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Microgravity influences maintenance of the human muscle stem/progenitor cell pool

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ABSTRACT

Microgravity induces skeletal muscle atrophy; however, the underlying mechanism is not clarified. In particular, the influence of microgravity on human skeletal muscle stem/progenitor cells (SMPCs) is not well understood. In this study, we used induced pluripotent stem cell-derived human SMPCs to investigate the effect of microgravity on maintenance of the stem/progenitor cell pool. Human SMPCs were induced by free-floating spherical aggregation culture, and derivatized-SMPC spheres were maintained in a microgravity condition (10^{-3} G) for 2 weeks using a clinostat rotation system. Microgravity culture deformed the SMPC spheres, with no signs of apoptosis. The most obvious change from microgravity culture was a significant decrease in the expression level of Pax7 in the SMPC spheres, with reduced numbers of myotubes in adhesion culture. Pax7 expression also decreased in the presence of the proteasome inhibitor MG132, indicating that the proteasomal degradation of Pax7 protein is not critical for its reduced expression in microgravity culture. Moreover, microgravity culture decreased the expression level of tumor necrosis factor receptor-associated factor 6 (TRAF6) and phosphorylation of its downstream molecule extracellular-related kinase (ERK) in SMPC spheres. Therefore, microgravity negatively regulates Pax7 expression in human SMPCs possibly through inhibition of the TRAF6/ERK pathway to consequently dysregulate SMPC pool maintenance. Overall, these results suggest that skeletal muscle atrophy is caused by microgravity-induced exhaustion of the stem cell pool.

1. Introduction

Skeletal muscle is one of the largest tissues in the body in addition to the bones, and is maintained at a steady-state condition for appropriate size (mass) and functions. This homeostatic maintenance of the skeletal muscle is assumed to mainly be driven by dynamics in protein turnover and through the progenies from resident muscle stem/progenitor cells (SMPCs). Muscle mass and functions are influenced by physical changes such as aging and gravity, which can dysregulate the maintenance system, resulting in muscle atrophy. For example, age-related muscle atrophy is associated with a reduced number of SMPCs due to dysregulation of stem cell pool maintenance in the mouse skeletal muscle [1]. However, the molecular mechanism underlying such physical change-mediated muscle atrophy remains poorly understood, and it is not yet clear whether different types of changes (i.e., aging and gravity) lead to muscle atrophy via a common mechanism.

Microgravity induces adverse effects on protein turnover in the muscle and in the behavior of myogenic cells. For example, microgravity promotes the degradation of muscle structural proteins and inhibits the terminal differentiation of myoblasts, the progeny of SMPCs [2,3], suggesting direct effects of microgravity on the skeletal muscle that consequently lead to muscle atrophy. However, many of the previous studies related to the skeletal muscle have mainly focused on differentiated myogenic cells such as muscle fibers, and the potential influences of microgravity on SMPCs have generally been overlooked. One possible reason for this limitation is the technical difficulty in expanding and isolating the rare SMPCs of the postnatal skeletal muscle [4]. Therefore, establishing effective methods for isolating and maintaining a large

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number of SMPCs will be helpful for investigating the effects of microgravity on stem cells in the skeletal muscle.

Pluripotent stem cells (PSCs) such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) have been recognized as valuable sources for preparing desired cell types. In addition to other cell types, myogenic cells, including SMPCs, can be isolated from both mouse and human PSCs [5], although the derivation efficiency in many of the established protocols could be improved. We recently established a novel derivation protocol for SMPCs from human PSCs based on a spherical aggregation technique [6]. Using this culture system, we can efficiently isolate a relatively large number of $Pax7^+$ SMPCs from both human ESCs and iPSCs. Importantly, undifferentiated SMPCs can be maintained for at least for 2 weeks in the spheres after their derivation from PSCs, suggesting that this protocol is useful for short-term investigations to clarify the effects of physical changes such as microgravity on SMPCs.

In this study, we used this sphere-based culture method to investigate whether microgravity influences maintenance of the iPSC-derived human muscle stem cell pool. Human SMPCs were isolated from iPSCs, and then spheres including SMPCs were cultivated in a simulated microgravity (10^{-3} G) condition. Using this culture system, we tested the effects of microgravity on sphere morphology, cell survival, and maintenance of the stem cell pool. We further evaluated the effect of microgravity on Pax7 expression and the tumor necrosis factor receptor-associated factor 6 (TRAF6) pathway, which has been reported as a potential regulator of Pax7 expression in the mouse skeletal muscle [7]. In general, it has been reported that microgravity conditions such as those experienced during space flight could induce the degradation of structural proteins in the muscle fiber via the ubiquitin/proteasome pathway [16]. Therefore, we further checked whether simulated microgravity culture induced the ubiquitin/proteasome pathway-dependent protein degradation in SMPC spheres. These findings should provide new insights into the effects of microgravity on the maintenance of stem/progenitor cells in the skeletal muscle.

2. Materials and methods

2.1. Derivation of human SMPCs from iPSCs

To prepare human SMPCs, we used the human iPSC cell line 201B7 [8]. In brief, human iPSCs were cultivated in Stemline Neural Stem Cell Expansion Medium (Stemline; Sigma-Aldrich) with 100 ng/ml basic fibroblast growth factor (ReproCell) and epidermal growth factor (Sigma-Aldrich), and maintained in free-floating culture condition for 4–6 weeks [6]. Aggregated PSCs, i.e., the SMPC spheres, were passed by a mechanical chopping technique every week [9]. Derivation of SMPCs from human PSCs was validated by detecting the expression of Pax7 protein (Fig. 1A) and the myogenic differentiation capacity in adhesion culture.

2.2. Microgravity culture

The SMPC spheres generated by free-floating culture for 6 weeks in standard gravity condition (1 G) as described above were further cultivated in microgravity condition (10^{-3} G). SMPC spheres were applied onto a clinostat rotation system (Zeromo; Kitagawa Iron Works Co. Ltd.) and rotated for 2 weeks. During rotated cultivation, the excessive condensation could degrade the SMPC spheres. Therefore, to keep the spheres afloat, a small amount of the polymer FP001 (FCeM-series; Nissan Chemical Industries, Ltd.) was added to the Stemline medium, which blocks condensation. The spheres were mechanically chopped for passaging at the middle of the culture period. The proteasome inhibitor MG132 (5 μM, Enzo Life Sciences) was added to the Stemline medium in the microgravity culture condition.

2.3. Immunocytochemistry

Cells or spheres were processed for immunocytochemistry by fixing with 4% paraformaldehyde and staining as described previously [10]. In brief, cells were permeabilized with 0.1% Triton-X100 in blocking solution (DAKO). After rinsing with phosphate-buffered saline (PBS), the cells were incubated with primary antibodies against Nanog (1:200; ReproCell), Pax7 (1:50; Developmental Studies Hybridoma Bank), and myosin heavy chain (MF20, 1:50; eBioscience). To evaluate actin expression in SMPC spheres, an anti-actin antibody conjugated with Alexa-555 (1:250; Cytoskeleton) was used. After incubation with the primary antibodies, the cultures were rinsed with PBS and then incubated with the secondary antibody conjugated with Alexa Fluor-555 (1:1000; Invitrogen). Cell nuclei were then stained with DAPI (PureBlu, Bio-Rad) and immunofluorescence images were acquired using the BIOREVO microscopy system (BZ-9000, Keyence).

2.4. Western blot analysis

Spheres cultured in standard gravity or microgravity condition were dissolved in RIPA buffer containing a protease/phosphatase inhibitor cocktail, and 20 μg of proteins were applied onto polyacrylamide gels. After electroblotting the proteins onto polyvinylidene fluoride membranes, they were incubated with primary antibodies against Numb (1:1000; Cell Signaling Technology), caspase 3 (1:1000; Cell Signaling Technology), β-actin conjugated with horseradish peroxidase (HRP; 1:4000; Novus Bioscience), Akt (1:1000; Cell Signaling Technology), phosphorylated (p)Akt (1:1000; Cell Signaling Technology), Pax7 (1:500; Developmental Studies Hybridoma Bank), ubiquitin (1:200; Santa Cruz), Cbl-b (1:200; Santa Cruz), TRAF6 (1:200; Santa Cruz), ERK (1:1000; Cell Signaling Technology), and pERK (1:1000; Cell Signaling Technology). After incubation with the primary antibodies overnight, the membranes were incubated with secondary antibodies conjugated with HRP against the rabbit polyclonal or mouse monoclonal primary antibodies, and rinsed with Tris-buffered saline including Tween-20. Proteins were visualized with HRP substrate (ECL Prime Western Blotting Detection System, GE Healthcare), and band intensities were quantified using the ImageJ software package (National Institutes of Health).

2.5. Adhesion culture of SMPC sphere-derived cells

SMPC spheres were gently dissociated with TrypLE reagent (Life Technologies), and plated onto cell culture plates coated with Geltrex matrix (Life Technologies). The cells were cultured in Dulbecco's modified Eagle medium (Life Technologies) containing 20% fetal bovine serum and antibiotics. At day 1 and 14 after plating, the cells were fixed with 4% paraformaldehyde for immunocytochemistry.

2.6. Statistical analysis

All values are expressed as mean ± standard deviation. A two-tailed Student's t-test was performed to compare the band intensities and cellular positivity rates between groups. Statistical analysis was performed using SPSS Statistics 20 (IBM Japan). *P*-values less than 0.05 or 0.01 were considered statistically significant.

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