^4 cpm) in a final volume of 20 μl. The binding reaction was performed without any reducing agent for 20 min at 25 °C, and 2 μl of a dye solution, containing 0.2% bromophenol blue, 0.2% xylene cyanol and 50% glycerol, was added. The mixtures were loaded onto 4% native polyacrylamide gel in 0.5×TBE (45 mM Tris–borate buffer, pH 8.3, containing 1 mM EDTA) at 4 °C. For competition analysis, 0.1 ng of the labeled probe was mixed with an excessive amount of unlabeled competitor oligonucleotide before the binding reaction was begun. Electrophoresis was carried out at 4 °C for 2–3 h at 140 V, and then the gels were dried and exposed to X-ray films for an appropriate time.

2.8. Other assays

Protein concentration was determined by Lowry's method with bovine serum albumin as a standard [23]. Partial pressure of oxygen in media was measured with a

blood gas analyzer (OPTI-CCA, Osmetech Inc., Kobe, Japan).

2.9. Statistical analysis

All data are expressed as means±S.D. for three to five individual samples per group and were analyzed by one-way analysis of variance using SPSS (release 6.1, SPSS Japan, Tokyo). Differences between values of two groups were tested by Scheffé's test. A P value of <0.05 was considered to be statistically significant.

3. Results

3.1. Impaired myotube formation of L6 cells cultured with the 3D-clinorotation

Non-rotated L6 myoblastic cells cultured in the proliferation medium (in the presence of 10% fetal calf serum) proliferated and spontaneously differentiated into myotubes after confluence (Fig. 1A). The 3D-clinorotation completely prevented the myotube formation of L6 cells (Fig. 1A): the number of myotubes in L6 cells with or without the 3D-clinorotation for 7 days were 4±1/hpf (n=5 flasks) and 1±1/hpf (n=5 flasks), respectively, although the 3D-clinorotation did not change the proliferation of L6 cells in this condition (Fig. 1C). We also examined effects of the 3D-clinorotation on myotube formation of L6 cells cultured in the differentiation medium (in the presence of 2% horse serum). The 3D-clinorotation partially inhibited the myotube formation of L6 cells (Fig. 1B), although myotube formation occurred in rotated and non-rotated conditions. The day when myotube formation started in the rotated L6 cells was delayed, compared with that in the non-rotated control cells. The number of myonuclei incorporated into a myotube in the 3D-clinorotated L6 cells was significantly less than those in the non-rotation control culture (Fig. 1D).

3.2. Effects of the 3D-clinorotation on expression of myogenesis-associated proteins

We examined the expression of myogenesis-associated proteins, such as MHC and myogenin, in L6 cells cultured with differentiation media. L6 cells without the 3D-clinorotation increased the levels of MHC mRNA and proteins in association with myotube formation of L6 cells (Fig. 2A and B). Prior to the increased MHC expression, the levels of myogenin mRNA and proteins were up-regulated in L6 cells cultured without the rotation. The 3D-clinorotation significantly prevented the up-regulated expression of these myogenesis-associated proteins at the mRNA and protein levels (Fig. 2A and B). In L6 cells cultured with the 3D-clinorotation, the expression of these proteins increased on Day 3 and returned to the basal levels on Day 7. The decreased expression of the myogenesis-associated proteins in the 3D-clinorotation was consistent with the reduced formation of myotubes (Fig. 1B). The decreases in concentration of MHC and myogenin proteins were not due to their enhanced degradation, since epoxomicin, a potent inhibitor for their degradation [9], did not cause accumulation of these proteins even in 3D-clinorotated L6 cells (data not shown).

3.3. Effects of the 3D-clinorotation on protein-ubiquitination in L6 cells

To elucidate the involvement of the ubiquitin-dependent proteolytic pathway in the retardation of myotube formation, we examined the effects of the 3D-clinorotation on protein-ubiquitination in L6 cells cultured with proliferation or differentiation media. In spontaneously differentiating conditions (non-rotating culturing with proliferation media), ubiquitinated proteins, especially with high molecular masses of 200–300 kDa, in L6 cells accumulated in a time-dependent manner (Fig. 3A, lanes 1–6). Ubiquitination showed the peak on Day 3, when the L6 cells reached 100% confluence and started differentiation into myotubes. The 3D-clinorotation significantly suppressed this protein-ubiquitination (Fig. 3A, lanes 7–12). The amounts of ubiquitinated proteins in non-rotating culturing with differentiation media were more than those with proliferation media and increased in a time-dependent manner (Fig. 3B, lanes 1–6). In enhanced differentiation conditions, the 3D-clinorotation weakly suppressed the increase in ubiquitinated proteins until Day 1 (Fig. 3B, lanes 7–10). It significantly decreased the amounts of ubiquitinated proteins after the day (Fig. 3B, lanes 11 and 12). In both cases, the immunoblot analysis of β-actin and Coomassie brilliant blue staining showed that the protein content in each lane was almost the same (Fig. 3 and data not shown).

3.4. Amounts of IκB protein and mRNA in L6 cells cultured with the 3D-clinorotation

To determine whether the retardation of myotube formation in the 3D-clinorotation system was associated with NF-κB signaling, we first examined the amounts of IκB protein and mRNA in the rotated or non-rotated L6 cells. The IκB protein level decreased prior to myotube formation in the non-rotated L6 cells cultured with differentiation media (Fig. 4A), whereas the levels of IκB transcripts increased during the differentiation of the non-rotated L6 cells (Fig. 4B). This decline in the amount of IκB protein was completely inhibited by a proteasome inhibitor (Fig. 4A). These results were consistent with the previous report suggesting that the ubiquitin-dependent proteolytic pathway regulates the IκB concentration in the cells [24]. In contrast, the 3D-clinorotation significantly

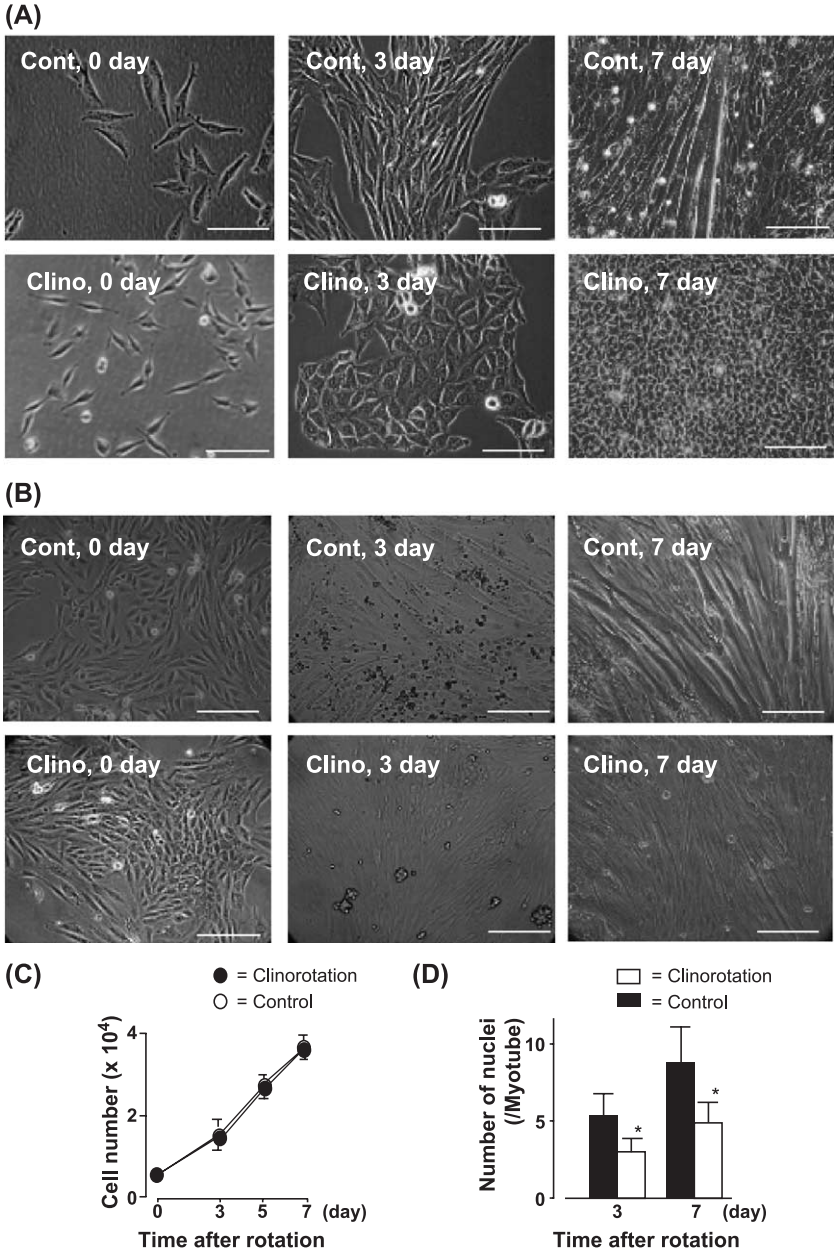

Fig. 1. Myotube formation of L6 cells cultured with a proliferating (A) or differentiating medium (B) during the 3D-clinorotation. (A, B) L6 cells (5×10^3/flask) were seeded into 25-cm^2 culture flasks and cultured to 30% or 75% confluence at 37 °C in a 5% CO_2 chamber. After the flasks were filled with the proliferation or differentiation medium, respectively, they were rotated at 37 °C on the 3D-clinostat apparatus in a 5% CO_2 chamber. Morphological changes in L6 cells before or after the rotation for 3 or 7 days were observed with an inverted optical microscope. Control cells cultured for 0, 3 or 7 days without the rotation were prepared in parallel. Each medium for the control or rotated cells was not changed after filling the culture flask with DMEM. The white lines in the figures indicate 100 μm. (C) Proliferation of L6 cells in the proliferation medium with or without the 3D-clinorotation was monitored by counting the number of cells stripped with 0.25% trypsin. Day 0 indicates the day of starting rotation. The values are means±S.D. (n=3). (D) Myotube formation of L6 cells in the differentiation medium with or without the 3D-clinorotation was assessed by counting the number of myoblasts incorporated into myotube. L6 cells cultured with or without the 3D-clinorotation for the indicated times were fixed with formalin, and the nuclei were stained with Hoechst-33342 (Dojindo, Kumamoto, Japan). Optical and fluorescence microscopic images were merged on a PC. The number of myonuclei in 20 myotubes/flask was counted and averaged per a myotube. Cont, control; Clino, clinorotation. The values are means±S.D. (n=3 flasks). *Significantly different compared with the values on non-rotated cells at the same times, $P<0.05$.

suppressed the decline in IκB protein level of L6 cells, as observed in epoxomicin-treated and non-rotated L6 cells (Fig. 4A). The amounts of IκB transcripts in rotated L6 cells also sustained at lower levels, compared with those in non-rotated L6 cells (Fig. 4B), suggesting that the 3D-clinorotation may inhibit a decrease in IκB protein level during differentiation at the posttranscriptional levels, as well as a proteasome inhibitor. The reduced levels of IκB

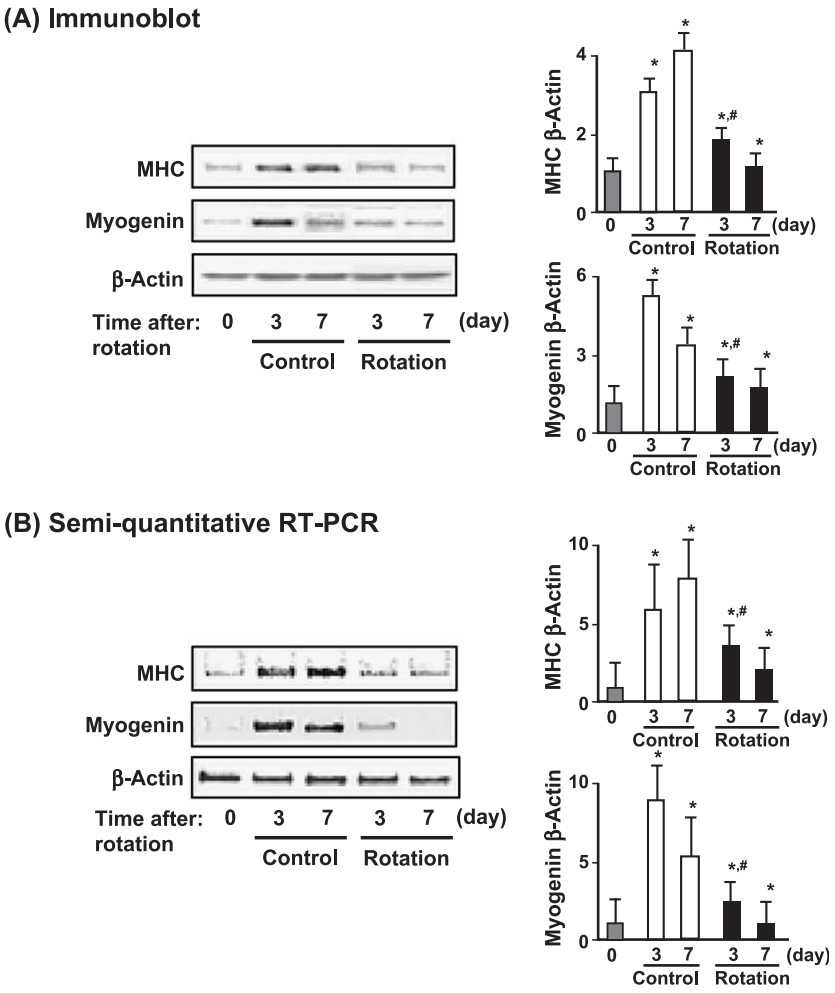

Fig. 2. Expression of MHC and myogenin in L6 cells with or without the 3D-clinorotation. (A, B) After flasks were filled with the differentiation medium, L6 cells were subjected to the 3D-clinorotation and rotated for the indicated days as described in the legend for Fig. 1. Control L6 cells were prepared in the same manner. Protein and total RNA were extracted from the rotated or control L6 cells with an acid guanidinium thiocyanate–phenol–chloroform mixture according to the standard protocol [12]. Immunoblotting (A) and semi-quantitative RT-PCR (B) for MHC, myogenin or β-actin were performed, and the intensity ratios of protein and cDNA of a target gene to β-actin were calculated as described in Materials and methods. The values are mean±S.D. (n=3). *,# Significantly different compared with the values on Day 0 and on non-rotated (control) cells, respectively, P<0.05.

mRNA in rotated or epoxomicin-treated L6 cells indicated the down-regulated NF-κB signaling, since the expression of IκB itself is regulated by NF-κB [25].

3.5. NF-κB-binding activities in L6 cells cultured with the 3D-clinorotation

We next examined whether the rotation-mediated inhibition of decrease in IκB protein level might affect the NF-κB signaling. The gel mobility shift assay showed that NF-κB-binding activities in L6 cells cultured without the 3D-clinorotation increased during their differentiation (Fig. 5, lanes 1 and 3). The 3D-clinorotation significantly suppressed these NF-κB-binding activities (Fig. 5, lanes 2 and 4). After three separate experiments were done, the intensities of the NF-κB-binding activities were quantified by densitometric analysis. The 3D-clinorotation decreased the differentiation-mediating NF-κB activation to 10–30% of the control value at each time point. The binding activities were inhibited by addition of an excessive amount of unlabeled NF-κB oligonucleotide, but not by the mutant oligonucleotide (Fig. 5, lanes 5–10).

3.6. Suppressive effects of the 3D-clinorotation on IκB-ubiquitination in transfected Cos cells

We did not detect any ubiquitinated IκB proteins in the precipitates of L6 cells by using an antibody against IκB or ubiquitin (data not shown). Finally, to confirm a suppressive effect of the 3D-clinorotation on IκB ubiquitination, Cos7 cells were cotransfected with IκB and FLAG-tagged ubiquitin and subjected to the 3D-clinorotation. The immunoprecipitation analysis showed that the amounts of ubiquitinated IκB proteins increased in the non-rotated and transfected Cos7 cells in a time-dependent manner. The rotated Cos7 cells at each point

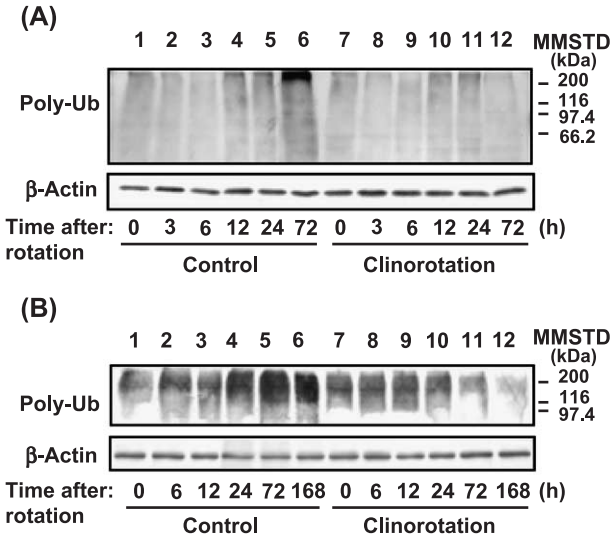

Fig. 3. Effects of the 3D-clinorotation on protein-ubiquitination in L6 cells. (A, B) Flasks containing L6 cells of 30% and 75% confluence were filled with the proliferation (A) or differentiation medium (B), respectively, and were rotated at 37 °C for the indicated times in a 5% CO_2 chamber. The respective control cells without the rotation were prepared in parallel. Protein was prepared as described in the legend for Fig. 2. Immunoblottings for ubiquitinated proteins were performed as described in Materials and methods. β-Actin was used as an internal standard. Similar results were obtained in three separate experiments. Poly-Ub, polyubiquitination; MMSTD, molecular mass standards.

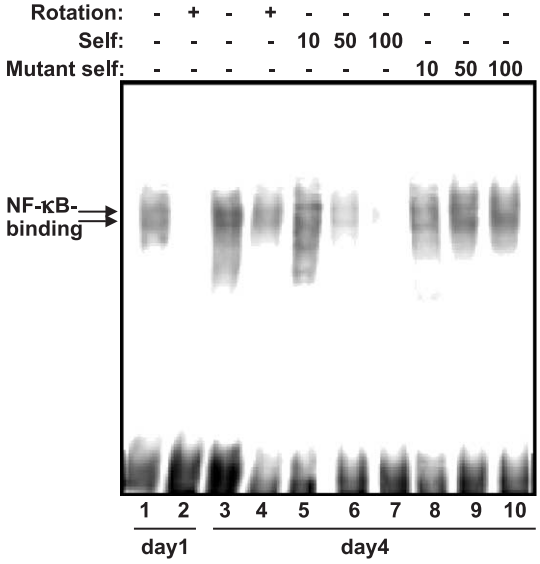

Fig. 5. Effects of the 3D-clinorotation on NF-κB-binding activity in L6 cells. Flasks containing L6 cells of 75% confluence were filled with the differentiation medium and were rotated at 37 °C for the indicated times in a 5% CO_2 chamber (lanes 2 and 4). The respective control cells without the rotation were prepared in parallel (lanes 1, 3, 5–10). Whole cell proteins were extracted on the indicated days, and a gel mobility shift assay was performed as described in Materials and methods. Lanes 5–7 contained a 10-, 50- or 100-fold molar excess of the unlabeled NF-κB oligonucleotides, respectively. Lanes 8–10 contained a 10-, 50- or 100-fold molar excess of the unlabeled mutant NF-κB oligonucleotides, respectively. Similar results were obtained in three separate experiments.

contained a lower concentration of ubiquitinated IκB proteins than the non-rotated cells (Fig. 6). The suppressive effect of the 3D-clinorotation on ubiquitination was observed in unidentified muscle proteins, besides IκB. The Western blot analysis using an anti-FLAG antibody showed that many ubiquitinated proteins accumulated in the transfected and non-rotated Cos7 cells, whereas the

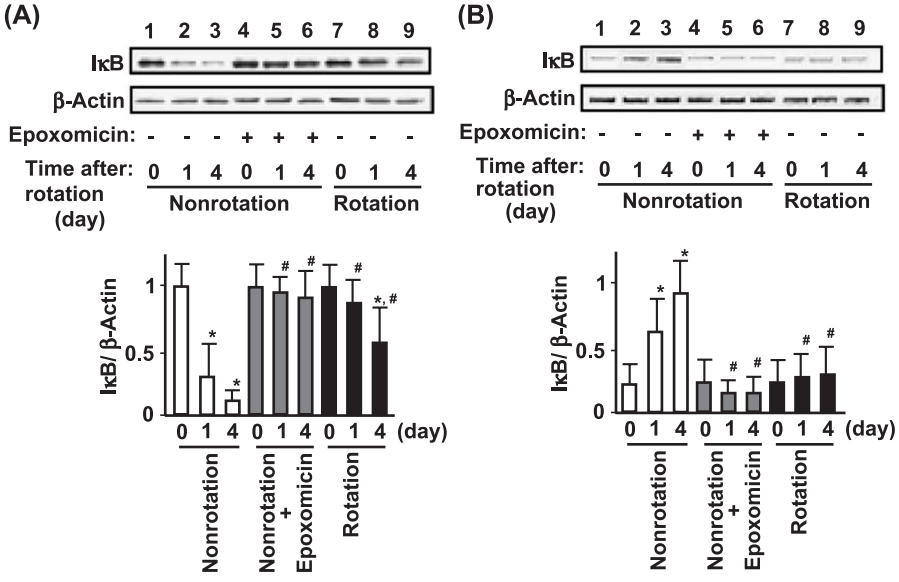

Fig. 4. Amounts of IκB protein and mRNA in rotated or epoxomicin-treated L6 cells. (A, B) Flasks containing L6 cells of 75% confluence were filled with the differentiation medium and were rotated at 37 °C for the indicated times in a 5% CO_2 chamber. The respective control cells without the rotation were prepared in parallel. Some flasks were filled with the differentiation medium containing 10 nM epoxomicin (Peptide Institute, Osaka, Japan) and cultured without the rotation in the similar conditions. Protein and total RNA were prepared as described in the legend for Fig. 2. Immunoblottings (A) and semi-quantitative RT-PCR (B) for IκB or β-actin were performed. The intensity ratios of protein and cDNA of IκB to β-actin were calculated as described in Materials and methods. The values are mean±S.D. (n=4). *, #Significantly different compared with the values on Day 0 and on non-rotated (control) cells, respectively, P<0.05.

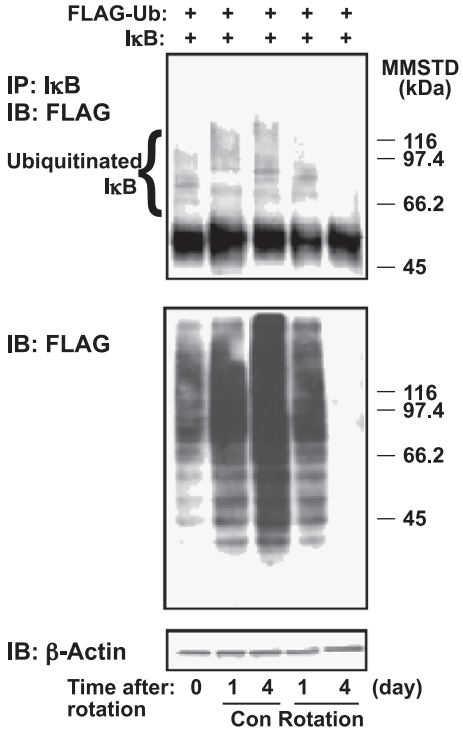

Fig. 6. Effects of the 3D-clinorotation on IκB ubiquitination in Cos7 cells. Cos7 cells (5×10^3/flask) were seeded into 25-cm^2 culture flasks and cultured overnight at 37 °C in a 5% CO_2 chamber. Cos7 cells were cotransfected with plasmids containing IκB and FLAG-tagged ubiquitin by using a non-viral vector FuGene6. Flasks containing the IκB- and ubiquitin-expressing Cos7 cells were filled with the proliferation medium 24 h after transfection and were rotated at 37 °C for the indicated days. Protein (100 μg/lane) extracted from the transfected Cos7 cells was subjected to the immunoprecipitation analysis as described in Materials and methods. Protein (40 μg/lane) was also subjected to the immunoblotting for FLAG (ubiquitin) and β-actin. Similar results were obtained in three separate experiments. Con, control; IP, immunoprecipitation; IB, immunoblotting; MMSTD, molecular mass standards.

3D-clinorotation significantly decreased their amounts (Fig. 6).

3.7. Effects of the 3D-clinorotation on other myogenesis-regulatory factors

We examined four other myogenesis-regulatory factors, UBP 45/65 deubiquitinating enzymes, β-TrCP, cyclin E and oxygen levels in media [5,17,19,26], to examine whether these factors were involved in impaired differentiation in the rotated L6 cells. The levels of UBP 45 or 69 deubiquitinase transcripts in the non-rotated L6 cells increased time-dependently, while the 3D-clinorotation reduced their expression (Fig. 7A). The mRNA amount of β-TrCP, a component of Skp1/Cul 1/F-box protein-ubiquitin ligase for IκB [19], tentatively increased during the myotube formation, but the expression profile was not changed by the 3D-clinorotation (Fig. 7A). The amounts of cyclin E protein, a regulator of cell cycle of myoblasts [5], in the rotated L6 cells decreased similarly as those in the non-rotated control cells (Fig. 7B). In addition, there was no significant difference in the oxygen level of media, another regulatory factor for expression of MyoD family proteins [26], between rotated and non-rotated flasks, although cell culturing decreased the oxygen level in both flasks in a time-dependent manner. The partial pressures of oxygen in media of the 7-day culturing with or without the 3D-clinorotation were 74.0±10.5 mm Hg (n=4) and 77.5±8.2 mm Hg (n=4), respectively.

4. Discussion

In this study, we examined the proliferation and differentiation of L6 cells cultured with the 3D-clinorotation, a model of simulated microgravity, to elucidate the association between microgravity and myoblast functions. Our results showed that myotube formation and expression of MHC and myogenin were inhibited by the 3D-clinorotation, whereas the 3D-clinorotation did not affect the proliferation of myoblasts. These data support the previous reports that culturing under microgravity and its simulated conditions are associated with the disturbance of myoblast differentiation [1,2]; however, the mechanism of this association is not well understood.

To elucidate the mechanism, we focused on the NF-κB pathway in L6 cells cultured with the 3D-clinorotation, since a lot of investigations showed that the activation of the NF-κB signaling is necessary for the differentiation of human, rat and chick myoblasts [27–29]. We found that the 3D-clinorotation significantly reduced accumulation of ubiquitinated proteins and blocked ubiquitination and degradation of IκB. Furthermore, it prevented NF-κB signaling, which is estimated by a NF-κB-binding activity, in L6 cells. Our present results suggest that the impaired differentiation of skeletal muscle cells exposed to microgravity or its simulated conditions may be mediated, at least in part, by inhibiting NF-κB signaling.

The present findings of L6 cells on the 3D-clinorotation do not directly lead to muscle atrophy during prolonged weightlessness, since whole skeletal muscle fibers are already differentiated. Michell and Pavlath [3] suggest that the differentiation and fusion of muscle satellite cells to muscle fibers in 2 weeks are necessary after the atrophic conditions to recover muscle mass to the normal value. Based on these findings, muscle cell differentiation impaired by the 3D-clinorotation may mean the loss of fusion of satellite cells to myofibers, which are necessary for recovering muscle volume after weightlessness. In fact, microgravity-mediated muscle atrophy in astronauts does not always recover even several weeks after landing [30,31]. Thus, stimulating NF-κB signaling of satellite cells during an early recovery period may be an effective treatment for astronauts or patients resistant to recovery from muscle atrophy.

Repression of IκB ubiquitination by clinorotation could be caused by an increase in deubiquitinase content or decrease in its responsible ubiquitin ligase (Skp1–Cullin–F-box complex) content. Therefore, we examined the amounts of deubiquitinases UBP 45/69 [17] and β-TrCP (a compo-

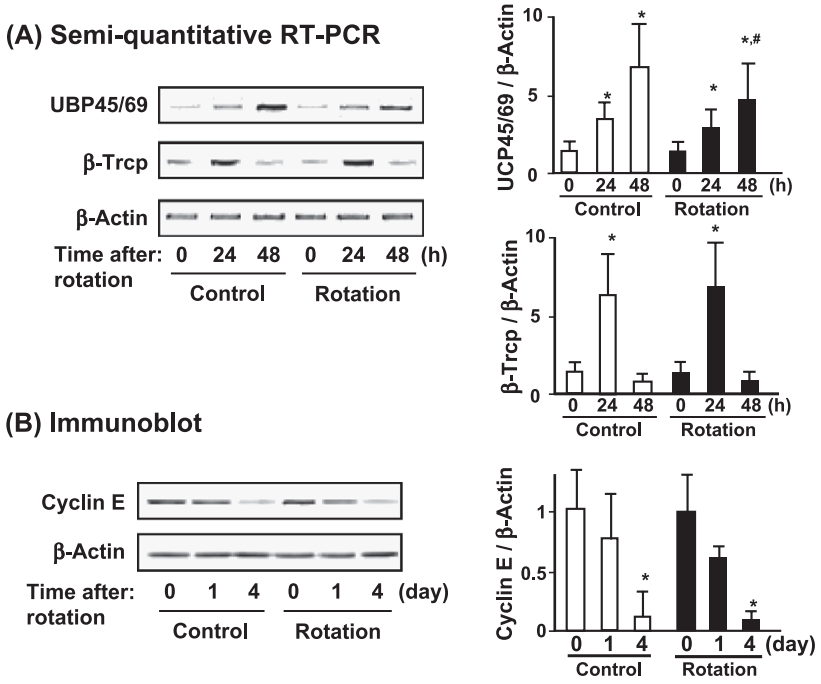

Fig. 7. Effects of the 3D-clinorotation on myogenesis-regulatory factors in L6 cells. (A, B) Flasks containing L6 cells of 75% confluence were filled with the differentiation medium and subjected to the 3D-clinorotation at 37 °C in a 5% CO_2 chamber. After the rotation for the indicated times, total RNA was extracted from the whole cells, as described in the legend for Fig. 2. The semi-quantitative RT-PCR for deubiquitinases UBP 45/69 and β-TrCP (A) was performed as described in Materials and methods. Since UBP 45 and 69 were derived from alternative splicing of the primary transcript [17], we used primers with similar sequences for their amplification. The ratio of cDNA intensity of a target gene to β-actin was calculated as described. Protein (40 μg/lane) was also subjected to immunoblotting for cyclin E and β-actin (B). The ratio of protein intensity of cyclin E to β-actin was calculated. The values are mean±S.D. (n=3). *, #Significantly different compared with the values on Day 0 and on non-rotated (control) cells, respectively, P<0.05.

nent of Skp1–Cullin–F-box complex) transcripts [19]. However, the formers were decreased or the latter were unchanged by the 3D-clinorotation (Fig. 7A), indicating that their expression profiles were not correlated with changes in IκB ubiquitination by the clinorotation. In contrast, previous reports from our and other laboratories have demonstrated that actual microgravity (spaceflight) and its simulation preferentially disturbed expression of cytoskeletal genes in skeletal muscle cells, osteoblasts and lymphocytes, suggesting that alternation of cytoskeleton may be the primary response to weightlessness [32–34]. Furthermore, a recent report showed that microtubule depolymerization correlates with the activation of IκB kinases (IKKs) α and β [35]. These findings may provide a clue to the mechanism involved in our present findings. The altered cytoskeletal network may disturb the trafficking or activation of IKKs for IκB ubiquitination.

Besides NF-κB signaling, we examined other regulatory factors for muscle cell differentiation, such as cyclin E and oxygen level in media. Cyclin E/cyclin-dependent kinase 2 complex has been reported to play an important role in triggering myogenesis; ubiquitin-dependent degradation of cyclin E is necessary for expression of the MyoD family proteins [5]. The present results showed that cyclin E protein levels similarly decreased in the non-rotated and rotated L6 cells. In contrast, oxygen concentration in media has also been reported to affect the proliferation of satellite cells and survival of mature fibers [26]. The physiological oxygen level (6%) increased the proliferation of the satellite cells more than the non-physiological oxygen level (20%) used in a traditional cell culture system. The 7-day culturing with or without the 3D-clinorotation significantly decreased oxygen level in media, but both the oxygen levels were similar. Based on these findings, cyclin E and oxygen were unlikely to be involved in the retardation of L6 cell differentiation caused by the 3D-clinorotation. However, the 3D-clinorotation used in this study suppressed ubiquitination of many proteins as well as IκB in L6 and Cos7 cells (Figs. 3 and 6). We cannot exclude the possibility that other ubiquitin-dependently degraded proteins are associated with the reduced differentiation of myoblastic cells cultured with the 3D-clinorotation. Further examinations are necessary to identify these proteins.

Using an in vivo or ex vivo system, two recent and interesting studies have reported that both unloading and loading activate the NF-κB pathways in skeletal muscle; unloading leads to the activation of the alternative NF-κB pathway that requires IKKα activation, whereas loading activates the canonical NF-κB pathway [36,37]. We used an in vitro system (i.e., 3D-clinorotation) that is free of neuronal and hormonal effects to investigate the effect of a mimic microgravity environment on differentiation of myoblastic cells. Skeletal muscle cells in tissue are well differentiated and are completely different from myoblastic

cells like L6 cells. Therefore, the discrepancy in activation of NF-κB signaling may be associated with differences between in vivo and in vitro responses of skeletal muscle cells to microgravity. This hypothesis may be supported by several in vitro investigations showing that clinorotation significantly repressed TNF-α-dependent activation of NF-κB in human osteoblastic cells [38]. In addition, we found similar discrepancy between in vivo and in vitro responses to microgravity in protein-ubiquitination. We previously reported that in rats exposed to an actual spaceflight (STS-90), degradation of skeletal muscle proteins was enhanced in association with activation of the ubiquitin-proteasome pathway [9], whereas our present results showed that the 3D-clinorotation suppressed protein-ubiquitination in myoblastic L6 cells. There is a possibility that these mysterious responses of myoblastic cells, including NF-κB activation and protein-ubiquitination, were due to the artificial effects of the 3D-clinorotation, since the clinorotation culture system provides simulated microgravity conditions, but not actual weightlessness, to cells. To clarify this issue, our proposal for a space experiment using L6 cells on the International Space Station has been accepted.

Besides clinostat, the environment created on the Earth within a rotating wall vessel is often referred to as "simulated microgravity". Recently, several investigations have showed that culturing of mouse C2C12 cells in a rotating wall vessel increased cellular proliferation and slightly decreased the sarcomeric myosin protein expression [39,40]. Unlike our present results in a three dimensional (3D)-clinostat system, a simulated microgravity created with a rotating wall vessel seemed not to have a drastic impact on myoblast differentiation. The intended applications of these two devices differ considerably (see Ref. [41] for review). A clinostat can reproduce a near-quiescent fluid environment similar to that which would be experienced with an unstirred container in actual weightlessness. On the other hand, the rotating wall vessel bioreactor simulates weightlessness to a lesser degree, considering relative cell motionlessness and reduced extracellular mass transport. The rotation wall vessel bioreactor ideally creates a low-shear, but necessarily mixed, fluid environment that is optimized for suspension culture and tissue growth. Distinct characteristics of these two devices may produce the discrepancy, although we cannot provide the rational reason at the present time.

The 3D-clinorotation suppressed the ubiquitination of proteins such as IκB in Cos7 cells as well as in L6 cells, indicating that simulated microgravity could suppress the NF-κB signaling in various kinds of cells. Recently many kinds of stem cells have been used to reproduce human tissues, but there are several difficulties associated with putting them to practical use. For example, it is difficult to keep stem cells immature for a long time [42]. Our present results provide the rational suggestion that the 3D-clinorotation is a useful tool to maintain stem cells in the primitive state by deubiquitinating IκB. In fact, we found and maintained novel stem cells for chondrocytes from human bone marrow-derived cells with our present 3D-clinorotation system for a long time (unpublished data, manuscript in preparation).

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