Doctoral Dissertation

Study on Effects of Isotropic Gravity on Neurogenic Differentiation Potential in Human Mesenchymal Stem Cells

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Contents

Contents (cont.)

Abstract

Human mesenchymal stem cells (hMSCs) are considered to be capable of responding to environmental changes induced by gravity. In cell expansion process, the maintenance of differentiation potential is one of the most important consideration for the efficacy of cell-based therapy. Although reactions to unidirectional gravity culture have been intensively studied in conventional culture conditions, little is known about the cellular adaptation to isotropic gravity culture conditions. Thus, this study investigated the effects of isotropic gravity on hMSCs using a 3D-clinostat (Gravite®), which generates a simultaneous rotation on two axes with constant angular velocity during the culture. This study focused on changes of neurogenic differentiation capacity in passaged hMSCs between the cells cultured under unidirectional gravity conditions and isotropic gravity conditions. Moreover, this study also examined alterations in dynamic cell behavior, mechanotransduction, and histone methylation in hMSCs under different gravitational culture conditions in order to understand the mechanism of cellular modification involved in their differentiation potential changes.

Chapter 1 shows the influence of isotropic gravity on modulated behavior mechanism and structural components relating to migration and mechanotransduction of hMSCs. Time-lapse observation revealed that cells cultured under unidirectional gravity conditions had unidirectional migration, while cells cultured under isotropic gravity conditions had multidirectional migration with active extension of leading edge and partially contraction of cell rear. Cells cultured under unidirectional gravity presented the maintenance of their spindle-shape via fibronectin fibril formation in their bodies as well as stabilization of focal adhesions with enriched apical actin stress fibers over the nucleus. However, cells cultured under isotropic gravity conditions had more intense of phosphorylated paxillin in regions of leading and trailing edges together with the up-regulation of MT1-MMP expression. Moreover, cells in isotropic gravity conditions showed fibronectin mainly as aggregate structure with less focal adhesions

and few apical actin stress fibers. Lastly, cells cultured under unidirectional gravity conditions mostly presented a basal-to-apical polarization of mechano-sensitive nuclear lamin A/C, but cells cultured under isotropic gravity conditions mainly showed the non-polarization of lamin A/C. Therefore, this chapter demonstrates that isotropic gravity-driven fibronectin assembly affects nuclear lamina organization through the spatial reorganization of actin cytoskeleton influencing on the alterations in cell behavior and mechanotransduction.

Chapter 2 presents the effects of isotropic gravity on nucleoskeleton, epigenetics, and neurogenic differentiation potential of passaged hMSCs. During serial cultivation with hMSC growth medium, the analysis of lamin organization and histone modifications at promoters of neurogenic lineage genes indicated that cells passaged under isotropic gravity conditions sustained the ratio between lamin A/C to lamin B together with preservation of histone methylation during passage culture. However, the lamin ratio and H3K27me3 enrichment significantly increased in cells grown under unidirectional gravity conditions against their increasing passage numbers. In neurogenic induction culture, differentiated cells from the cells cultured under isotropic gravity conditions expressed higher neurogenic levels than those cultured under unidirectional gravity conditions. The levels of neurogenic markers in the cells from isotropic gravity conditions were consistent during an increase in their passage numbers, while cells from unidirectional gravity conditions exhibited a reduction of the neurogenic levels against the increased passage numbers. These results demonstrate that isotropic gravity-coordinated lamin organization leads to the suppression of histone modification associated with maintenance of neurogenic differentiation potential in passaged hMSCs.

Taken together, this study is the first time to provide an insight into the effects of isotropic gravity on neurogenic differentiation potential in passaged hMSCs. The pioneered data of cell behavior, mechanotransduction, and organization of nuclear lamins as well as histone modification promote the directivity of neurogenic differentiation in hMSCs during cell expansion that would be the supportive information for applications in cell-based therapy.

General introduction

Expansion of human mesenchymal stem cells

Human mesenchymal stem cells (hMSCs) are currently major stem cells for cell-based therapy that have been utilized in a wide range of clinical applications for approximately 15 years (Wei, X. *et al.*, 2013). Mesenchymal stem cells were discovered by Friedenstein et al. who found a fibroblastic cell type derived from mouse bone marrow, which could generate some clonal colonies that were able to construct bone tissue when activation by heterotypical transplantation (Friedenstein, A.J. *et al.*, 1966). hMSCs are multipotent adult stem cells that are located in multiple human tissues such as bone marrow, umbilical cord, and fat tissue (Abdallah, B.M. *et al.*, 2008). Bone marrow–derived hMSCs are referred as bone marrow stromal cells, or multipotent mesenchymal stromal cells suggested by the International Society for Cell & Gene Therapy (ISCT) (Dominici, M. *et al.*, 2006). hMSCs have self-renewal ability and potency to multilineage differentiation into mesoderm-type cells such as osteoblast, chondrocyte, adipocyte (Bianco, P. *et al.*, 2001), and possibly to other non-mesoderm-type cells, which are neuron (Dezawa, M. *et al.*, 2004) and hepatocyte (Luk, J.M. *et al.*, 2005). Furthermore, hMSCs also support hematopoietic stem cells in terms of their growth and differentiation (hematopoiesis) (Dexter, T.M., 1979).

Due to the innate properties of hMSCs, this cell type has become an attractive source for various applications in regenerative medicine as they can be isolated from removable human tissues and can be expanded under appropriate culture conditions *in vitro*. In addition, hMSCs has ability to modulate immune responses that makes them feasible for allogeneic transplantation without risk of immune rejection (Aggarwal, S. *et al.*, 2005). To illustrate, Fig. 1 shows the overview of processes in cell-based therapy by using hMSCs. Starting with the

human tissue is harvested from patient for isolation of hMSCs, and then the isolated cells are expanded for increasing populations under appropriate culture conditions to make the sufficient cell numbers. Finally, the hMSCs are transplanted back to the patient for the treatment by functions of injected cells (Golpanian, S., *et al.*, 2016). Until now, several researchers have well documented about the therapeutic potentials by using hMSCs in many clinical trials of cellular applications such as diabetes mellitus, neurogenic diseases, cardiac diseases, liver diseases, kidney diseases, bone diseases, and autoimmune diseases (Patel, D.M. *et al.*, 2013).

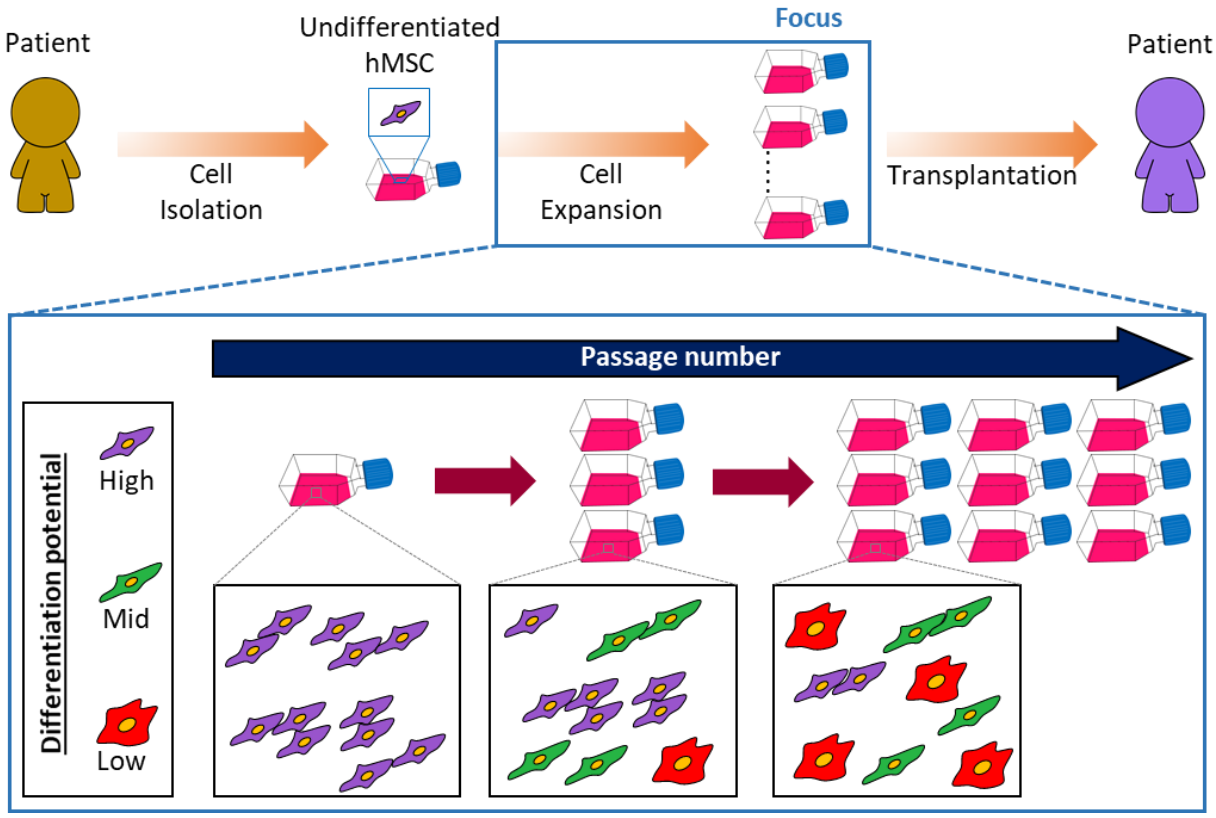

Fig. 1 Overview of processes in cell-based therapy and changes in differentiation potential in passaged hMSCs during cell expansion process.

In this study, the cell expansion process was focused. A bioprocess of mammalian cells has more than 50 years in experience since the cultures of CHO cells (Wurm, F.M., 2013). The evolution of the regenerative medicine has raised and hMSCs have been several investigated for many years. During the cell expansion, rate of proliferation and differentiation potentials in the passaged hMSCs are two main points that should be considered to maintain the performance of the cell processing and quality of the cell products. hMSCs have a limited lifespan similar to other normal somatic cells. After several cell divisions, hMSCs decrease the rate of proliferation and enter senescence that stop of proliferation. This phenomenon is called "Hayflick limit" as described by Leonard Hayflick (Hayflick, L., 1965). Current studies have indicated the critical point that hMSCs exhibit a reduction in differentiation potentials when increasing their population doublings or passage numbers during *in vitro* culture under unidirectional gravity conditions (Bonab, M.M. *et al.*, 2006; Noer, A. *et al.*, 2007; Yang, Y.H.K. *et al.*, 2018). Many factors have been presented to influence the senescence and decrease in differentiation potentials such as stress (Fridlyanskaya, I. *et al.*, 2015), changes in nuclear lamina (Raz, V. *et al.*, 2008), alterations in chromosomal structure (Hänzelmann, S. *et al.*, 2015), and telomere attrition (López-Otín, C. *et al.*, 2013). These cellular modulations possibly limit their abilities and qualities in therapeutic applications due to lack of their differentiation potentials. Therefore, development of culture process is requisite and important for maintenance of cellular characteristics, which is the highest considering parameters in cell expansion process to achieve the specificities of cell-based therapy.

Isotropic gravity in cell culture

Changes in the physical environment, such as the gravity level, gravitational direction and dynamics of the surrounding, influence on cell responses through indirect mechanisms such as alterations in gravity-dependent convection, buoyancy force, and sedimentation (Todd, P.,

1989; van Loon, J.J.W.A., 2007). In the literature, reaction diffusion systems have been extensively referred as fundamental models of spatio-temporal dynamics of various biochemical reactions, including enzyme kinetics and the assembly of complex protein networks (D Murray, J., 2003). Remarkably, it has been reported that gravity affects these reaction diffusion systems in both theoretical calculations (Kondepudi, D.K. *et al.*, 1981) and practical experiments, proving that gravity influences the traveling velocity based on the convection of target patterns relating to their interactions (Fujieda, S. *et al.*, 2001; Vailati, A. *et al.*, 2011). Moreover, it has been reported that microgravity disturbed the microtubule assembly compared with those under unidirectional gravity conditions (Papaseit, C. *et al.*, 2000). As space-flight experiment is limited, several ground-based facilities for simulating microgravity environment have been widely developed, such as diamagnetic levitation systems, rotating wall vessel (RWV), and random positioning machine (RPM) (Herranz, R. *et al.*, 2013). For cultured cells, a three-dimensional (3D)-clinostat is a recently common culture device used to provide an isotropic gravity environment for simulating the microgravity culture conditions by rotations of the culture system in two axes with constant angular velocity.

To understand the physical phenomena in different gravitational environment, Fig. 2 presents the comparison of particle movement induced by the gravity, whereas particles have higher density than surroundings. Under space environment with zero gravity or microgravity, the particles have no movement or very tiny motion due to lack or less of gravity driving force, respectively. However, on the Earth environment, the particles always sense to the gravity, which unchanged of the gravity level. Although during the operation of this 3D-clinostat, an average gravity-vector is approximately at 10^{-3} G but the 3D-clinostat also creates the circular translocation of particles due to occurrence of sedimentation motion by change of gravitational direction during the rotating operation on the Earth (Brungs, S. *et al.*, 2016). Nevertheless, in case of unidirectional gravity, which is a conventional gravity condition for cell culture on the

Earth, it presents that the particles are sedimented to the bottom side due to the direction of gravity under 1 G condition.

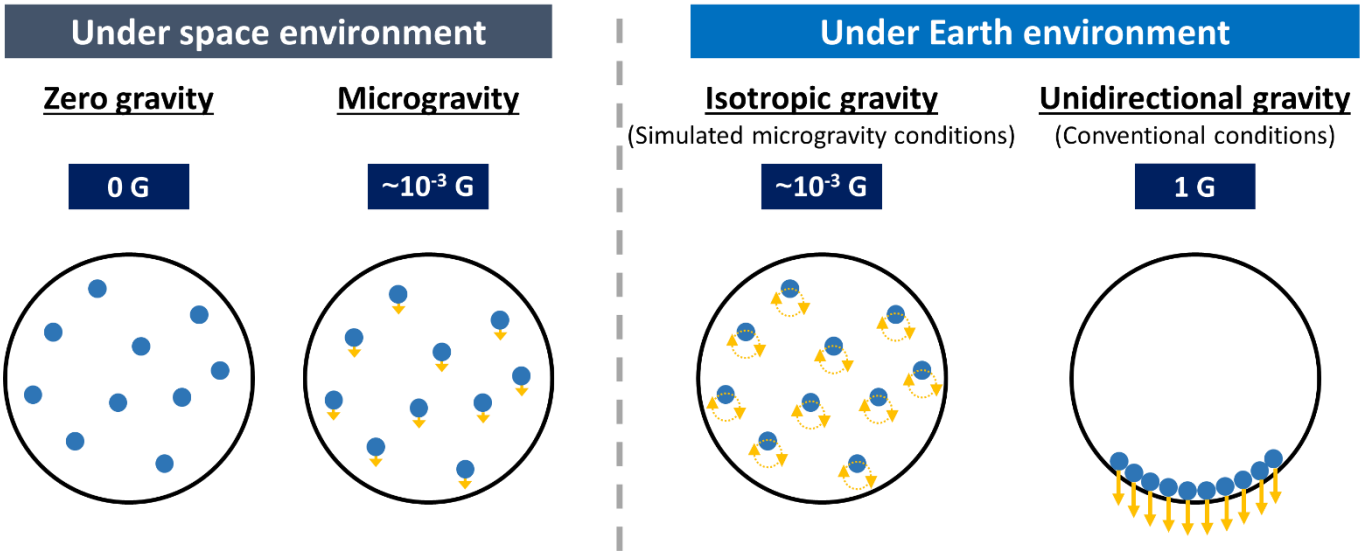

Fig. 2 Comparison the effects of different gravitational environment on the physical phenomena of particle motion induced by the gravity, whereas particles have higher density than surroundings.

In this study was focusing on the culture system on the Earth that compared between unidirectional gravity and isotropic gravity. In case of unidirectional gravity with face-up type or conventional culture, the cells are grown under static unidirectional gravity conditions and the cells sense to the compressive gravity force with unchanged of gravitational direction (Fig. 3). Moreover, under unidirectional gravity with upside-down type, the cells sense to the unidirectional gravity which produces the tension force to the cells. On the other hand, under isotropic gravity conditions, which can be established by the operation of 3D-clinostat with constant rotation in in *X*- and *Z*-axis (Gravite®, Fig. 3). The cells experience the dynamic multidirectional gravity, which is isotropic gravity during the rotations. As this phenomenon, cells sense isotropic tension force during the culture with operation of 3D-clinostat. Although

the advent and improvement of this culture process has allowed the effects of isotropic gravity to be studied *in vitro*, the isotropic gravity by 3D-clinostat has been rarely applied to evaluate the responses from a number of cell types especially in hMSCs.

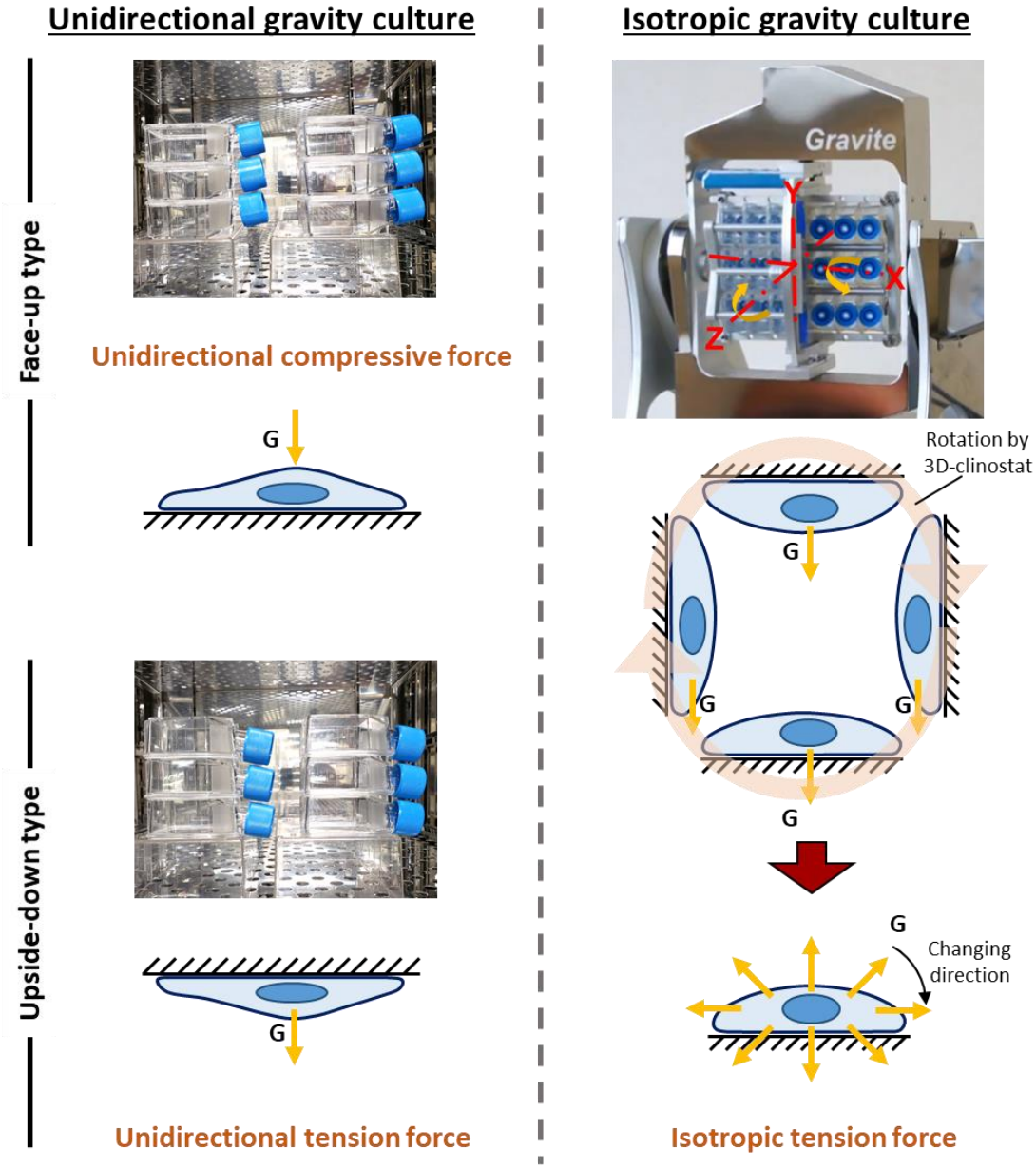

Fig. 3 Culture systems using in this study, which are unidirectional gravity culture (left) with face-up type and upside-down type, and isotropic gravity culture (right) by operation of a 3D-clinostat (Gravite®) that generate the isotropic gravity culture conditions by rotations in *X*- and *Z*-axis with constant angular velocity.

Research outline

This study aims to understand the influence of isotropic gravity on neurogenic differentiation potential in passaged hMSCs during cell expansion process. This study considers the changes in migration behavior, mechanotransduction, and histone modification within the cells between those cultured under unidirectional gravity conditions and isotropic gravity conditions. This thesis is divided into 3 chapters, at the preface section as introduction. This section describes an overview of the usage of hMSCs in regenerative medicine and illustrates the changes in cellular properties during cell expansion with increasing passage numbers that influences the quality of cells for therapeutic applications. Lastly, this section gives information about the influence of gravity on biological phenomena and principle of isotropic gravity device for cell culture system *in vitro*.

Chapter 1 focuses on investigation of cellular mechanisms responsible for modulating hMSC behaviors under isotropic gravity conditions. Cell behavior is one of the important indicators that are influenced by several phenomena in the cells such as cell-substrate adhesion, cell-cell interaction, and dynamic change of cell morphology, which relates to various cell signalings. This chapter shows mechano-adaptation in hMSCs under altered gravitational culture conditions by remodeling of cytoskeleton and nucleoskeleton organization as well as changes in fibronectin assembly and difference in matrix-degrading enzyme activity regarding the stability of cell-substrate adhesion and migration behavior of hMSCs. The results of this chapter could be applied for further investigation about influence of mechanotransduction on differentiation phenotypes in passaged hMSCs shown in next chapter.

Chapter 2 demonstrates an application of isotropic gravity on cell expansion process. This section focuses on effects of isotropic gravity on nuclear adaptation and neurogenic differentiation potential in passaged hMSCs. Organization of nuclear lamins involves the mechanotransduction and chromatin structure in their nucleus. The chromatin compaction

relates to histone modification, which is a kind of epigenetic memory that is heritable and affecting their daughter cells. These histone methylations at specific regions regulates their particular gene expressions influencing differentiation phenotypes of the cells.

Chapter 3 presents the summary of isotropic gravity effects on hMSCs that the results of this study are capable of proposing an alternative culture process, which possibly maintains the quality of hMSCs during serial cultivation in terms of differentiation capacity into neural lineage. Furthermore, the perspective of implementation of isotropic gravity for further cellular investigations is also be illustrated.

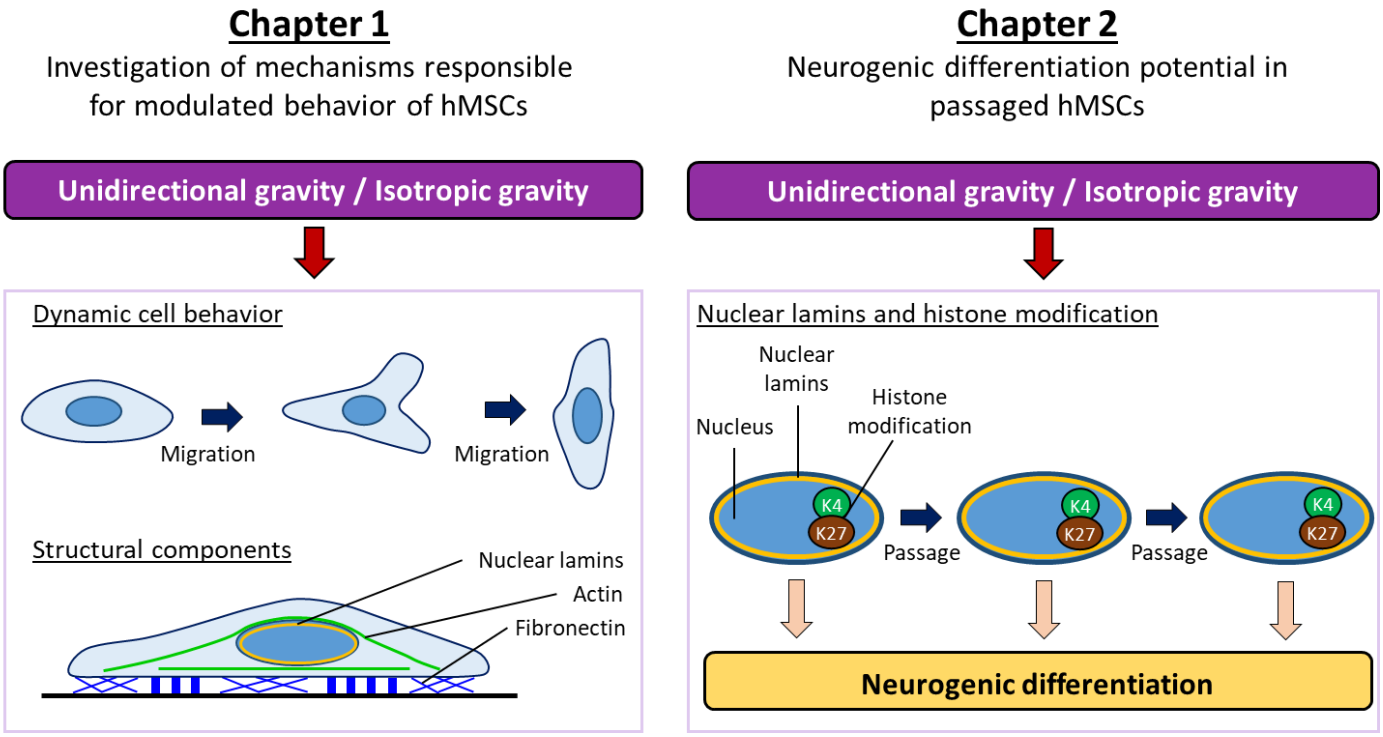

Fig. 3 Schematic diagram showing an outline of this study.

Chapter 1

Investigation of mechanisms responsible for modulated behavior of hMSCs under isotropic gravity culture

1.1 Introduction

Biological living organisms are able to adapt to changes in surrounding physical environment (Albrecht-Buehler, G., 1991; Mesland, D.A.M., 1992). Dynamic movement of cell morphology is greatly affected by the culture boundaries governed by that cells (Lehnert, D. *et al.*, 2004; Gasiorowski, J.Z. *et al.*, 2013). Alterations in cell behavior influence the balance of mechanical loadings at the cell membrane, which is the interaction between intercellular and extracellular components of the cells (Lauffenburger, D.A. *et al.*, 1996; De Pascalis, C. *et al.*, 2017). The biophysical forces on the cell membrane are the major outputs from actin cytoskeleton reorganization together with myosin contraction (Hall, A. *et al.*, 2000). This mechanotransduction occurs between extracellular matrix (ECM) and nucleus via integrin, focal adhesion proteins, cytoskeleton, and linker of nucleoskeleton and cytoskeleton (LINC) complex (Wang, N. *et al.*, 2009). These physical cytoplasmic reactions involve the response of the nucleus in both of nuclear stiffness and several gene transcriptions (Enyedi, B. *et al.*, 2016) due to changes in nuclear lamina organization (Dahl, K.N. *et al.*, 2008). Until now, the effects of altered gravitational culture conditions on migration behavior and mechano-adaptation in the cells, especially in ECM assembly and nucleoskeleton structure are not fully understood and need to be more comprehensively examined.

Therefore, the aim of this chapter was to investigate the influence of isotropic gravity on dynamic cell behavior with rearrangement of actin cytoskeleton and fibronectin assembly in

hMSCs. Moreover, structural organization of nuclear lamina was also evaluated in order to understand the changes in mechanotransduction in the hMSCs cultured under unidirectional gravity conditions and isotropic gravity conditions. This chapter presents the fundamental mechanism of isotropic gravity underlying the biophysical phenomena involving the interaction between ECM and nuclear lamina via reorganization of actin cytoskeleton.

1.2 Materials and methods

1.2.1 Cells and culture conditions

Bone marrow-derived hMSCs (Lot no. 0000374385; Lonza Walkersville Inc., Walkersville, MD, USA) were cultured according to the manufacturer's instructions. Routine subcultures of hMSCs were maintained with hMSC growth medium (Lonza) at 37 °C containing 5% CO_2 in a humidified atmosphere. At 70% confluency, the cells were detached by treatment with a 0.1% trypsin/0.02% EDTA enzymatic solution (Sigma-Aldrich, St. Louis MO, USA) and then cultured in a new culture vessel. In this study, cells at their fifth passage were used the following experiments.

For all experiments in this chapter except staining of F-actin and lamin A/C (Fig. 1-9), hMSCs were expanded for the specified number of days in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) at a depth of 4 mm supplemented with 10% fetal bovine serum (FBS; Life Technologies, Carlsbad, CA, USA) and antibiotics (Life Technologies) in the T-flask culture vessels (Corning, Tewksbury, MA, USA). The seeding density was 5.0×10^3 cells/cm^2 calculated using the number of viable cells that was estimated by a trypan blue exclusion test with a hemocytometer via the direct counting of cell suspension.

In the case of F-actin and lamin A/C staining (Fig. 1-9), cryopreserved bone marrow-derived hMSCs (Lot no. 0000654251; Lonza) at second passage cultured with hMSC growth medium (Lonza) were used for investigation of F-actin and lamin A/C structure.

1.2.2 Cell culture under isotropic gravity conditions

The Gravite®, a culture device used for generating isotropic gravity environment, was produced by Space Bio-Laboratories Co., Ltd. (Hiroshima, Japan). Using a simultaneous rotation with constant angular velocity on two axes, the Gravite® abrogates the cumulative gravity at the center of the machine, producing a simulated microgravity conditions of 10^{-3} G in 8 min after the operation estimated by an attached acceleration sensor in the Gravite®. This is achieved by rotation of the culture chamber located at the center of the device that is possible to disperse the gravity direction uniformly within a spherical boundary. In the culture, on day 0, hMSCs were seeded into a 12.5-cm^2 T-flask with vented filter cap (Corning). After the attachment for a day, the medium was removed and completely filled with fresh medium without any bubbles. Then, the cells were cultured under isotropic gravity environment at the center of the Gravite® until day 3 without any medium change. For the time-lapse observations, a phase contrast microscope with a 10× objective lens has been installed into the Gravite®. After setting position of the culture flask above the objective lens, the machine started rotations and the images were automatically obtained every 10 min during this machine's operation continuously. For control condition, on day 1 after fully filled with fresh medium into the T-flask without any bubbles, the cells were cultured under unidirectional gravity with face-up condition under static environment.

1.2.3 Immunofluorescent staining

Immunostaining was carried out as described previously (Kim, M.H. et al., 2010). Briefly, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS; Wako Pure Chemical Industries, Tokyo, Japan) for 10 min at room temperature. Then, the cells were incubated in PBS with 0.5% polyoxyethylene octylphenyl ether for 10 min. After that the samples were masked the non-specific proteins at 4 °C overnight with Block Ace (Dainippon

Sumitomo Pharma Co., Ltd., Osaka, Japan) and subsequently incubated with primary antibodies at 4 °C overnight. This study used primary antibodies against fibronectin (Santa Cruz Biotechnology, Dallas, TX, USA), total paxillin (Millipore, Billerica, MA, USA), phosphorylated paxillin (Cell signaling technology Inc., Danvers, MA, USA), lamin A/C (Santa Cruz), and lamin B (Abcam, Cambridge, UK). All antibodies were adequately diluted in deionized water containing 10% Block Ace. After primary staining, the samples were washed twice with Tris-buffered saline (TBS; Dako, Glostrup, Denmark) and immunolabeled with the designed secondary antibodies (Alexa Flour 488-conjugated anti-rabbit, anti-mouse, or anti-goat IgG or Alexa Flour 594-conjugated anti-mouse IgG; both from Life Technologies) for 1 h at room temperature. The samples were stained with 4′,6-diamidino-2-phenylindole (DAPI, Life Technologies) and fluorescence-rhodamine-phalloidin (Life Technologies) for nuclei and F-actin, respectively. The fluorescent staining images were taken using a confocal laser scanning microscope (FV-1000; Olympus, Tokyo, Japan) through 60× or 100× objective lenses.

1.2.4 Quantitative real-time reverse transcription–polymerase chain reaction (qRT-PCR)

RNA isolation and qRT-PCR assays were carried out following an referring report (Kim, M.H. et al., 2014). Briefly, total RNA was isolated from the cells using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to company instruction. Then, the isolated RNA was reverse transcribed using a PrimeScript® RT reagent kit (Takara Bio Inc., Shiga, Japan). The qRT-PCR assays were conducted using SYBR® Premix Ex Taq (Takara Bio Inc.) and carried out on a real-time PCR system (Applied Biosystems 7300, Thermo Fisher Scientific, Waltham, MA, USA). This study utilized the following specific primers according to other reports shown as Table 1-1 (Gilles, C. et al., 2001; Ogawa, Y. et al., 2015). Relative mRNA expression of the target genes was evaluated by the cycle threshold value (C_t) and normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using the $2^{-\Delta\Delta Ct}$ method.

Table 1-1 Primer sequences used in gene expression analysis in chapter 1

Genes	Forward sequence (5' → 3')	Reverse sequence (5' → 3')
MT1-MMP	CCATTGGGCATCCAGAAGAGAGC	GGATACCCAATGCCCATTGGCCA
Fibronectin	GGTGACACTTATGAGCGTCCTAAA	AACATGTAACCACCAGTCTCATGTG
GAPDH	CAACGGATTTGGTCGTATTGG	GCCATGGGTGGAATCATATTG

1.2.5 Protein extraction and western blot analysis

Protein extraction and western blot analysis were conducted following a referring report (Kim, M.H. et al., 2014). Briefly, cells were rinsed with ice-cold PBS twice and then total protein in the cells was extracted using RIPA lysis buffer (Sigma-Aldrich) and a Halt™ protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific) on ice for 30 min. Protein concentration was estimated using a BCA protein assay kit (Thermo Fisher Scientific). Equal amounts of total lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to immun-Blot low fluorescence PVDF membrane (Bio-rad, Hercules, CA, USA). The membrane was blocked with ECL™ blocking agent (GE Healthcare, Chicago, IL, USA) for 1 h at room temperature and then incubated with primary antibodies against total paxillin (Millipore), phosphorylated paxillin (Cell signaling technology), and GAPDH (Sigma-Aldrich) overnight at 4 °C. Later, the membrane was incubated with secondary antibodies, DyLight® 800 anti-Rabbit IgG (Thermo Fisher Scientific), Starbright™ blue 700 anti-mouse IgG (Bio-Rad), for 1 h at room temperature. Fluorescent signals were displayed with a ChemiDoc™ MP Imaging system (Bio-Rad). The optical intensity of protein signals was quantified using an image processing and analytical software (Image Lab™, Bio-Rad).

1.2.6 Statistical analysis

All quantitative data were obtained from triplicate samples, which are expressed as the means ± standard deviation (SD). Comparisons between the groups were evaluated by one-way analysis of variance (ANOVA) and the Tukey's honest significance test. *P*-values less than 0.01 were considered statistically significant.

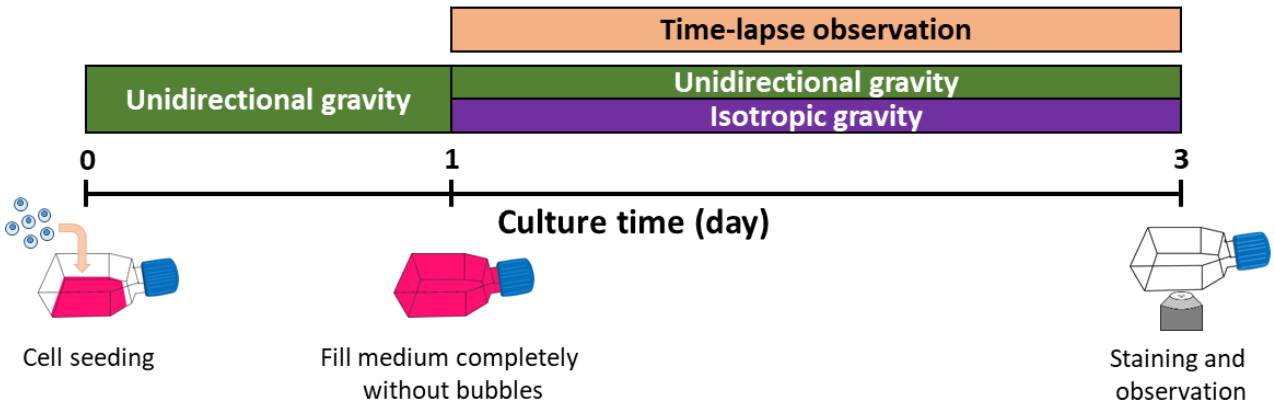

Fig. 1-1 Schematic diagram showing an experimental plan in chapter 1.

1.3 Results

1.3.1 Changes in behavior of hMSCs

To examine the influence of isotropic gravity on dynamic morphological changes in hMSCs, the cells were cultured under unidirectional gravity conditions for a day. Then, a Gravite® was used to generate isotropic gravity conditions in the culture environment, and the changes in cell behavior were monitored. Notably, the time-lapse observing images show that the cells presented dynamic protrusions and extensions of cell boundary during the cultures under both gravitational conditions. Cells cultured under unidirectional gravity conditions presented the spindle-shaped cells with unidirectional migration through extension of the leading edge following by contraction of the trailing edge (Fig. 1-2A). On the other hand, cells cultured under isotropic gravity conditions showed multidirectional migration by active

extension of leading edge with partial contraction of cell rear (Fig. 1-2A). Furthermore, cells under isotropic gravity conditions exhibited higher frequency of directional changes of migration than those under unidirectional gravity culture conditions (Fig. 1-2B).

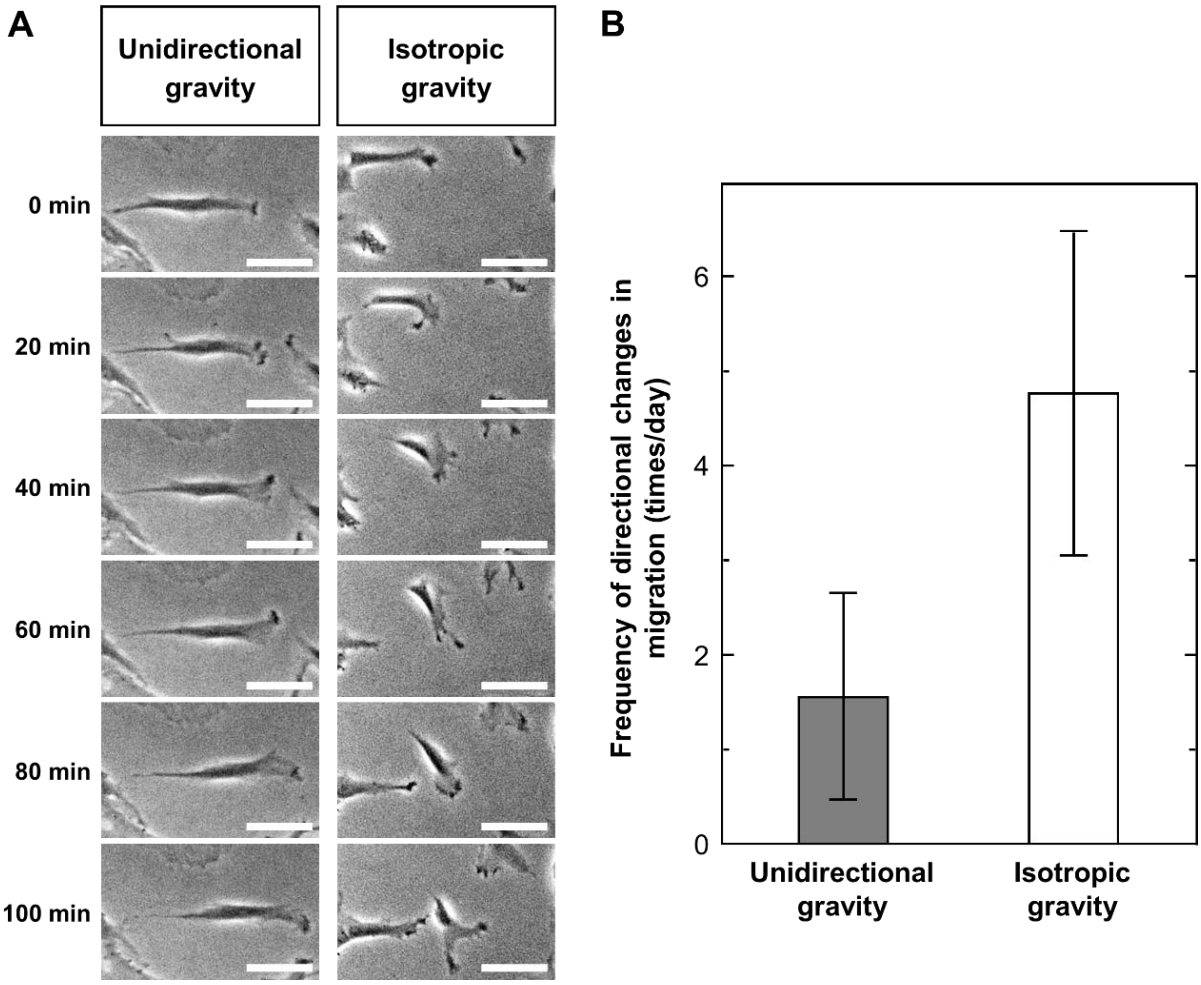

Fig. 1-2 Representative snapshot images showing migration behavior of hMSCs under unidirectional gravity conditions and isotropic gravity conditions (A). Scale bars: 50 μm. Quantitative analysis of cell migration having directional changes in the movement evaluated from time-lapse observations (B). The directional change in migration was defined by a cell movement with change in the direction that was greater than 90 degrees from its initial direction (n = 20).

1.3.2 Cytoskeletal structure and focal contact formation

To clarify actin cytoskeletal structure, hMSCs cultured under unidirectional gravity conditions and isotropic gravity conditions were fluorescent stained with F-actin at days 1 and 3 of the cultures. The cells under unidirectional gravity culture conditions on day 1 (Fig. 1-3A) and day 3 (Fig. 1-3B) had longitudinal actin stress fibers along both of the apical side and basal side. However, the cells grown under isotropic gravity conditions showed broader cortical actin fibers in the filopodia and lamellipodia regions and had few stress fibers mainly located at the basal side but not apical side (Fig. 1-3C). Furthermore, paxillin, a focal contact protein, was also investigated in cells under both gravitational conditions at the same time points. These observing results indicate that paxillin spots located at the central and peripheral regions of the cells cultured under both gravitational conditions, with intensive staining at the end of stress fibers with the longitudinal direction in a scattered manner. In addition, cells under isotropic gravity conditions showed more paxillin aggregation, especially at the leading edges of the cells (Fig. 1-3C). In contrast, cells cultured under unidirectional gravity conditions exhibited the dispersion of paxillin at both of the cell edges and bodies (Fig. 1-3A, 1-3B).

To examine dynamics of focal contact protein at the migration state, hMSCs on day 3 were evaluated by immunostaining and western blot analysis of total paxillin and phosphorylated paxillin. Based on immunostaining observations, cells cultured under unidirectional gravity conditions, phosphorylated paxillin was mainly co-localized with paxillin in the central and peripheral regions (Fig. 1-4A). However, cells cultured under isotropic gravity conditions had phosphorylated paxillin with more intensive formation at the cell edges (Fig. 1-4B1) compared with those grown under unidirectional gravity conditions (Fig. 1-4A1). In the case of western blot analysis, although the relative fluorescent levels of total paxillin against GAPDH were not significantly different between two culture conditions. Nevertheless, average intensity ratio of phosphorylated paxillin per total paxillin in cells cultured under isotropic

gravity conditions was significantly higher than those grown under unidirectional gravity conditions (Fig. 1-4C and Fig. 1-4D), which demonstrates the enhancement of paxillin phosphorylation at edges of the cells under isotropic gravity conditions.

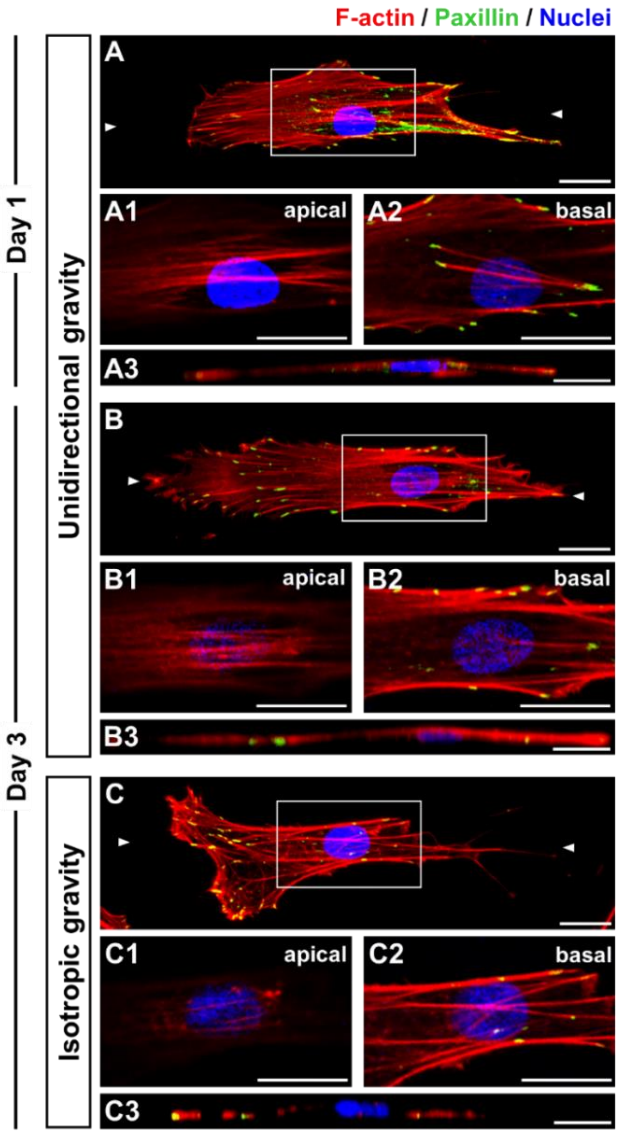

Fig. 1-3 Actin cytoskeleton structure and focal adhesion formation in hMSCs. Representative images showing F-actin (red) and paxillin (green) organization as well as the nuclei (blue) in cells on day 1 (A) and day 3 (B) under unidirectional gravity conditions and on day 3 in cells cultured under isotropic gravity conditions (C). Panels A1–C2 present enlargements of the boxed areas in panels A–C, respectively. Panels A1, B1, and C1 show the apical sides, while panels A2, B2, and C2 present the basal sides. Panels A3, B3, and C3 indicate the vertical cross-sectional views between the arrows in panels A–C, respectively. Scale bars: 20 μm.

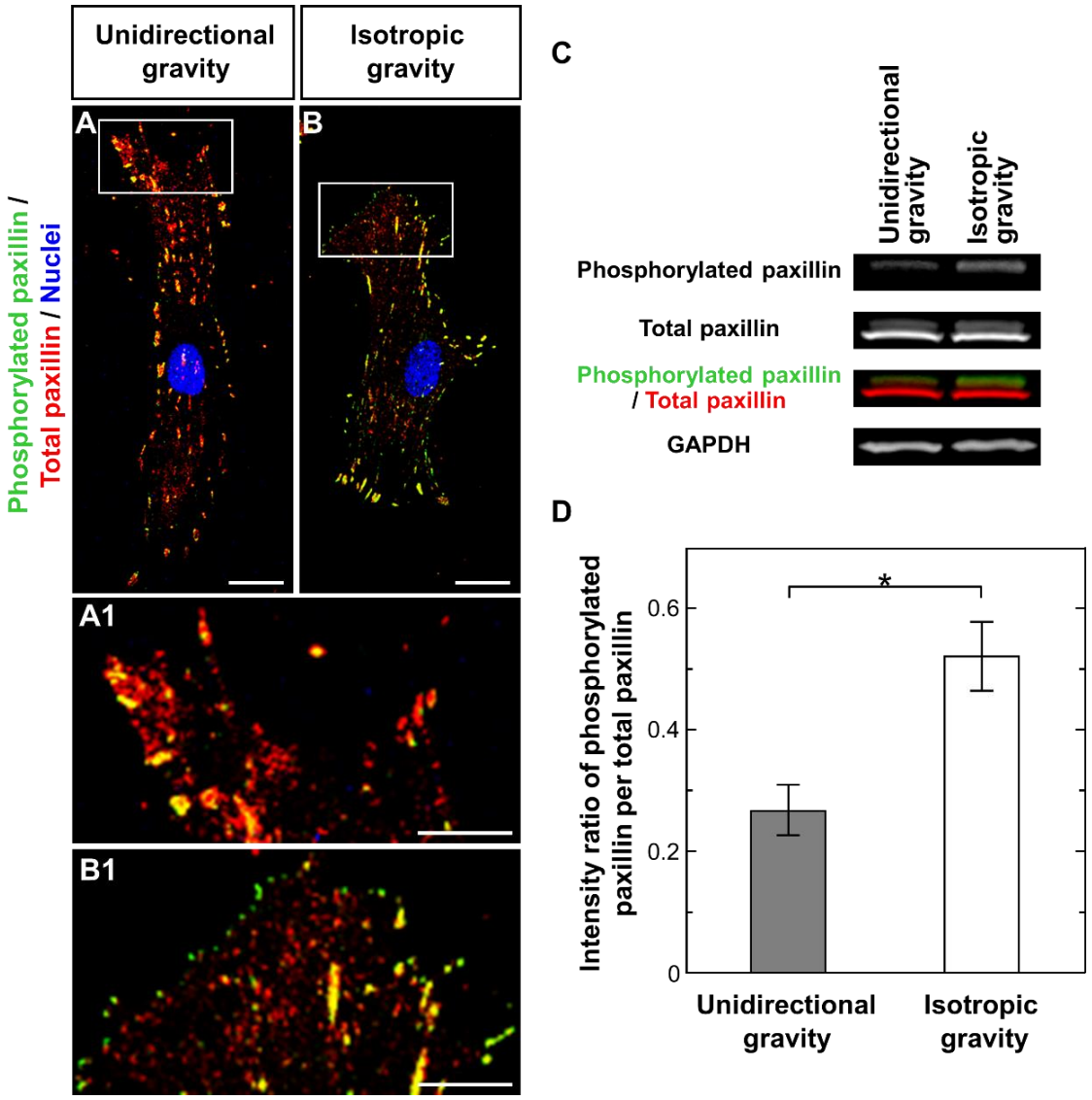

Fig. 1-4 Phosphorylation of focal contact protein in hMSCs. Representative images showing phosphorylated paxillin (green), total paxillin (red), and nuclei (blue) on day 3 in cells cultured under unidirectional gravity conditions (A) or isotropic gravity conditions (B). Panels A1 and B1 show enlargements of the boxed areas in panels A and B, respectively. Scale bars: 20 μm (A and B) and 10 μm (A1 and B1). Representative images of western blot analysis of total paxillin and phosphorylated paxillin levels in hMSCs on day 3 (C). Quantitative analysis of level ratio of phosphorylated paxillin to the total paxillin in hMSCs on day 3 (D) (n = 3). *p < 0.01.

1.2.3 Fibronectin assembly and activity of enzymatic matrix degradation

Fibronectin assembly and actin cytoskeletal formation were observed by fluorescent staining of fibronectin and actin cytoskeleton in the cells grown under both gravitational culture conditions at day 1 and day 3 as adhesion state and migration stage, respectively. In both culture types of gravity-treated cells, concentrated fibronectin was detected at the interface between the cells and culture surface. On day 1, fibronectin with fibril structures were not observed at the region of cell periphery (Fig. 1-5A1), but few were detected in the longitudinal direction under cell bodies with stress fibers (Fig. 1-5A2). On day 3, the cells cultured under unidirectional gravity conditions had fibronectin fibrillogenesis under the cell bodies indicated by an increase of fibronectin fibrils (Fig. 1-5B2) from those on day 1 (Fig. 1-5A2). On the other hand, cells cultured under isotropic gravity conditions on day 3 were observed with fewer fibronectin fibrils under the cell bodies (Fig. 1-5C2), but exhibiting more aggregated structure than those grown under unidirectional gravity conditions (Fig. 1-5B2). In addition, cells cultured under isotropic gravity conditions had significantly lower mRNA expression of fibronectin than the cells grown under unidirectional gravity conditions (Fig. 1-6A).

To investigate activity of enzymatic matrix degradation, the expression of membrane-type-1 matrix metalloproteinase (MT1-MMP or MMP14), which is a surface enzyme responsible for fibronectin degradation, was evaluated in cells grown under both gravitational culture conditions at days 1 and 3 by qRT-PCR assay. As shown in Fig. 1-6B, the relative mRNA expression of MT1-MMP in cells cultured under unidirectional gravity conditions was significantly lower on day 3 than their expression on day 1. However, cells cultured under isotropic gravity conditions presented the significantly higher MT1-MMP expression on day 3 than that detected on day 1. Moreover, MT1-MMP expression in cells on day 3 cultured under isotropic gravity conditions was approximately 3-fold higher than those grown under unidirectional gravity culture conditions.

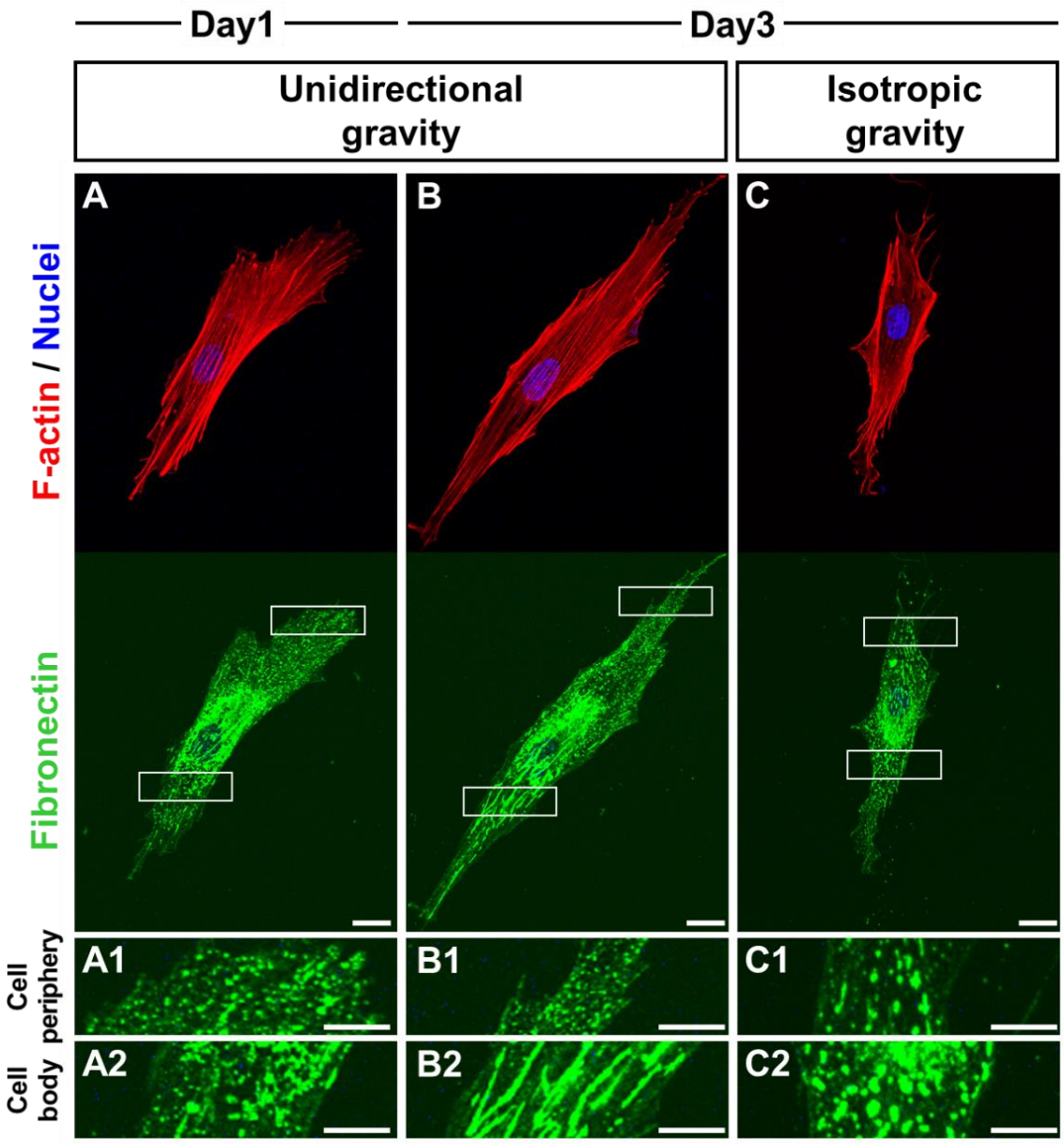

Fig. 1-5 Fibronectin assembly and cytoskeleton formation in hMSCs. Representative images showing F-actin (red), fibronectin (green), and nuclei (blue) on day 1 (A) and day 3 (B) in cells cultured under unidirectional gravity conditions and on day 3 in cells cultured under isotropic gravity conditions (C). Panels A1–C2 present enlargements of the boxed areas in panels A–C, respectively. Panels A1, B1, and C1 show the cell peripheries, while panels A2, B2, and C2 present the cell bodies. Scale bars: 20 μm (A–C) and 10 μm (A1–C2)

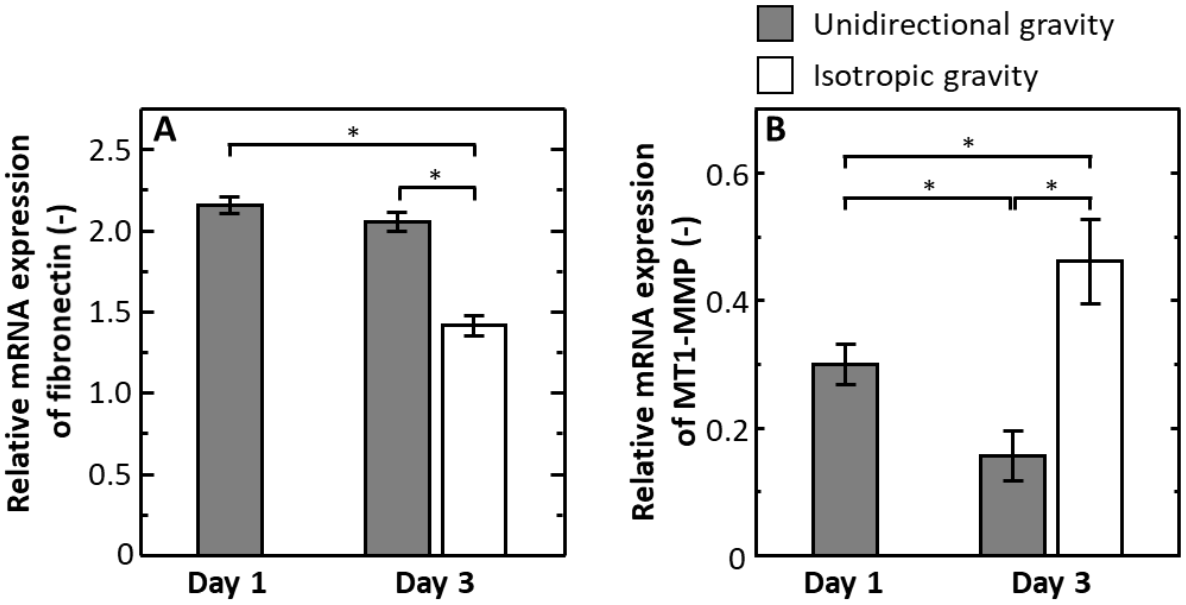

Fig. 1-6 Quantitative RT-PCR analysis of fibronectin (A) and MT1-MMP (B) expressions in hMSCs cultured under unidirectional gravity conditions (shaded bars) on day 1 and day 3 as well as isotropic gravity conditions (open bar) on day 3. (n = 3). *p < 0.01.

1.3.4 Structural organization of nuclear lamina

This study further investigated the structural organization of nuclear lamins (types A/C and B), which are major components of the nuclear lamina in the nuclei of cells on day 3 cultured under both gravitational conditions. Immunostaining observations show that lamin A/C and lamin B were detected mostly at the periphery of the nuclei in all culture conditions (Fig. 1-7, Fig. 1-8). However, cells cultured under isotropic gravity conditions presented more concentrated lamin B at periphery than those grown under unidirectional gravity conditions. Cells cultured under unidirectional gravity conditions showed lamin A/C mainly located at the apical side, indicating a basal-to-apical polarization of lamin A/C (Fig. 1-7A1a). Remarkably, cell cultured under isotropic gravity had lamin A/C localized on both apical and basal sides (Fig. 1-7B1a), suggesting that polarization of lamin A/C has less occurrence in the cells cultured under isotropic gravity conditions.

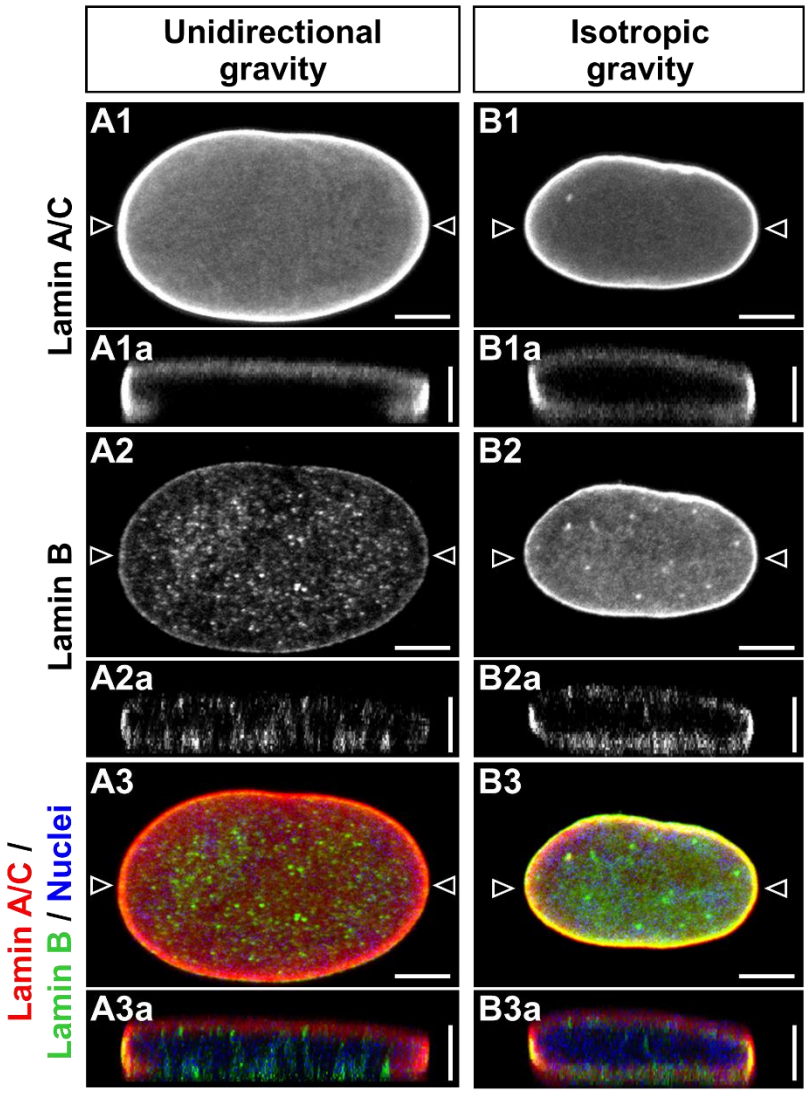

Fig. 1-7 Structural organization of nuclear lamins in hMSCs. Representative images shows lamin A/C (panels A1-B1) and lamin B (panels A2-B2) as well as the merged images (panels A3 and B3) represent lamin A/C (red), lamin B (green), and nuclei (blue) on day 3 in cells cultured under unidirectional gravity conditions (A1 - A3) or isotropic gravity conditions (B1-B3). Panels A1a-B3a indicate the vertical cross-sectional views between the arrows in panels A1 - B3, respectively. Scale bars: 5 μm.

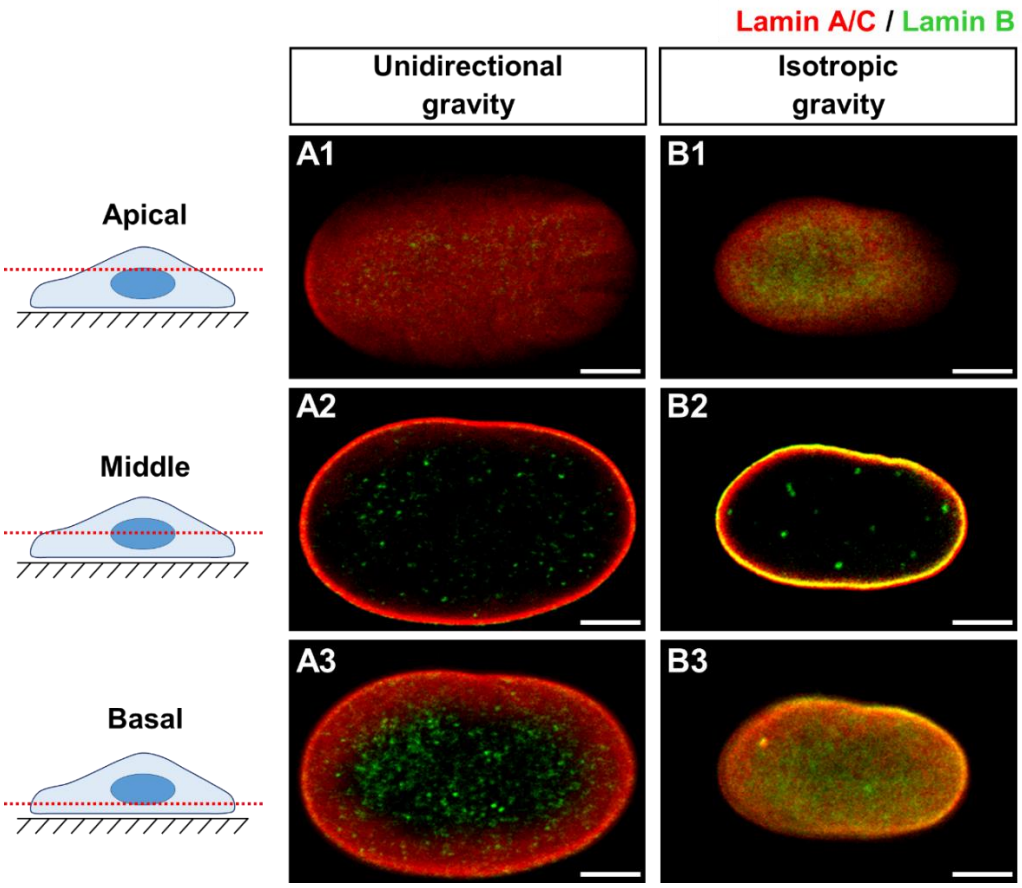

Fig. 1-8 Vertical distribution of nuclear lamins in hMSCs. Representative images show lamin A/C (red) and lamin B (green) in cells on day 3 cultured under unidirectional gravity conditions (A1-A3) or isotropic gravity conditions (B1-B3). Panels A1/B1, A2/B2, and A3/B3 present the apical, middle, and basal sides of the nucleus, respectively. Scale bars: 5 μm.

To confirm, the relationship between apical actin stress fibers and a basal-to-apical polarization of lamin A/C, fluorescent staining of F-actin and mechanosensitive nuclear lamin A/C was conducted in cells cultured under unidirectional gravity and isotropic gravity for 3 days. Cells cultured under unidirectional gravity under face-up condition and upside-down condition exhibited apical stress fibers over the nucleus together with appearance of a polarization of lamin A/C. However, cells grown under isotropic gravity conditions mostly presented less apical stress fibers that mainly along the basal side, with fewer cells having a polarization of lamin A/C than those grown under unidirectional gravity conditions (Fig. 1-9).

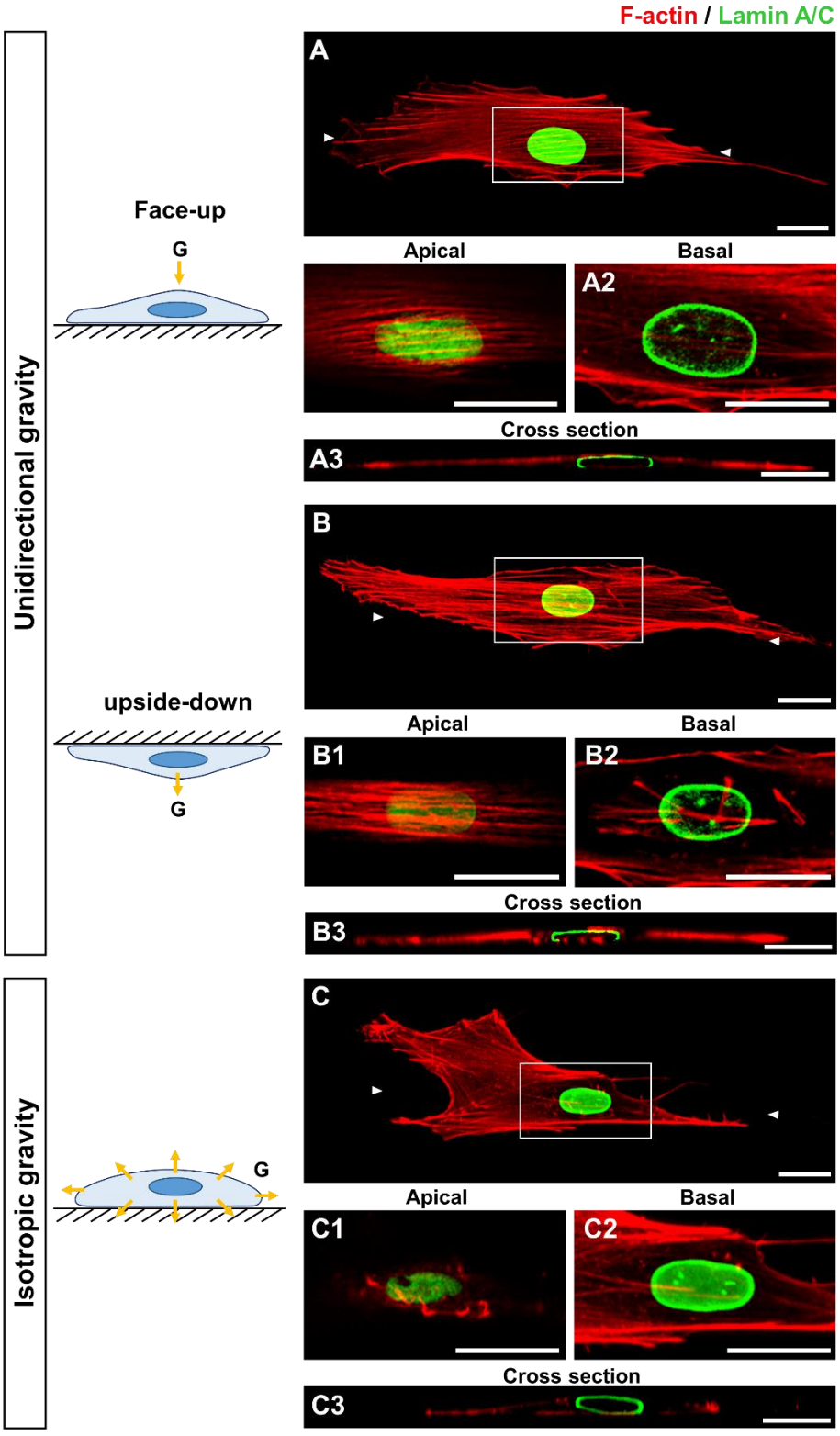

Fig. 1-9 Cytoskeleton and nucleoskeleton organization in hMSCs on day 3. Fluorescent staining images show F-actin (red) and lamin A/C (green) in the cells cultured under unidirectional gravity under face-up condition (A), upside-down condition (B) or isotropic gravity conditions (C). Panels A1-C2 present the enlargements of the boxed areas [Continued on the next page]

1.4 Discussion

Alterations in culture environment surrounding the cells are extensively noted to influence their behavior. However, the mechanism of cellular adaption to the different gravitational conditions has been largely unknown. Cells have an attachment and spreading to the culture surface by interacting with the adsorbed surface proteins via integrins that stabilizes the cell adhesion (Doyle, A.D. *et al.*, 2012). The interactions between integrins and surface protein controlled by surface adsorption and conformation of ECM proteins such as fibronectin (Bergkvist, M. *et al.*, 2003). Thereby, variations in mechanical surface properties or structural organization of ECM affect the generation of cellular tractional forces at the integrins (Ingber, D.E., 2006). Adhesion between cells and culture substrate are involved in cell migration due to formation and turnover of focal contact proteins as well as remodeling of cytoskeleton formation (Gardel, M.L. *et al.*, 2010). Phosphorylation of paxillin conducts formation of active lamellipodium protrusions (Zaidel-Bar, R. *et al.*, 2007) and is frequently used to indicate the dynamics of focal contact turnover (Nagano, M. *et al.*, 2012). Several researchers have documented that degradation of ECM is mediated by various factors including activity of matrix metallopeptidase (MMP) (Westermarck, J. *et al.*, 1999). Notably, the dynamics and distribution of focal contacts regulated by MT1-MMP (Bravo-Cordero, J.J. *et al.*, 2016) that the MT1-MMP plays as an important role in fibrillar fibronectin degradation (Shi, F. *et al.*, 2011). Moreover, cellular response through the interaction between ECM, focal adhesion, cytoskeleton, and nucleus is one of the most important consideration for mechanotransduction systems in the cells (Wang, N. *et al.*, 1993).

Fig. 1-9 [Continued from previous page] in panels A-C, respectively. Panels A1–C1 showing the apical sides, while panels A2-C2 present the basal sides. Panels A3-C3 indicate the vertical cross-sectional views between the arrows in panels A-C, respectively. Scale bars: 20 μm.

Nuclear lamins are widely known to interface membrane-associated proteins to form nuclear lamina at the inner of nuclear membrane (Vlcek, S. *et al.*, 2007). Actin cytoskeleton, which is linked to the nuclear lamina associated by LINC complex, has recently been reported to affect the remodeling of chromatin structure and expressions of transcriptions (Graham, D.M. *et al.*, 2016). The biophysical connection provides a structural fundamental for mechanical transduction influencing the chromatin compaction via the actin cytoskeleton (Gieni, R.S. *et al.*, 2008). Current reports also have provided the critical information about the significant contribution of nuclear lamins to mechanical properties and genome organization in the nucleus (Lammerding, J. *et al.*, 2004; Swift, J. *et al.*, 2013; Kim, J.K. *et al.*, 2017). For example, nuclear lamin A/C contributes to nuclear stiffness (Lammerding, J. *et al.*, 2006; Chen, L. *et al.*, 2018) and is influenced by apical actin formation (Kim, J.K. *et al.*, 2017), which is an essential aspect of signalings from mechanical loadings between the ECM and nucleus (Kim, D.H. *et al.*, 2015). Notably, Ihalainen et al. (Ihalainen, T.O. *et al.*, 2015) have well documented that a basal-to-apical polarization of lamin A/C is mediated by compression to the nucleus. This information presents that differential basal-to-apical polarization of lamin A/C epitopes in the nuclear lamina are controlled by mechanical forces in response to environmental changes due to alteration in actin cytoskeletal contraction.

This study investigated changes in cytoskeleton and nuclear lamina in hMSCs cultured under different gravitational conditions. Fig. 1-10 shows a schematic diagram about the effects of isotropic gravity on cell-surface interfaces with changes in fibronectin assembly, construction of focal adhesions, and formation of actin cytoskeleton network. The recent culture system for simulation of microgravity environment, 3D-clinostat (Gravite®), has enabled the study of the effects of gravity on cell behavior. In unidirectional gravity culture condition, Cells had a stretched morphology and unidirectional migration (Fig. 1-2 A) with the maintenance of their spindle-shape via fibronectin fibril formation in their bodies and stabilization of focal

adhesions with enriched apical actin stress fibers (Fig. 1-3 and Fig. 1-5). On the other hand, cells cultured under isotropic gravity conditions had a multidirectional migration with active extension of leading edge (Fig. 1-2 A) and were partially contracted as well as more degradation of fibronectin fibrils at the cell rear (Fig. 1-3 and Fig. 1-5). These phenomena were supported by immunostainings of phosphorylated paxillin and total paxillin, which presented the higher intensity ratio of the phosphorylated form at cell edges in isotropic gravity conditions (Fig. 1-4). The results of this study suggest that cells cultured under isotropic gravity have active extension of their leading edges together with the inhibition of fibronectin fibrillogenesis by up-regulation of MT1-MMP expression (Fig. 1-6B). This enzymatic activity causes the release of focal adhesions and suppress maturation of stress fiber formation, which influence on the migration behavior, similarly to previously findings of hMSCs grown on dendrimer-immobilized surfaces (Ogawa, Y. *et al.*, 2015). Moreover, another similar phenomenon is also reported in the study on osteoblastic cells that microgravity environment affected their fibronectin assembly (Guignandon, A. *et al.*, 2014). In addition, the promotion of phosphorylated paxillin mediated active cell migration together with turnover of focal contacts have also been documented (Wozniak, M.A. *et al.*, 2004). Accordingly, based on the results of this study and those of other related studies, isotropic gravity has induced the changes in fibronectin assembly by up-regulation of MT1-MMP expression, which the fibronectin is mainly responsible for the construction of focal adhesions and formation of cytoskeleton by cell-substrate interactions. This activation of focal adhesion could determine cell behavior by initiation of intracellular signaling cascades such as signaling by Rho family small GTPases (Boudreau, N.J. *et al.*, 1999). To be acknowledged, this is the first time to investigate the effects of isotropic gravity on the behavior of hMSCs together with illustration of altered cellular mechanism involving with modulation in ECM assembly, stabilization of focal adhesions, and cytoskeleton reorganization that respond to the gravitational environment.

This chapter also reports the alteration in nuclear lamina-cytoskeleton interaction in hMSCs mediated by isotropic gravity-driven fibronectin assembly. This study found apical stress fibers together with a basal-to-apical polarization of lamin A/C in hMSCs cultured under unidirectional gravity conditions (both of face-up and up-side down conditions) but these apical actin stress fibers and the polarization was much lower in cells that were cultured under isotropic gravity conditions (Fig. 1-9). It is plausible that apical actin stress fibers that link to apical site of the nuclear surface, continuously generate compressive forces on the nucleus. Inhibition of apical actin stress fiber formation decreases the level of compressive pressure applied to the nuclear lamins and enforces polarization of lamin A/C meshwork at apical site to remodel back to less tightly packed state as non-polarization (Kim, D.H. *et al.*, 2015). Owing to other report (Ihalainen, T.O. *et al.*, 2015), it is possible that alternations of surrounding environments including gravity, might induce changes in the cytoskeleton-nuclear lamina interactions via the modification of ECM-cytoskeleton organization. These current results present that isotropic gravity environment induces changes in mechanotransduction in hMSCs through the loss of lamin A/C polarization due to disruption of the apical stress fiber formation. Alteration in mechanotransduction in hMSCs observed under isotropic gravity conditions seemed to be similar to the concept of "force isotropy" documented by Nava et al showing that the cytoskeletal forces were relevant to the extracellular loadings (Nava, M.M. *et al.*, 2012). According to their work, isotropic tension in cytoskeleton is mediated by focal adhesions and traction forces in cytoskeleton with similar magnitude at varying orientation, indicating roundish nuclear morphology. Therefore, it is likely that isotropic gravity induces isotropic properties which demonstrates an isotropic force distribution in term of position and momentum in the cells. For further investigation on the applications of isotropic gravity in cell-based therapy, the next chapter will present the influence of altered mechanotransduction by isotropic gravity on epigenetic modification and differentiation potential in passaged hMSCs.

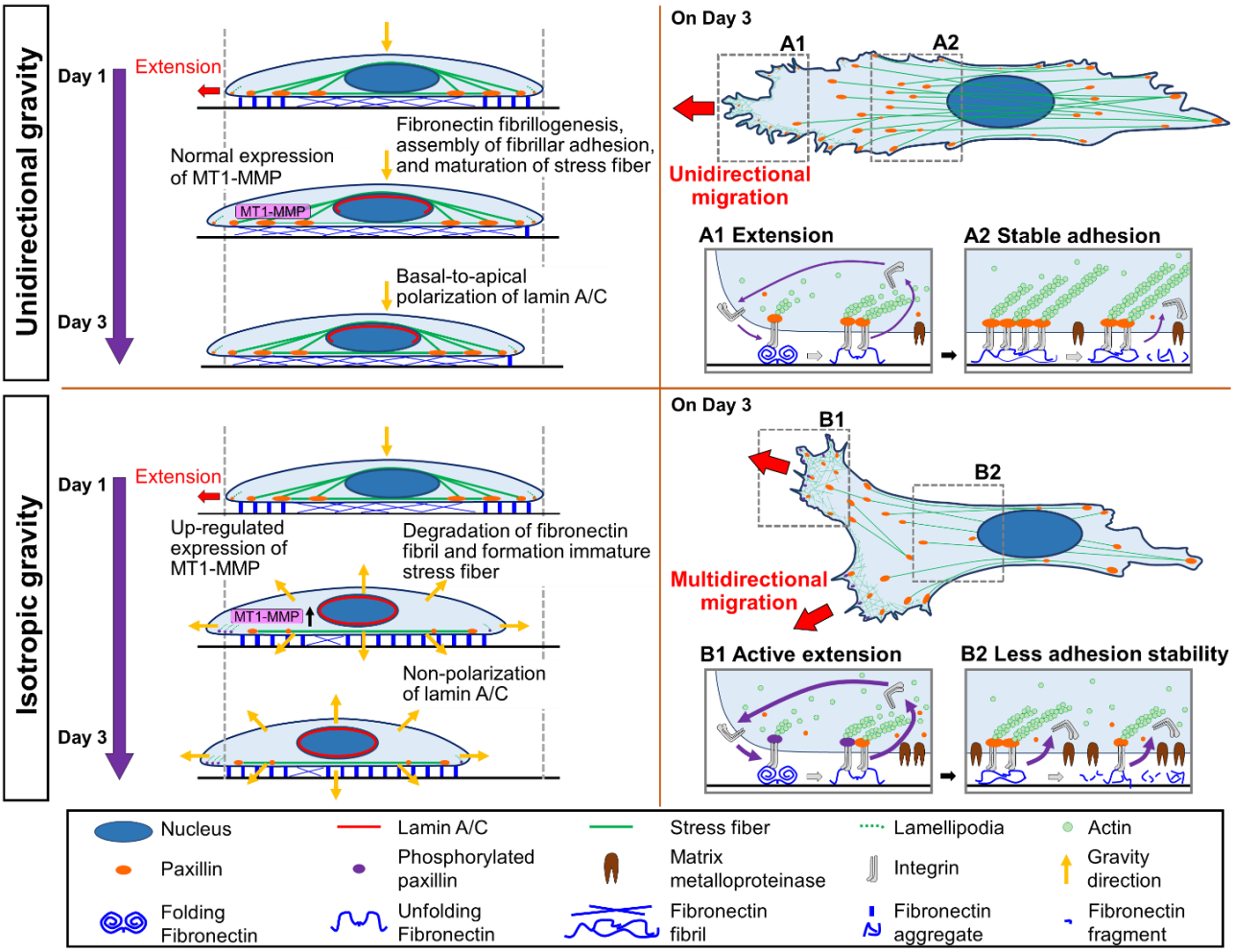

Fig. 1-10 Schematic illustration showing the processes of fibronectin assembly, focal adhesion construction, and actin cytoskeleton formation together with structural changes in the nuclear lamina in hMSCs suggested by the finding in this chapter. When cells cultured under unidirectional gravity conditions, the cells undergo unidirectional migration with extension at the cell edge (A1) and show the stable adhesion of the cell body along with fibronectin fibril formation, focal adhesion assembly, and stress fiber maturation (A2). These characteristics subsequently influence on a basal-to-apical polarization of lamin A/C. However, cells cultured under isotropic gravity conditions showed multidirectional migration with active extension of the cell edge (B1) and have less adhesion stability due to fibronectin aggregation resulting from the upregulation of MT1-MMP expression (B2). These changes suppressed focal adhesion assembly and caused formation of immature stress fiber influencing to non-polarization of lamin A/C due to lack of mechanotransduction in the cells.

1.5 Summary

This chapter presents the influence of isotropic gravity on mechanism of modulation in cell behavior with changes in cytoskeletal and nucleoskeletal organization in hMSCs. These results indicate that the observed changes in migration are largely occurs due to disruption of the fibronectin assembly. The altered fibronectin structure induced by MMPs are widely known to play an important role in the reorganization of cell-substrate interactions, the subsequent remodeling of actin cytoskeleton and changes in nucleoskeleton structure. Taken together, this study provides significant insight into the functional mechanism of gravity that affects cell behavior and mechanotransduction in hMSCs.

Chapter 2

Neurogenic differentiation potential in passaged hMSCs under isotropic gravity culture

2.1 Introduction

Human mesenchymal stem cells (hMSCs) have self-renewal and differentiation capacities that are important characteristics to apply in tissue engineering and regenerative medicine (Ullah, I. *et al.,* 2015). Differentiation potentials of hMSCs into multiple lineages have promoted the possibility of autologous transplantation of hMSCs as therapy for human diseases (Bang, O.Y. *et al.,* 2005). Central nervous system (CNS), composed of brain and spinal cord, is the most important of regulation panel in human body. CNS disorders including neurodegenerative diseases, traumatic brain injury (TBI), spinal cord injury (SCI), and stroke present significant therapeutic challenges because an effective treatment is rarely achieved due to limitation of their regeneration (Kim, S.U. *et al.*, 2009). An utilization of human embryonic stem cells (hESCs) has been suggested as one of the novel techniques for treatment of neurological diseases due to their great ability in differentiation (Lee, H. *et al.,* 2007). However, there has been concerns about the risk of immune rejection (Barker, R.A. *et al.*, 2004), limited availability of the cells, and ethics (McLaren, A., 2001) that were the major barriers for their clinical applications. As hMSCs that have multipotency to differentiate into ectodermal lineage such as neurons and secretion of signaling molecules, many researchers have presented that hMSCs could be used as autologous transplantation to patients with middle cerebral artery occlusion, which improved functionality and lessened cerebral atrophy (Bang, O.Y. *et al.,* 2005). Moreover, many attempts to cure the CNS disorders by using hMSCs have been

subsequently reported (Cho, S.R. *et al.*, 2009; Joyce, N. *et al.*, 2010; Momin, E.N. *et al.*, 2010) and some examination presented that cell-based therapy had better functional outcomes than drug therapy in clinical trials (Lindvall, O. *et al.*, 2010; Lo Furno, D. *et al.*, 2018). As a result, hMSCs present an impressive potential in cell-based therapy of neurogenic disorders that could improve the performance and quality of therapeutic applications.

The properties of expanded hMSCs is one of the essential aspects to consider in order to assess the efficacy of their therapeutic applications for the treatment of human illnesses (Ikebe, C. *et al.*, 2014). However, during *in vitro* expansion under unidirectional gravity culture conditions, hMSCs with higher passage numbers tend to lose their proliferation ability and capacity of multilineage differentiation (Kretlow, J.D. *et al.*, 2008; Wagner, W. *et al.*, 2008; Zaim, M. *et al.*, 2012), as well as a reduction in neurogenic gene expressions in the differentiated neural cells (Shall, G. *et al.*, 2018). Moreover, the differentiation of hMSCs into neurogenic lineage is still challenging point to make the high level of the differentiation comparing to the other differentiation into mesodermal lineage (Zhang, Q. et al., 2018). Due to these phenomena, alternative culture system with appropriate culture strategies should be developed for cell preparation and maintenance, which allow them to preserve their undifferentiated state or direct the differentiation into the desired fate.

According to the understanding of isotropic gravity effects on cellular adaptation in hMSCs presented in chapter 1, the physical culture environment is well known to be a critical signal that leads to cellular growth and differentiation (Guilak, F. *et al.*, 2009). Since the primary examination in the micro gravitational space environment, a number of specific instruments have been developed for cell culture system under the microgravity conditions (Kern, V.D. *et al.*, 2001). The growing investigations on the cellular adaptation to altered gravity environment has shown changes in perception and transduction of biophysical loadings via networks of actin filaments, and also the alterations in the activity of enzyme kinetics and

self-assembly of protein networks (Bizzarri, M. *et al.*, 2014). Mechanical forces occurring at intercellular and intracellular regions affect the response of cell nucleus due to transduction of the mechanical signalings via actin cytoskeleton and lamin nucleoskeleton, which influence the genome expression and organization (Stephens, A.D. *et al.*, 2017).

Modification in epigenetic marks in chromatin, such as histone methylation, is well known to play an essential role in the promotion or inhibition of specific transcriptional pathways without any changes in the DNA sequence (Bonasio, R. *et al.*, 2010). Histone methylation at promoter region is a critical point of the initiation state for transcription that controls the expression of promoter-related gene (Li, B. *et al.*, 2007). Therefore, this epigenetic modification is one of the important factors in the regulation of various cellular processes cascaded with numerous cellular phenotypes (Jaenisch, R. *et al.*, 2003). Moreover, microgravity exposure has been reported to affect chromatin re-modeling, which contributes to alteration in gene expressions (Casey, T. *et al.*, 2015). Nonetheless, mechanism of gravity cues to epigenetic modification is still unclear. The mechanotransduction from ECM to nucleus via cytoskeleton and nucleoskeleton may be an essential regulator for remodeling of chromatin structure by histone methylation in cells.

Recently, a 3D-clinostat is one of the common culture machines that have been used for simulating the microgravity culture conditions (Herranz, R. *et al.*, 2013) by isotropic gravity environment, and it has been shown to be an effective process on ground for attenuating effects of gravity similar to space-flight experiments (Tabony, J. *et al.*, 2007). Furthermore, the epigenetic mechanism of histone modification, which is inherited from cell expansion under isotropic gravity conditions, is still remaining elusive (Najrana, T. *et al.*, 2016). In addition, until now, it still requires better understanding about influence of passage culture during growth expansion of hMSCs on their differentiation capacity into neurons. Neurofilament heavy (NF-H) and microtubule-associated protein 2 (MAP2) are neuron-specific proteins in mature

neurons, which NF-H is a neuroskeleton supporting the transmission of electrical impulse, and MAP2 does the function as stabilization of microtubule for transporting the neurotransmitters (Paziana, K. *et al.*, 2015). Therefore, the objective of this chapter was to investigate the effects of isotropic gravity on neurogenic differentiation potential of passaged hMSCs during growth expansion culture. Moreover, this chapter also discussed the contribution of epigenetic memory of histone modifications on neurogenic differentiation capacity of hMSCs during serial cultivation. The results of this chapter are capable of proposing the alternative culture process, which could maintain the quality of hMSCs during passage culture for therapeutic applications with effective neurogenic differentiation.

2.2 Materials and methods

2.2.1 Cells and culture conditions

Human bone marrow-derived mesenchymal stem cells (Lot no. 0000654251; Lonza) were thawed and cultured accordingly to the manufacturer's protocol in hMSC growth medium (Lonza) at 37 °C under 5% CO_2 in a humidified atmosphere. At 70% confluency, the cells were enzymatically detached with a 0.1% trypsin/0.02% EDTA solution (Sigma-Aldrich). Then, the cells were seeded at a density of 4.0×10^3 cells/cm^2 in every passage of growth culture and a viable cell number was estimated by counting of suspended cells with a hemocytometer, by a trypan blue dye exclusion test.

For isotropic gravity culture, a 3D clinostat, Gravite® (Space Bio-Laboratories Co., Ltd.), was used which generates a simultaneous rotation on two axes with constant angular velocity. At the center of the rotating frames, the Gravite® cancels out the cumulative gravity and simulates microgravity environment of 10^{-3} G in 8 min after the operation estimated by an installed acceleration sensor. Thus, this is accomplished to scatter the gravitational vector identically within a spherical boundary during the cultures.

In every passage of growth culture, on day 0, hMSCs were seeded into a 12.5-cm^2 T-flask with a vented filter cap (Corning) and cultured under unidirectional gravity environment under face-up condition for a day in a manner in which cells are attached on the bottom culture surface. Then, on day 1, the medium was removed from the flask. Subsequently, the flask was fully filled with fresh medium without any bubbles. After that, the cells were cultured under unidirectional gravity conditions with face-up side or isotropic gravity conditions at the center of the Gravite® until day 5 without any medium change. On day 5 of every passage, the cells were subcultured into a new culture vessel, and the cells were cultured continuously for 3 passages for 15 days in total time.

2.2.2 Proliferation ability

In every passage of growth culture, adhesion efficiency and specific growth rate of the passaged hMSCs were evaluated for estimation of proliferation ability. After seeding for 24 h, the cells attached on the culture surface were monitored via a phase-contrast microscope with magnification at 4x. The attachment efficiency, α (-), was calculated as follows: $\alpha = X_{24}/X_0$, where X_{24} is the cell density after seeding for 24 h, and X_0 is the cell density at the seeding. The specific growth rate, μ (h^{-1}), in an exponential growth phase in each passage was determined as follows: $\mu = \ln(X_{120}/X_{24})/96$, where X_{120} represents the cell density after seeding for 120 h estimated by enzymatic cell detachment and a trypan blue dye exclusion test.

2.2.3 Fluorescent staining

Immunostaining was conducted as described previously (Kim, M.H. *et al.*, 2014). Concisely, the cells were washed twice with PBS and fixed for 10 min with 4% paraformaldehyde in PBS (Wako Pure Chemical Industries) at room temperature. After that,

the cells were permeabilized with 0.5 % polyoxyethylene octylphenyl ether dissolved in PBS for 10 min at room temperature. Then, the samples were masked the non-specific proteins by incubation overnight at 4 °C with Block Ace (Dainippon Sumitomo Pharma). Subsequently, the samples were incubated with primary antibodies at 4 °C overnight. This study used primary antibodies against neurofilament heavy (NF-H; Abcam), and microtubule-associated protein 2 (MAP2; Abcam) that were diluted in deionized water containing 10% Block Ace. After the incubation with primary antibodies, the samples were washed twice with TBS and then immunolabeled for 1 h at room temperature with the appropriate secondary antibody, Alexa Flour 488-conjugated anti-mouse IgG and Alexa Flour 594-conjugated anti-rabbit IgG (Life Technologies). The nuclei were stained with DAPI (Life Technologies), respectively. The fluorescent stained samples were observed manually by a confocal laser scanning microscope (FV-1000; Olympus) through a 20× objective lens.

2.2.4 Protein extraction and western blot analysis

The protein extraction and western blot analysis were performed as described previously (Kim, M.-H. *et al.*, 2019). Briefly, total protein was extracted from the cells by incubation on ice with a RIPA lysis buffer (Sigma-Aldrich) containing dithiothreitol (DTT, Abcam), a Halt™ protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific) for 30 min. Protein concentration was estimated using a BCA protein assay kit (Thermo Fisher Scientific). Equal amounts of the total loading proteins were separated by SDS-PAGE (Wako), and transferred to immuno-blot low fluorescence PVDF membrane (Bio-rad). The membrane was blocked the non-specific proteins with ECL™ blocking agent (GE Healthcare) for 1 h at room temperature. After that, the membrane was immunolabeled with primary antibodies at 4 °C overnight that were diluted in TBS containing ECL™ blocking agent. This protein analysis used the antibodies against lamin A/C (Santa Cruz), lamin B (Abcam), GAPDH (Sigma-Aldrich), H3K4me3,

H3K27me3, H3 (Cell signaling technology), NF-H, and MAP2 (Abcam). Then, fluorescent staining was conducted by incubation with secondary antibodies, DyLight® 800 anti-Rabbit IgG (Thermo Fisher Scientific) and Starbright™ blue 700 anti-mouse IgG (Bio-Rad) for 1 h at room temperature. Fluorescent signals were monitored using a ChemiDoc™ MP Imaging system (Bio-Rad). Finally, the optical intensity of protein signals was quantified using an image analysis software (Image Lab™, Bio-Rad).

2.2.5 Quantitative real-time reverse transcription–polymerase chain reaction (qRT-PCR)

RNA isolation and qRT-PCR were conducted as described previously (Ogawa, Y. *et al.*, 2015). Shortly, total RNA was isolated from the cells using an RNeasy Mini Kit (Qiagen, Hilden, Germany), according to a manufacturer's instruction. The RNA was then reverse transcribed to cDNA using a PrimeScript® RT reagent kit (Takara Bio Inc., Shiga, Japan). The qRT-PCR assays were performed using SYBR® Premix Ex Taq (Takara Bio Inc.) with specific primers listed in Table 2-1 for their sequences. The assay was executed on a real-time PCR system (Applied Biosystems 7300, Thermo Fisher Scientific) and the relative mRNA expression of the target genes was evaluated by the cycle threshold value (C_t) and normalized to that of GAPDH using the $2^{-\Delta\Delta Ct}$ method.

Table 2-1 Primer sequences used in gene expression analysis in chapter 2

Genes	Forward sequence (5' → 3')	Reverse sequence (5' → 3')
HDAC1	GGGACCTACGGGATATTGGG	GACCGCACTAGGCTGGAACA
HDAC2	TGGGAGGAGGTGGATACACAA	AGCTTGAAGTCCGGTCCAAA
EZH2	GCGGAAGCGTGTAAAATCAGA	CCTTCGCTGTTTCCAACTCTT
JMJD3	CGAGTGGAACGAGGTGAAGAA	CCTGGCAGTGCTTCATCGA
CD73	AGTTCGAGGTGTGGACATCGTG	ATCATCTGCGGTGACTATGAATGG
CD90	ATCGCTCTCCTGCTAACAGTC	CTCGTACTGGATGGGTGAACT
Oct4	AGCAAAACCCGGAGGAGT	CCACATCGGCCTGTGTATATC
Nanog	TGATTTGTGGGCCTGAAGAAAA	GAGGCATCTCAGCAGAAGACA
NF-H	CAGAGCTGGAGGCACTGAAA	CTGCTGAATGGCTTCCTGGT
MAP2	CGAAGCGCCAATGGATTCC	TGAACTATCCTTGCAGACACCT
GAPDH	CAACGGATTTGGTCGTATTGG	GCCATGGGTGGAATCATATTG

2.2.6 Chromatin Immunoprecipitation with quantitative polymerase chain reaction (ChIP-qPCR)

ChIP-qPCR was conducted using a SimpleChIP® Enzymatic Chromatin Immunoprecipitation Kit (Cell signaling technology), according to the manufacturer's procedure. This study used three antibodies against H3K4me3, H3K27me3, and IgG control (Cell signaling technology) for immunoprecipitation. Immunoselected DNA was quantified by RT-PCR method with the specific primers against the promoters of MAP2 and NF-H genes. The ChIP-qPCR primer sequence is listed in Table 2-2. The qRT-PCR assays were analyzed with SYBR® Premix Ex Taq (Takara Bio Inc.) on a real-time PCR thermal cyclers (Applied Biosystems 7300, Thermo Fisher Scientific). Cycle threshold value (C_t) in each condition was

used to calculate for relative fold enrichment of H3K4me3 and H3K27me3 at each specific promoter to condition of IgG control.

Table 2-2 Primer sequences used in ChIP analysis

Genes	Forward sequence (5' → 3')	Reverse sequence (5' → 3')
NF-H	CTCCTCCAGCGGCTTCCACTCGT	GCGCCTGCAGCTGCTCCTTCTCAC
MAP2	TAAGCGGTGTGTGTGTGTGC	ATGATGACAAGCCACTCAGC

2.2.7 Neurogenic differentiation potential

For neurogenic differentiation culture, hMSCs that have been cultured under unidirectional gravity conditions or isotropic gravity conditions on day 5 of every passage, were transferred into a new culture vessel coated with fibronectin (PromoCell, Germany) with seeding density of 5.0×10^3 cells/cm^2. The cells were incubated with hMSC growth medium for a day under unidirectional gravity environment. After that, the medium was replaced with neurogenic differentiation medium (PromoCell). Then, the cells were cultured under unidirectional gravity conditions for 7 days with the change of culture medium every 3 days.

2.2.8 Statistical analysis

All quantitative data was obtained from triplicate experiments, represented as the means ± standard deviation. Comparisons between the conditions were evaluated by one-way analysis of variance (ANOVA) with the Tukey's honest significance test. The statistical significance was considered at the *p*-values less than 0.05.

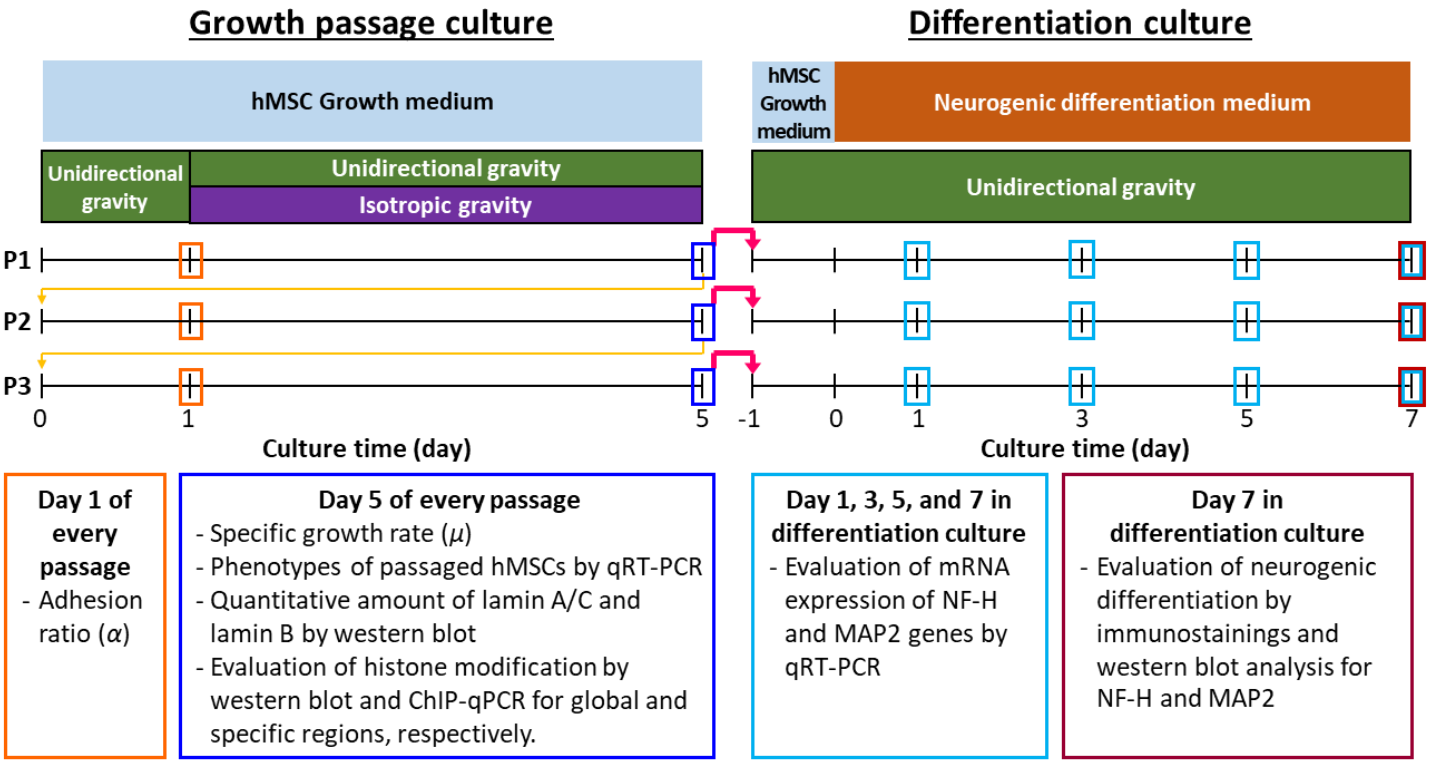

Fig. 2-1 Schematic diagram showing experimental plan in chapter 2. P: passage number.

2.3 Results

2.3.1 Proliferation ability

During the cultivation for three passages, on day 1 and day 5 of each passage, the cells were monitored by a phase contrast microscope for checking the proliferation and cellular confluency in the cultures. On day 1 of each passage, there were no significant difference about morphology of the cells from both gravitational culture conditions (Fig. 2-2A). On day 5 in each passage, the cells presented the proliferation by an increase of cell number and confluency in the culture. However, cells cultured under both gravitational culture conditions had no significant difference about their morphology (Fig. 2-2B).

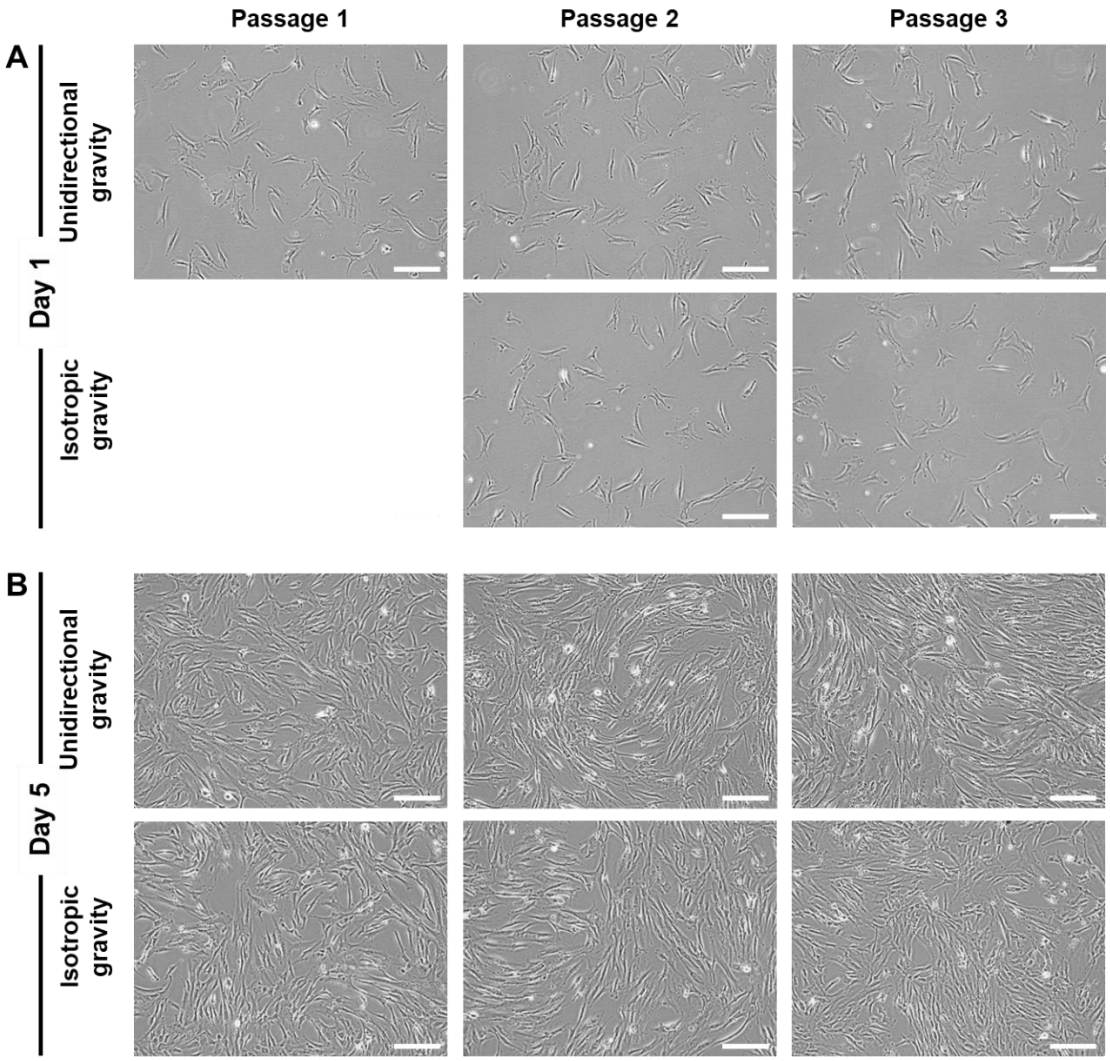

Fig. 2-2 Morphology of passaged hMSCs during the cultures under unidirectional gravity and isotropic gravity on day 1 (A) and day 5 (B) of each passage. Scale bars: 200 μm.

Proliferation indices were considered as adhesion efficiency (α) and specific growth rate (μ) evaluated during a growth passage culture under unidirectional gravity conditions and isotropic gravity conditions. The α and μ values of passaged hMSCs cultured under both gravitational conditions were consistent at least up to the three passages. There was no significant difference in adhesion efficiency and specific growth rate between the passaged cells cultured under both gravitational culture conditions (Fig. 2-3).

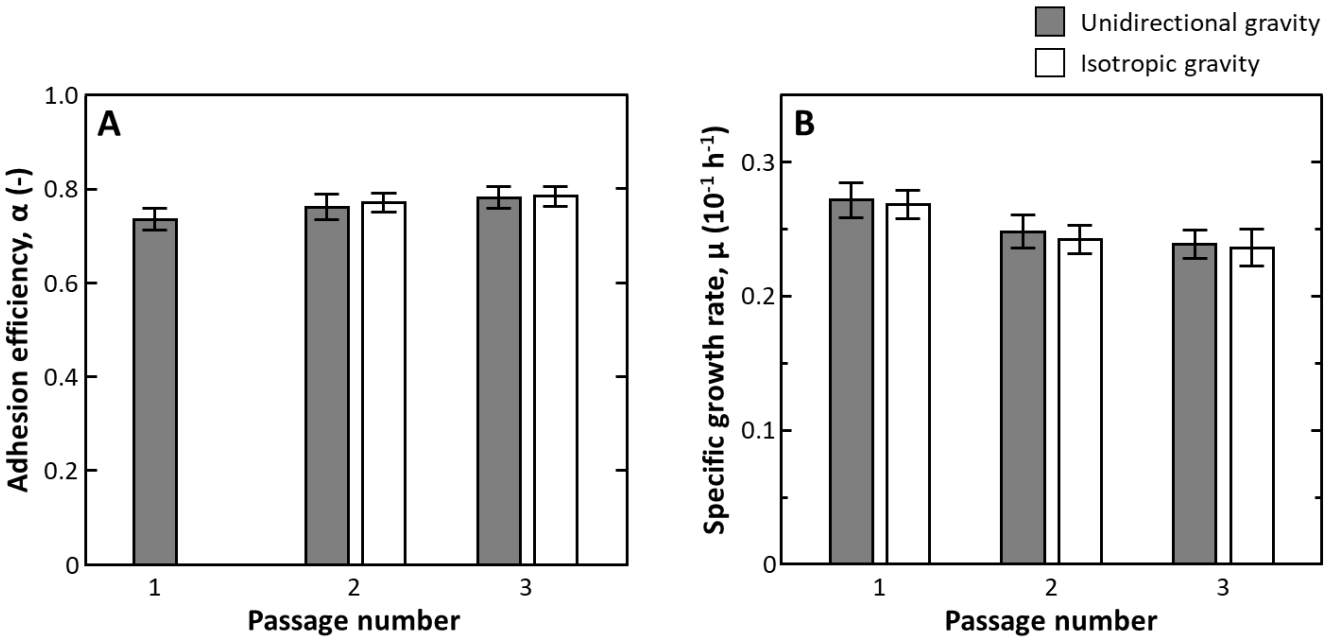

Fig. 2-3 Proliferation ability of hMSCs during growth passage culture under unidirectional gravity conditions (shaded bars) and isotropic gravity conditions (open bars) estimated by attachment efficiency (A) and specific growth rate (B) (n = 3).

2.3.2 Phenotypes of passaged hMSCs

To clarify the change in phenotypes of passaged hMSCs during growth expansion culture under both gravitational conditions, cells on day 5 of each passage were analyzed for hMSC-specific markers (CD73, CD90), pluripotent markers (Oct4, Nanog), and neurogenic markers (NF-H, MAP2). The expressions of CD73 and CD90 exhibited no significant difference between cells cultured under both gravitational conditions, which were maintained through serial cultivation (Fig. 2-4A-B). The expressions of Oct4 and Nanog in cells cultured under isotropic gravity conditions were higher than those grown under unidirectional gravity conditions and remained at a high level at least up to the three passages (Fig. 2-4A-B). However, these pluripotent levels in cells grown under unidirectional gravity conditions gradually

decreased against an increase of their passage numbers. At the third passage, the expressions of Oct4 and Nanog in the cells cultured under isotropic gravity conditions were 2.3 and 2.1-fold higher than those grown under unidirectional gravity conditions, respectively. In addition, the analysis of neurogenic markers during growth expansion culture revealed that cells passaged under both gravitational culture conditions had no significant difference in their neurogenic expressions (Fig. 2-4E-F).

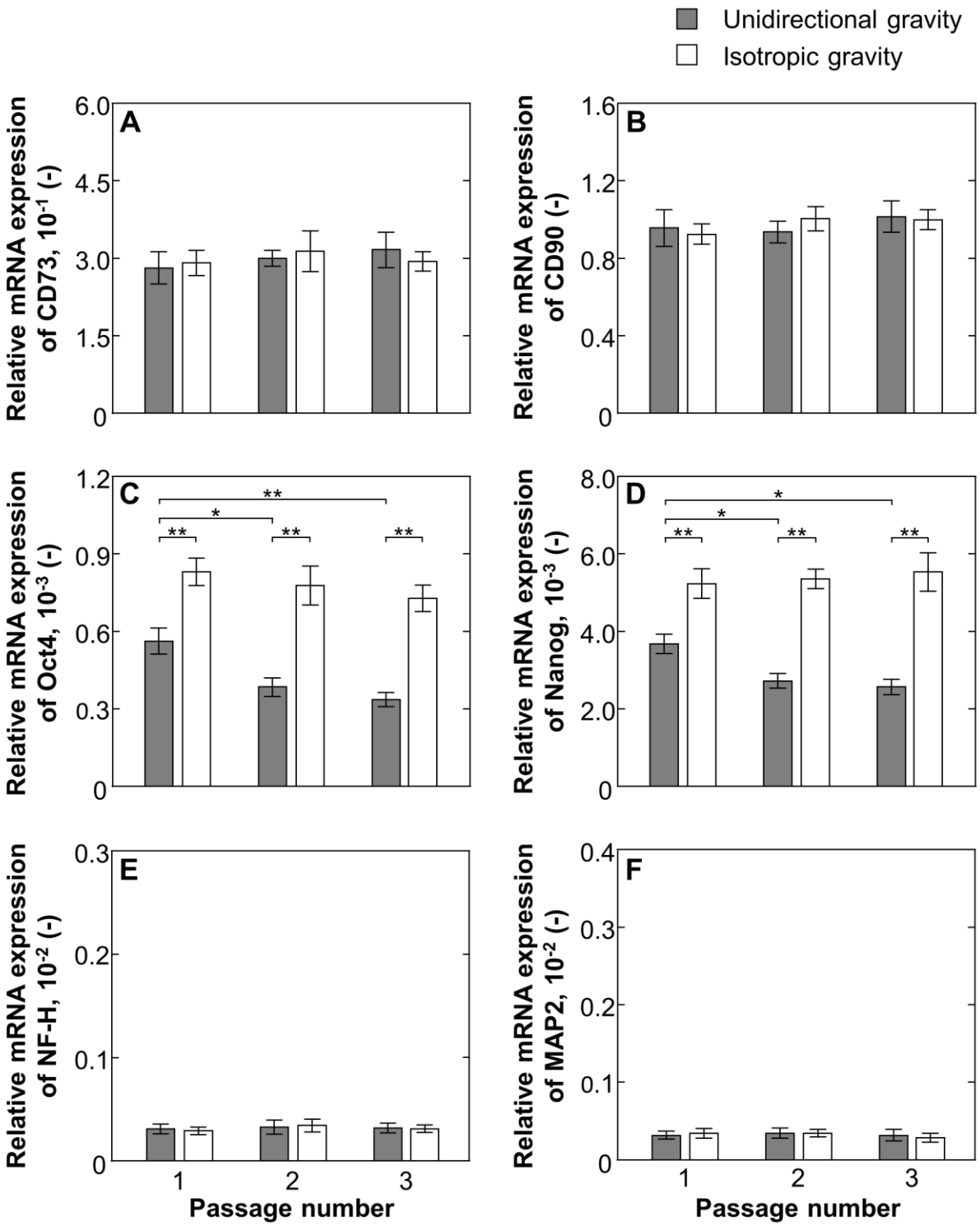

Fig. 2-4 Quantitative RT-PCR analysis of hMSC markers; CD73 (A), CD90 (B), pluripotent markers; Oct4 (C), Nanog (D), and neurogenic markers; NF-H (E), MAP2 (F) in passaged hMSCs during growth culture under conventional conditions (shaded bars) and isotropic gravity conditions (open bars) (n = 3). *p < 0.05, **p < 0.01.

2.3.3 Levels of nuclear lamina

To investigate the influence of isotropic gravity on nucleoskeletal organization in passaged hMSCs during growth expansion culture, cells cultured with hMSC growth medium under altered gravitational conditions were examined for the levels of nucleoskeletal proteins (lamin A/C and lamin B) by western blot analysis (Fig. 2-5A) and the intensity ratio of lamin A/C to lamin B, which relative to GAPDH was calculated (Fig. 2-5B). At the first passage, the lamin ratio in cells cultured under isotropic gravity conditions was 1.9-fold lower than those grown under unidirectional gravity culture conditions. Cells cultured under isotropic gravity conditions had no significant difference in the lamin ratio with an increase in their passage numbers. However, the lamin ratio in the cells grown under the unidirectional gravity culture conditions gradually increased with increasing passage numbers. At the third passage, the ratio of lamin A/C to lamin B in cells cultured under isotropic gravity conditions was 2.4-fold lower than those grown under unidirectional gravity conditions.

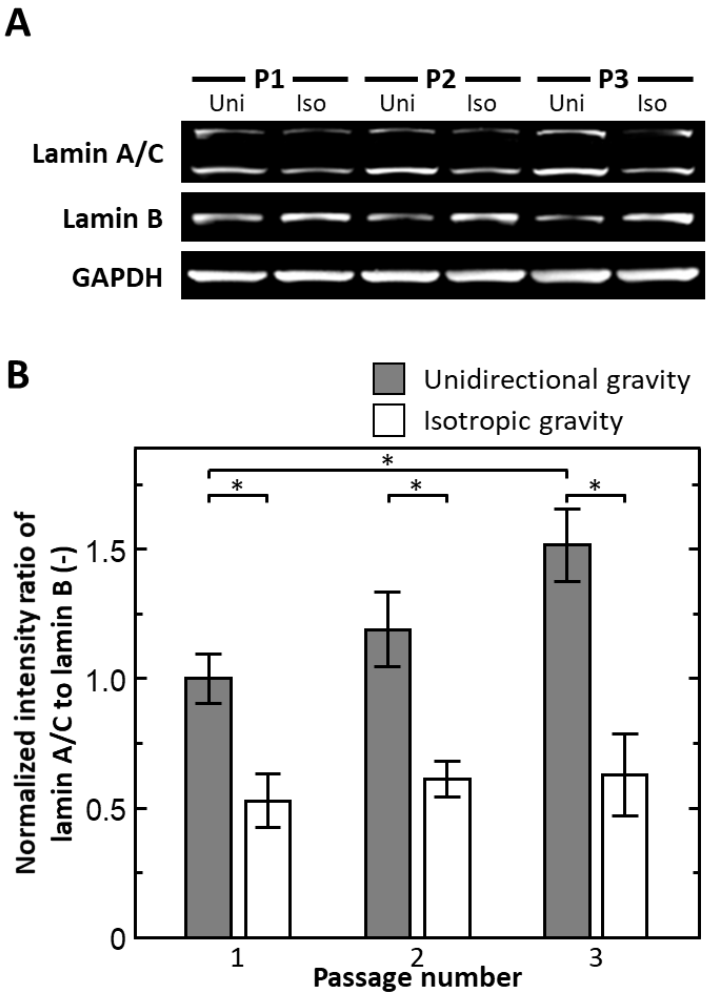

Fig. 2-5 Levels of nuclear lamins in passaged hMSCs estimated by western blot analysis. Representative image of western blot analysis (A). P: passage number, Uni: unidirectional gravity conditions, Iso: isotropic gravity conditions. Quantitative data showing as a normalized intensity ratio of lamin A/C to lamin B in western blotting images (B) (n = 3). *p < 0.01.

2.3.4 Histone modification

To investigate the changes of global histone modification in passaged hMSCs, cells cultured with hMSC growth medium under both gravitational conditions on day 5 of each passage were examined the expressions of enzymatic genes related to histone acetylation and methylation. From gene expression analysis, there was no significant difference in the passaged cells in terms of changes in passage numbers, and altered gravitational culture conditions (Fig. 2-6).

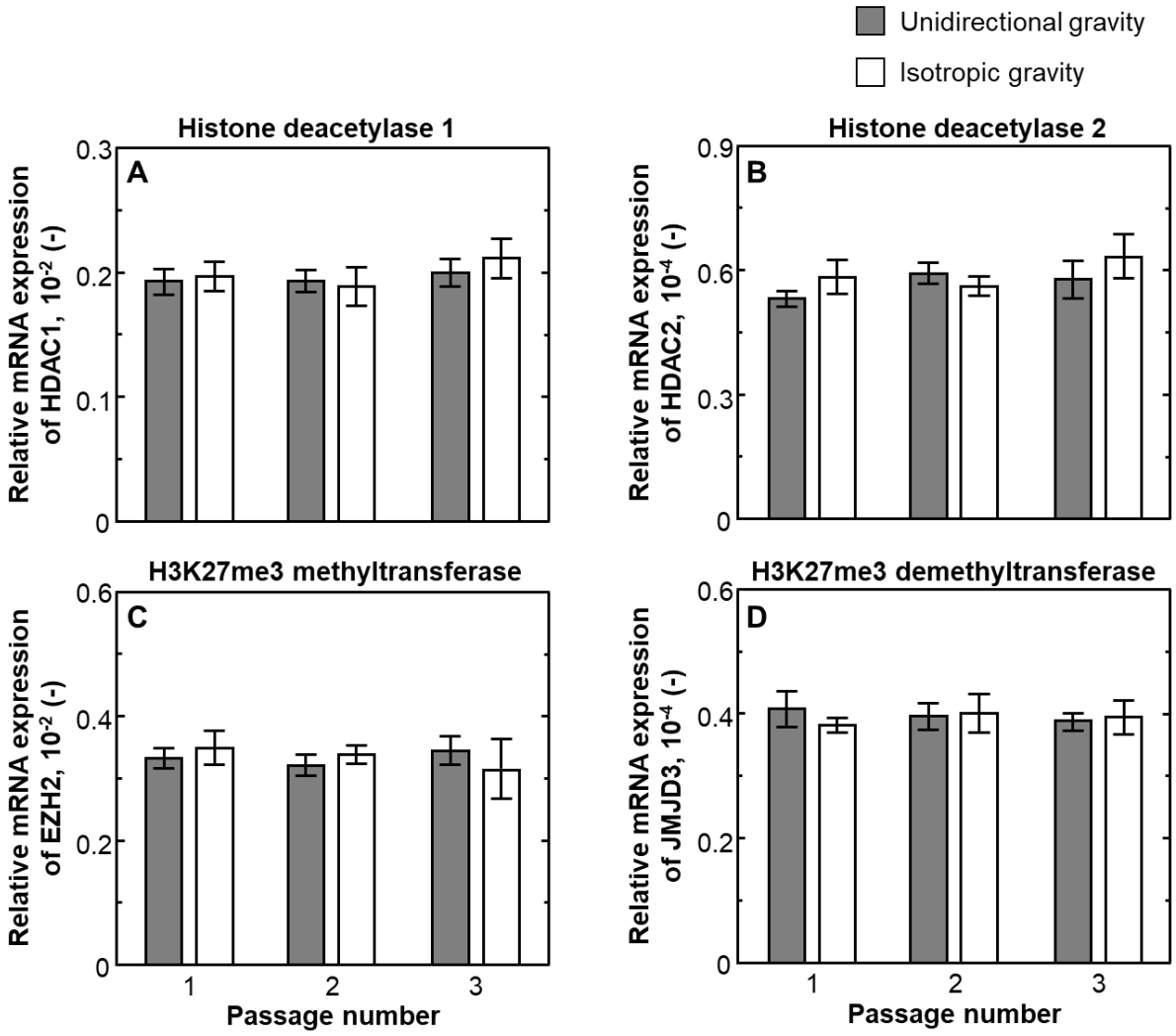

Fig. 2-6 Quantitative RT-PCR analysis of HDAC1 (A), HDAC2 (B), EZH2 (C), and JMJD3 (D) expressions in the cells cultured under unidirectional gravity conditions or isotropic gravity conditions during growth expansion culture (n = 3).

To examine changes in epigenetic chromatin marks, histone H3 trimethylation at lysine 4 (H3K4me3) and histone H3 trimethylation at lysine 27 (H3K27me3) in passaged hMSCs, cells cultured with hMSC growth medium under both gravitational conditions on day 5 in every passage were investigated by western blot analysis for analysis of global histone modification (Fig. 2-7A-B). By calculation of the relative intensity of H3K4me3 and H3K27me3 to H3, there was no significant difference in the passaged cells in terms of changes in passage numbers, and altered gravitational culture conditions (Fig. 2-7C-D).

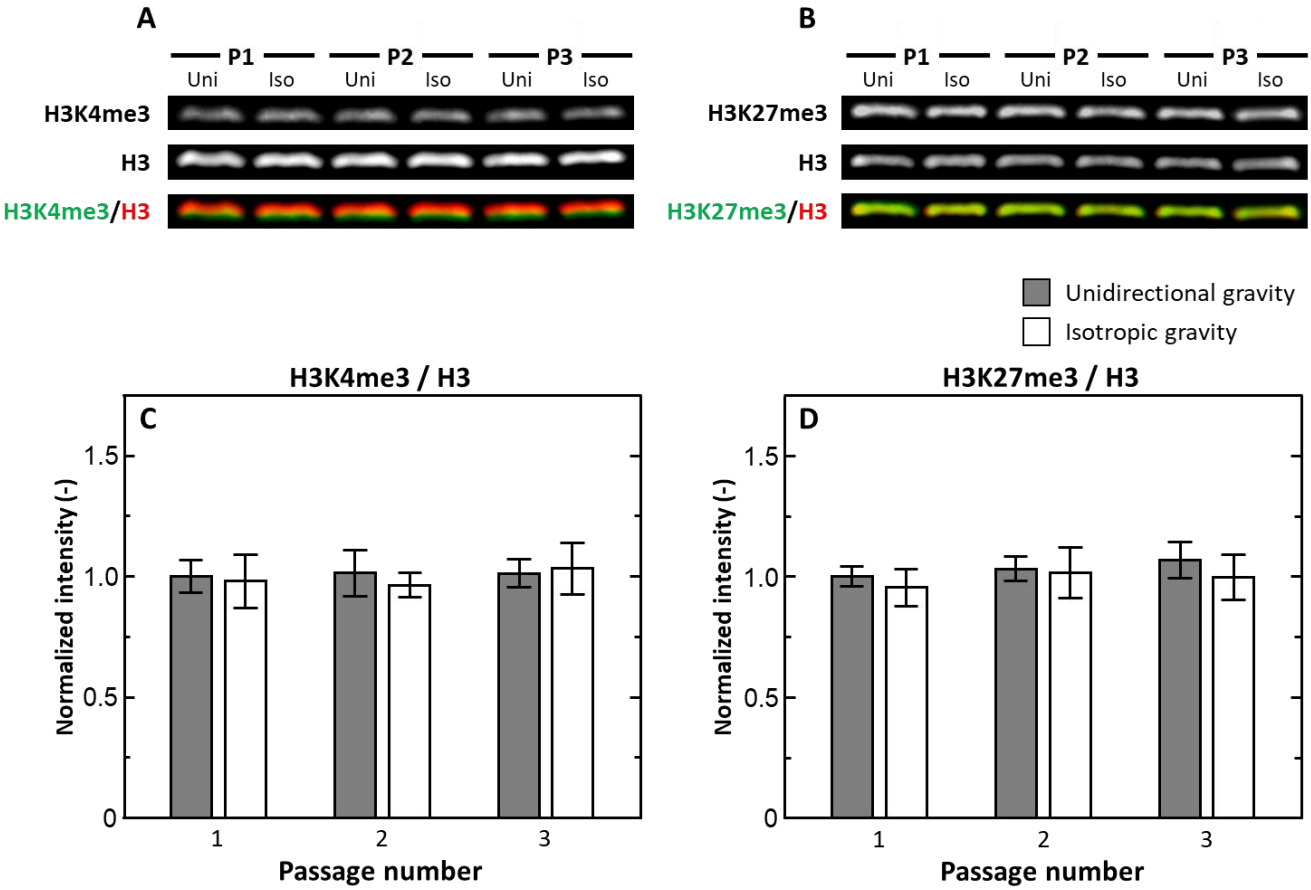

Fig. 2-7 Global histone modification in passaged hMSCs evaluated by western blot analysis. Representative image of western blot analysis of H3K4me3, H3K27me3, and H3 (A-B). P: passage number, Uni: unidirectional gravity conditions, Iso: isotropic gravity conditions. Quantitative data showing normalized intensities of H3K4me3 (C), and H3K27me3 (D) to H3 in western blotting images (n = 3).

To clarify the histone modification at specific regions, chromatin immunoprecipitation (ChIP) analysis of activation-associated H3K4me3 and repression-associated H3K27me3 at promoters of genes related to neuronal lineage, NF-H and MAP2, was conducted in passaged hMSCs cultured under both gravitational conditions on day 5 in every growth passage. In the case of H3K4me3 modification at NF-H and MAP2 promoters (Fig. 2-8A-B), there were no significant differences in the enrichment of H3K4me3 by variation in their passage numbers or altered gravitational culture conditions. However, H3K27me3 modification showed that at the first passage, cells cultured under isotropic gravity conditions exhibited the enrichment of H3K27me3 at promoters of NF-H and MAP2 that were 2.0 and 1.7-fold lower than those grown under unidirectional gravity conditions, respectively (Fig. 2-8C-D). Cells passaged under isotropic gravity conditions sustained the enrichment of H3K27me3 at the neuronal promoters during passaging. Nevertheless, those grown under unidirectional gravity conditions were significantly accumulated the enrichment of H3K27me3 with their increasing passage numbers. At the third passage, the enrichment level of H3K27me3 at promoters of NF-H and MAP2 in cells cultured under isotropic gravity conditions were 2.8 and 3.1-fold lower than those cultured under unidirectional gravity conditions, respectively.

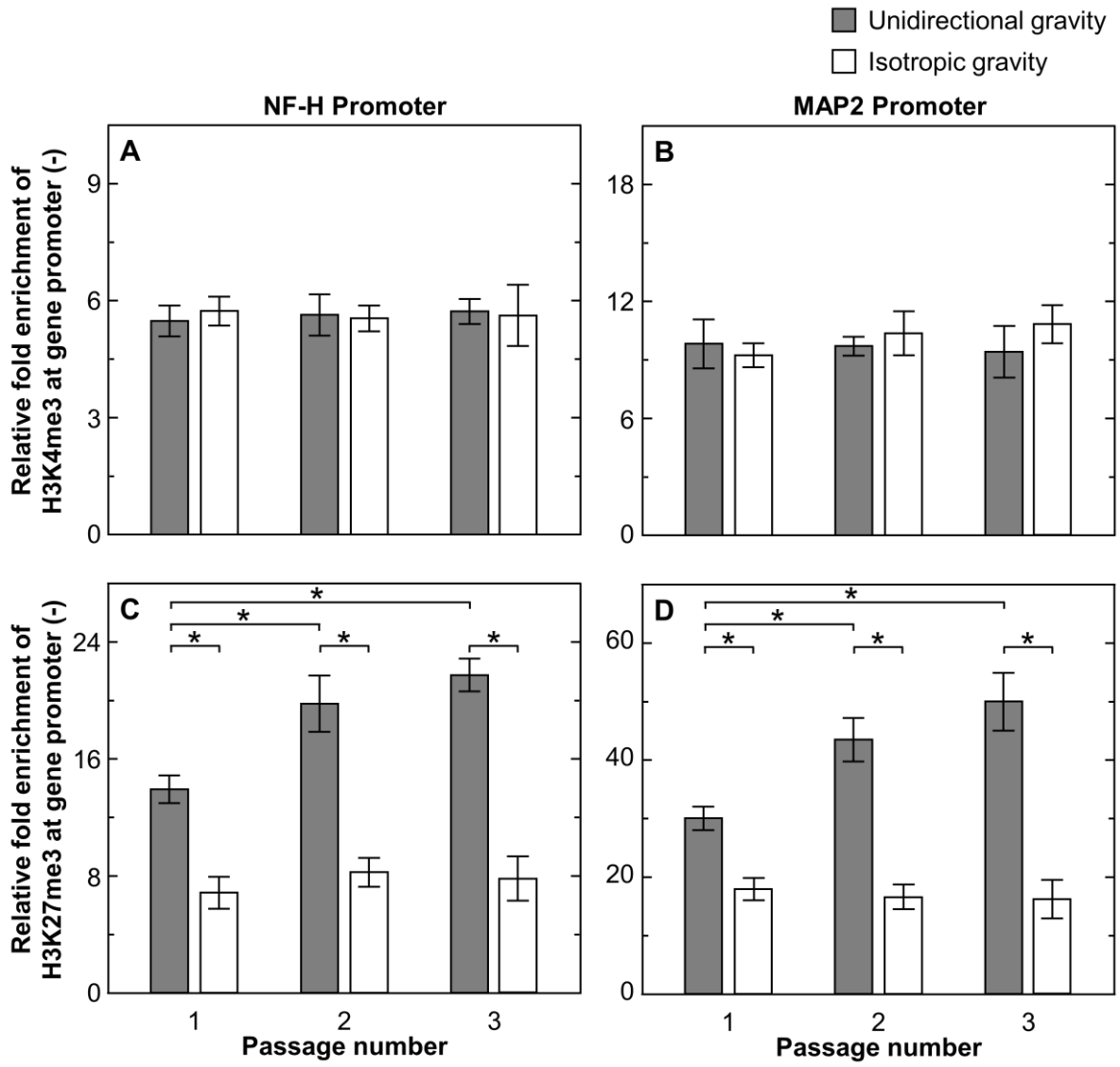

Fig. 2-8 Specific histone modification at neurogenic promoters in passaged hMSCs representing as relative fold enrichments of H3K4me3 (A, B) and H3K27me3 (C, D) at promoters of NF-H (A, C) and MAP2 (B, D) genes estimated by ChIP-quantitative PCR analysis ($n = 3$). *$p < 0.01$.

2.3.5 Neurogenic differentiation potential

To evaluate neurogenic differentiation potential in passaged hMSCs cultured under both gravitational conditions, the cells from each growth culture conditions were transferred into a new culture vessel and cultured with neurogenic differentiation medium for 7 days. The expressions of neurogenic markers (NF-H and MAP2) were evaluated by immunostaining, western blot, and qRT-PCR analyses. Fluorescent observations revealed that a large number of NF-H- and MAP2-positive cells were found in all cultures of passaged cells from both the gravitational conditions (Fig. 2-9). Western blot analysis of NF-H and MAP2 showed the prominent differences in their protein expression levels that were dependent on gravitational conditions and passage numbers (Fig. 2-10A). At the first passage of growth culture, the relative ratios of NF-H and MAP2 to GAPDH in the differentiated cells from the cells cultured under isotropic gravity conditions exhibited 1.3 and 1.4-fold higher than those from unidirectional gravity conditions, respectively (Fig. 2-10B). The relative levels of NF-H and MAP2 in the cells from the isotropic gravity conditions were consistent at least up to the three passages of growth culture. However, cells from unidirectional gravity conditions presented the reduction in NF-H and MAP2 levels in accordance with their increasing passage numbers; especially, the NF-H and MAP2 levels showed drastic decrease after the first passage of the growth culture. Lastly, at the third passage of growth culture, the relative levels of NF-H and MAP2 in cultures from passaged cells under isotropic gravity conditions showed 1.9 and 2.6-fold higher than those from unidirectional gravity conditions, respectively.

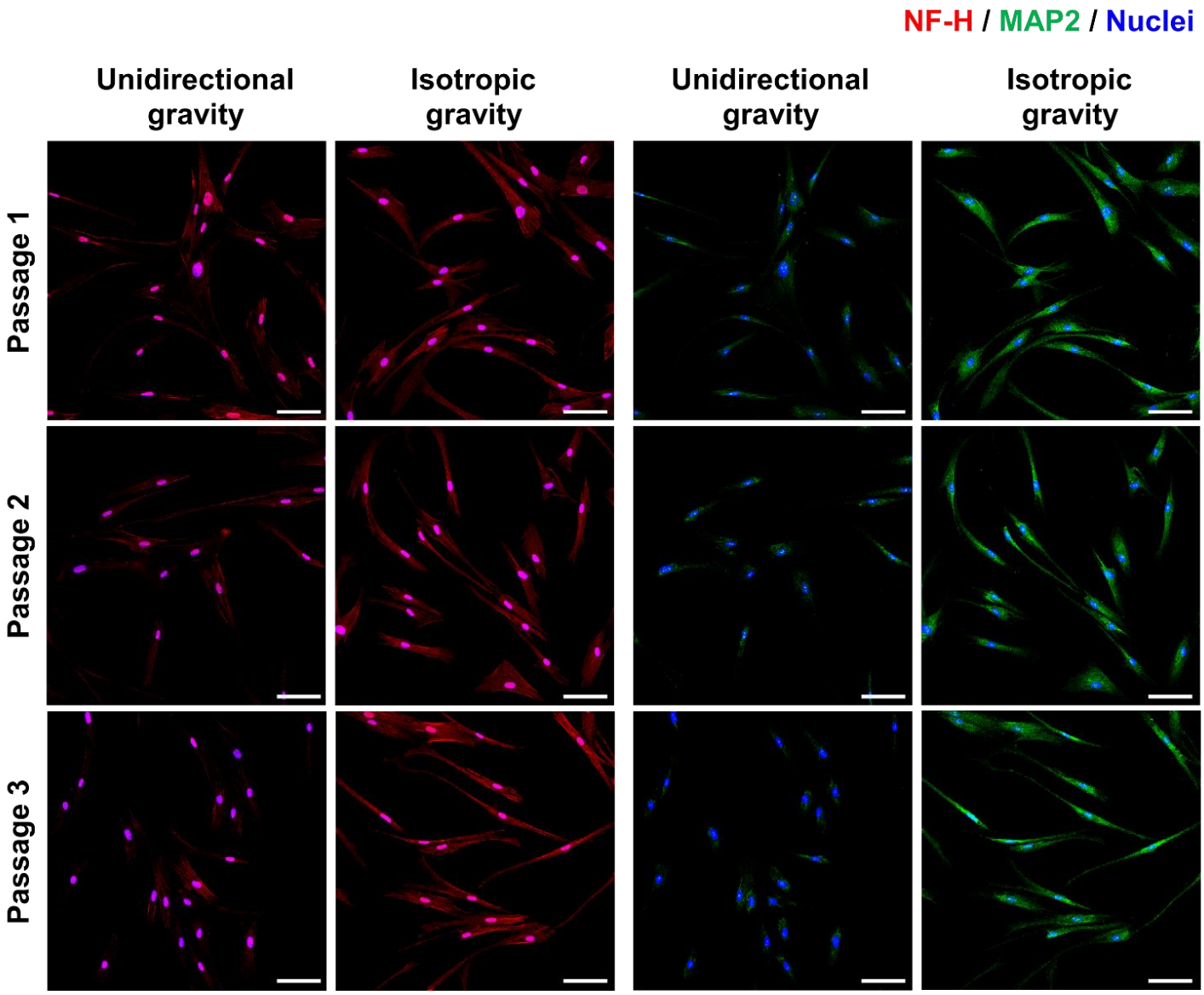

Fig. 2-9 Immunostainings of the differentiated cells after cultured in neurogenic differentiation medium for 7 days. Fluorescent staining images show NF-H (red), MAP2 (green), and nuclei (blue) in the cells from unidirectional gravity conditions or isotropic gravity conditions (A). Scale bars: 100 μm.

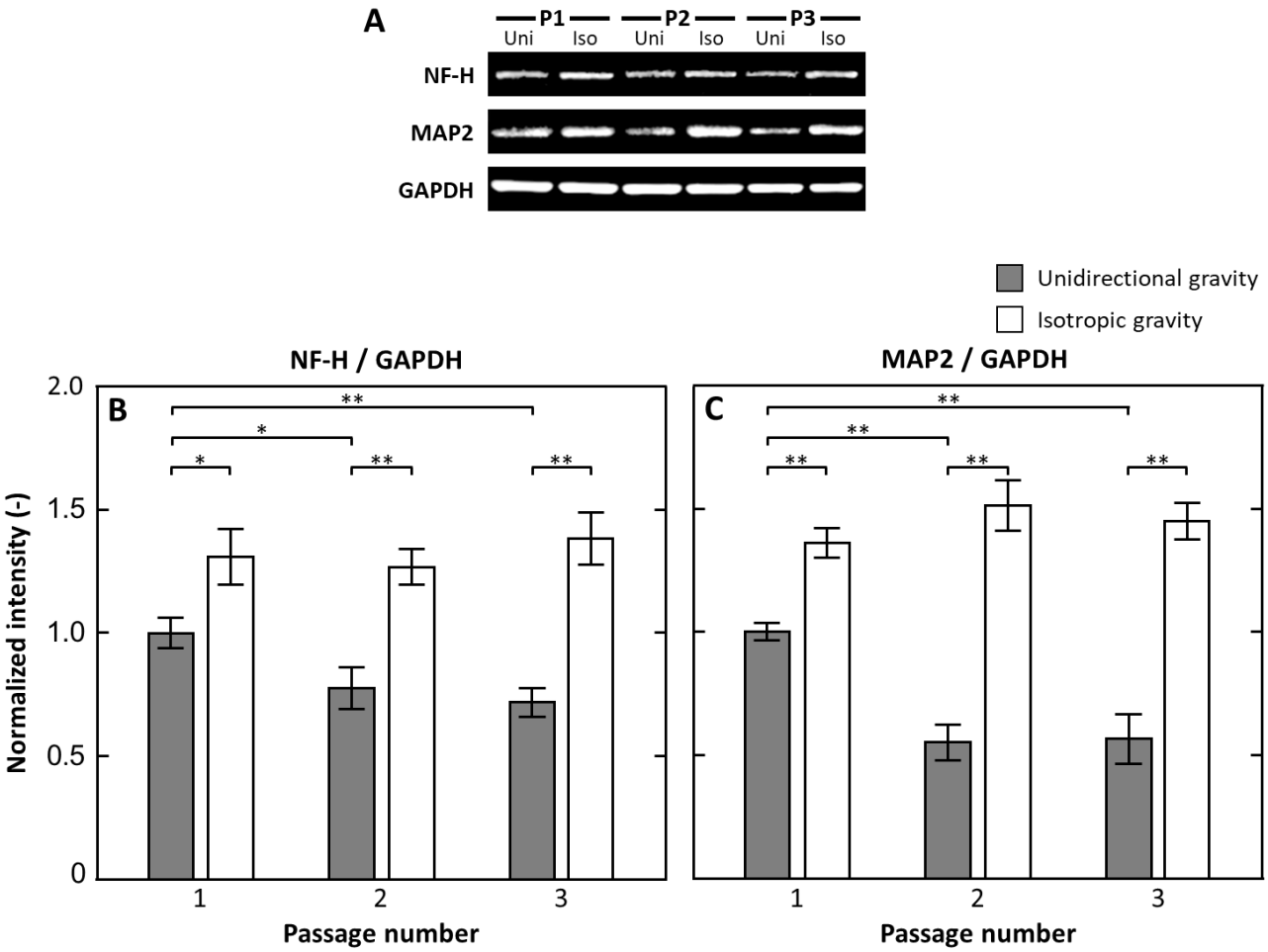

Fig. 2-10 Neurogenic differentiation potential of passaged hMSCs evaluated by western blot analysis of the differentiated cells after cultured in neurogenic differentiation medium cultured under unidirectional gravity conditions for 7 days. Representative image of western blot analysis for of NF-H, MAP2, and GAPDH (A), P: passage number, Uni: unidirectional gravity conditions, Iso: isotropic gravity conditions. Quantitative data showing normalized intensities of NF-H (B) and MAP2 (C) relatively to GAPDH in western blotting images ($n = 3$). *$p < 0.05$, **$p < 0.01$.

In addition, this study also examined the expressions of two neurogenic-related genes, NF-H and MAP2, in the cells during differentiation culture using qRT-PCR method. The relative gene expression levels of NF-H and MAP2 in cells from isotropic gravity conditions were higher than those from unidirectional gravity conditions, at the same differentiation culture time (Fig. 2-11). On day 7 of differentiation culture of cells from the first passage, cells from isotropic gravity conditions expressed the levels of NF-H and MAP2 that were 1.5 and 1.8-fold higher than those from unidirectional gravity conditions, respectively. By increasing the passage numbers in growth culture, cells from isotropic gravity conditions showed constant expression of the neural-related genes. However, cells from unidirectional gravity conditions decreased their expressions with higher passage numbers in growth culture. At the third passage, the expression levels of NF-H and MAP2 in cells from isotropic gravity conditions were 2.7 and 2.5-fold higher than those from unidirectional gravity culture conditions, respectively.

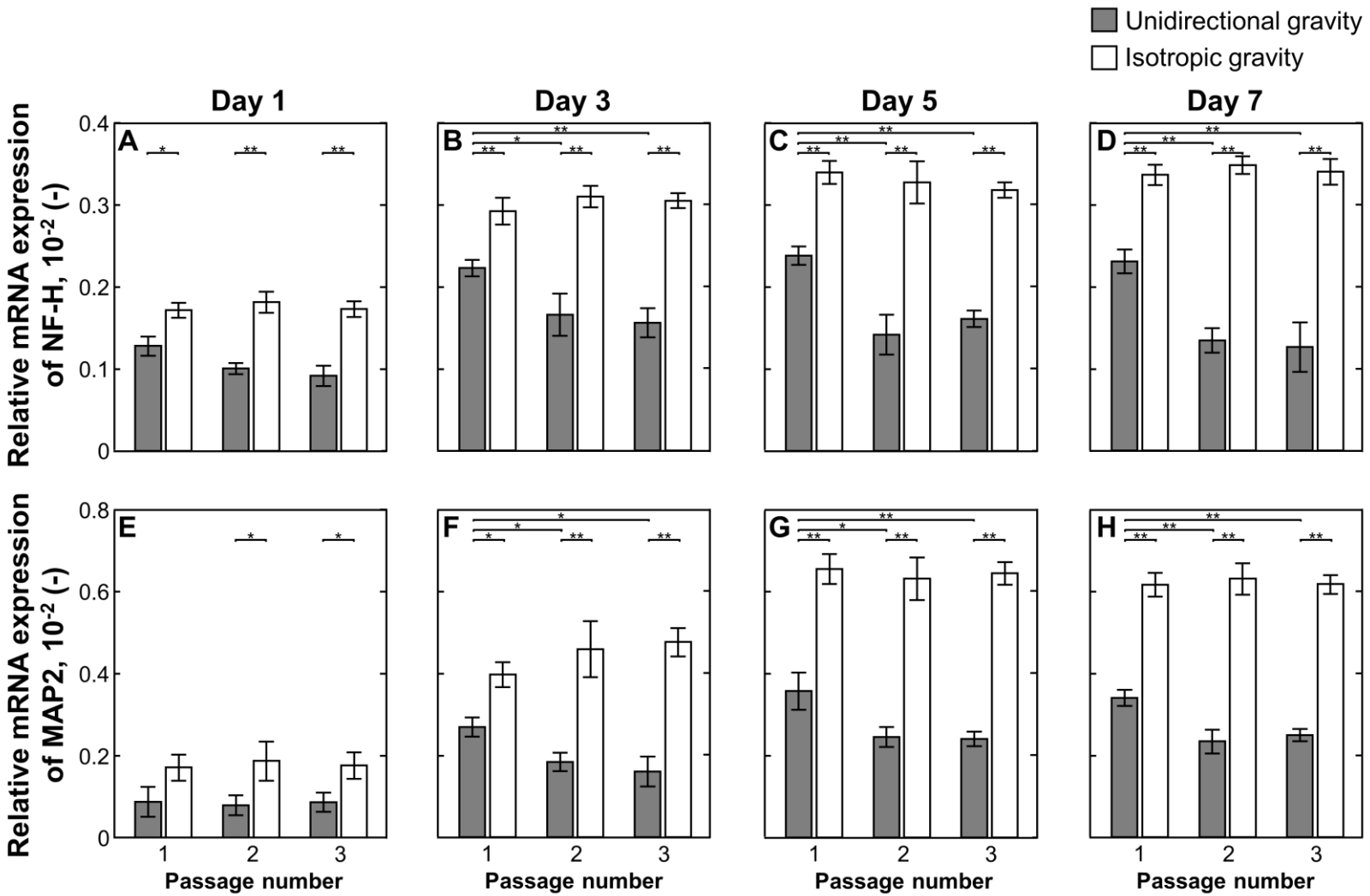

Fig. 2-11 Quantitative RT-PCR analysis of NF-H and MAP2 expressions in the cells from unidirectional gravity conditions or isotropic gravity conditions during neurogenic differentiation culture under unidirectional gravity conditions ($n = 3$). *$p < 0.05$, **$p < 0.01$.

2.4 Discussion

Gravity arising in the culture environment has a profound influence on proliferation and differentiation potency of passaged hMSCs in expansion culture and the cellular mechanisms need to be elucidated in order to control these cellular functions. Stem cell nucleus responds to physical cues from their surrounding environment, which potentially induce the diverse biological activities. Nuclear lamins are mechanosensitive proteins that are well known to regulate mechanical properties by the interaction between fibrillar networks at the inner nucleus and actin cytoskeleton (Dahl, K.N. *et al.*, 2008). Recent development of an isotropic gravity culture device for simulation of microgravity has motivated several studies aiming to elucidate cellular adaptation to the altered gravitational culture environment. The results in chapter 2 indicated that cells cultured under unidirectional gravity conditions exhibited a stretched morphology and undergo unidirectional migration, while cells cultured under isotropic gravity conditions presented multidirectional migration with high frequency of directional changes in their movement. Moreover, cells cultured under unidirectional gravity conditions maintained their spindle-shape via fibronectin fibril formation in cell bodies and stabilization of focal adhesion with enrichment of actin stress fibers. However, cells cultured under isotropic gravity conditions showed an active extension with more intense level of phosphorylated paxillin at cell edge. Moreover, cells cultured under isotropic gravity conditions also showed the inhibition of fibronectin fibrillogenesis by up-regulation of MT1-MMP resulting in the aggregate fibronectin structure in the cell bodies. Because of these phenomena, isotropic gravity influences on cell behavior and mechanotransduction in hMSCs with changes in ECM assembly and spatial reorganization of actin cytoskeleton (Koaykul, C. *et al.*, 2019).

Furthermore, this chapter was continuously seeking for the application of mechanotransduction change by evaluation of the neurogenic differentiation potential in passaged hMSCs under altered gravitational culture conditions. Fig. 2-12 shows a schematic

illustration of hypothetical mechanism based on the observed results in this study, which also addresses two major questions. The first question is how do change in cytoskeletal formations influence the epigenetic modification under isotropic gravity conditions? Second, how can these mechanisms maintain neural differentiation potency in passaged hMSC cultured under isotropic gravity conditions during a serial cultivation?

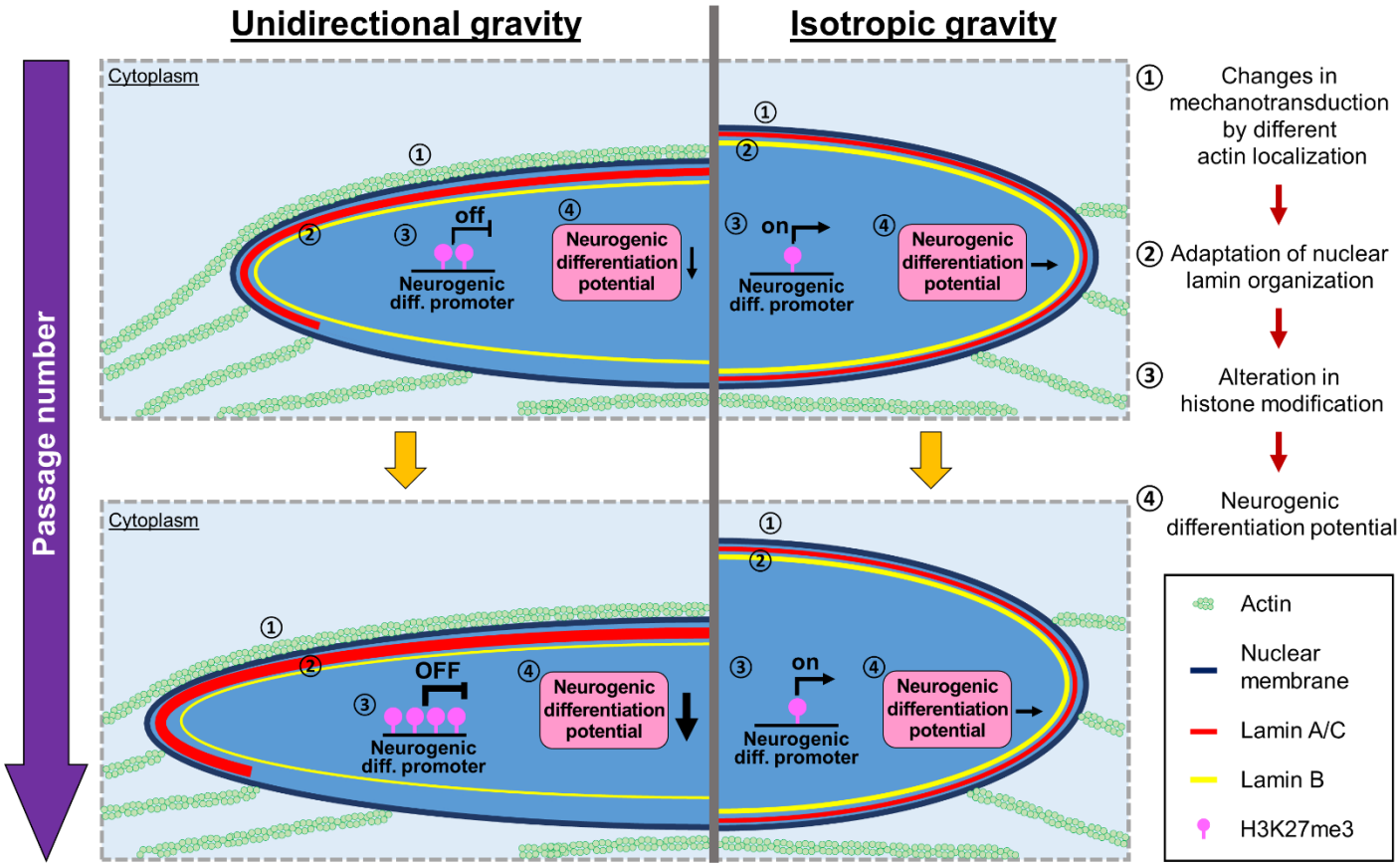

Fig. 2-12 Schematic illustration showing working hypothesis of changes in neurogenic differentiation potential in passaged hMSCs cultured under unidirectional gravity conditions and isotropic gravity conditions due to alterations in mechanotransduction, organization of nuclear lamins, and specific histone modification.

2.4.1 Isotropic gravity-induced remodeling of cytoskeleton and nucleoskeleton that contributes to suppression of epigenetic modification in hMSCs

Alterations in cell-substrate adhesion and cytoskeletal formation in response to environmental change are linked to epigenetic modification (Lelièvre, S.A., 2009). It has been suggested that the structural pathway linking between ECM and nucleus, also defined as the "mechanotransduction pathway", is an essential biophysical transduction pathway for translating the biophysical signalings into the activity within the nucleus (Tamiello, C. *et al.*, 2016). The study in chapter 1 found the cellular adaptation with differential structure of cytoskeleton and nucleoskeleton in hMSCs when cultured under altered gravitational culture conditions. The actin cytoskeleton forms a dome-like actin cap on top of nucleus, which has been shown to regulate nuclear morphology and nuclear shape-determining functions (Khatau, S.B. *et al.*, 2010). Several researchers have well documented that assembly of lamin A/C and lamin B is modified by mechanical signalings was affecting the nucleus (Swift, J. *et al.*, 2014; Osmanagic-Myers, S. *et al.*, 2015). This study presents the changes in mechanotransduction by altered gravitational culture conditions that influenced nucleoskeleton adaptation. Cells grown under unidirectional gravity culture conditions had apical stress fibers over the nucleus together with appearance of lamin A/C polarization (Fig. 1-9) and higher level ratio of lamin A/C to lamin B against an increase in their passage numbers. However, cells cultured under isotropic gravity conditions exhibited fewer apical stress fibers over the nucleus that repressed the lamin A/C polarization and sustained the ratio of lamin A/C to lamin B during the growth passage culture (Fig. 2-5).

Changes in organization of nuclear lamina in terms of both structure and type-levels affect nuclear lamina-chromatin attachments influencing on genome organization (Zheng, X. *et al.*, 2018). Moreover, epigenetic modifications also affect the alterations in gene expressions and subsequent to cellular phenotypes (Norouzitallab, P. *et al.*, 2019). It has been reported that

epigenetic switching was associated with histone methylation and demethylation at specific promoters of differentiation regions, which dictates the hMSC lineage determination and affects the characteristic and level of differentiated cells (Hemming, S. *et al.*, 2014). Moreover, it is also well documented that chromatin structure was associated with histone methylation of H3K4me3 and H3K27me3 at specific sites, which involves gene activation and repression of the transcriptions from those gene regions (Shilatifard, A., 2006). These histone marks can be remained and transmitted to daughter cells throughout DNA replication and mitotic cell division (Wang, F. *et al.*, 2013).

Transcriptions of neurogenic differentiation-related genes such as NF-H and MAP2 are triggered by neurotrophic factors presenting in the neurogenic differentiation medium (da Silva Meirelles, L. et al., 2008). In this chapter, by culture under neurogenic differentiation medium, cells passaged under isotropic gravity conditions presented significantly higher levels of NF-H and MAP2 proteins in the neurogenic-differentiated cells than those passaged under unidirectional gravity conditions (Fig. 2-10). In addition, the analysis of repressive modifications, H3K27me3, at promoters of NF-H and MAP2 indicated that cells from isotropic gravity conditions exhibited lower levels of this methylation enrichment than those cultured under unidirectional gravity conditions (Fig. 2-8). The discovery in this chapter on isotropic gravity-induced changes in nucleoskeleton organization affecting the epigenetic modification that might be a potential mechanistic basis for the alterations in the gene expression of various cell phenotypes.

2.4.2 Isotropic gravity-induced epigenetic memory conducts as a key role in the maintenance of neurogenic differentiation potential in passaged hMSCs

This chapter shows the examination of hMSC properties during passage culture under unidirectional gravity conditions and isotropic gravity conditions (Fig. 2-4). Cells grown under

both gravitational culture conditions maintained the expression of CD73 and CD90, which are the key markers of hMSCs (Dominici, M. *et al.*, 2006), and also had no significant expressions of neurogenic markers, NF-H and MAP2, in the cells during serial cultivation. Interestingly, cells passaged under isotropic gravity conditions consistently maintained their expression level of pluripotent markers, Oct4 and Nanog, which were significantly higher than those grown under unidirectional gravity conditions. It has been reported that Oct4 and Nanog were associated with the multipotency of hMSCs towards the mesoderm lineage through the regulation of Dnmt1 promoter (Tsai, C.C. *et al.*, 2012). Moreover, the up-regulated Oct4 and Nanog have also presented the enhancement of neurogenic expressions in hMSCs (Zhang, S. *et al.*, 2015). Therefore, these results support the capability of isotropic gravity culture that is able to preserve the undifferentiated state of hMSCs during serial cultivation.

Histone modification associated with epigenetic memory also impacts the stem cell differentiation (Feinberg, A.P. *et al.*, 2016). Following the discussion above, mechano-adaptation of the stem cell nucleus reacts with biophysical loading in association with the actin cytoskeleton. The stem cell nucleus responds to mechanical signalings by reorganizing their nucleoskeleton and chromatin architecture, and the mechanical loading also instills a mechanical memorization in nuclei through modification in chromatin condensation (Heo, S.J. *et al.*, 2015).

Exposure of physical input to the nucleus during long cultivation time, the force induces cellular modulation in the nucleus by synthesis of additional or new elements, which potentially controls subsequent mechanical signalings that are interpreted by the cells. Cellular memorization indicates a specific subdivision of mechanotransductive reactions. It is induced by mechanical dosage that is greater than some thresholds, and results in persistence of mechano-adaptive response, which further influences the expressions of cellular phenotypes (Heo, S.J. *et al.*, 2018). Another study presents that hMSCs isolated from different sources,

such as adipose tissue and bone marrow, have altered epigenetic memory that contributes to changes in differentiation capacities into specific fates (Xu, L. *et al.*, 2017). Thus, the culture condition during the cell expansion is the buildup of the remodeling of mechanical adaptation, which is stored as epigenetic memory in the cells.

This chapter presents the epigenetic mechanisms in passaged hMSCs, which maintained their phenotypes while growing under isotropic gravity conditions. Cells cultured under isotropic gravity conditions presented the maintenance of neurogenic differentiation potential during passage culture (Figs. 2-9, 2-10, 2-11). However, cells grown under unidirectional gravity conditions showed a reduction in the neurogenic differentiation potency due to accumulation of H3K27me3 at neurogenic promoter sites (Fig. 2-8). The mechano-sensitive systems, molecules, and signaling pathways are involved in the response and adaptation to isotropic gravity. Association between ECM-integrin-cytoskeleton is a key framework for mechanotransduction in the cells (Sims, J.R. *et al.*, 1992; Ingber, D.E., 2006; Elosegui-Artola, A. *et al.*, 2016). Isotropic gravity culture sustains the neurogenic differentiation capacity in passaged hMSCs by suppression of specific histone modification at the neurogenic promoters together with regulating organization of the cytoskeleton and nucleoskeleton during the serial cultivation. Thus, design of the culture system, which is capable of regulating the epigenetic modification in hMSCs, is a critical challenge for controlling the quality of cellular differentiation involving the epigenetic mechanism in their memory storage. Further elucidation of these mechano-adaption and mechanisms of memory storage is still required to completely explicate the relationship between the mechanical signalings and stem cell differentiation, which consequently promotes the directivity of commitment to desired phenotype specific to the targeted-applications.

2.5 Summary

This chapter examined the effects of isotropic gravity on neurogenic differentiation potential of passaged hMSCs during expansion culture. Isotropic gravity plays as an essential role in the changes in cytoskeletal and nucleoskeletal organization and the subsequent modification of epigenetic marks at specific regions, which influence their expressions of differentiation phenotypes. Furthermore, this study also discovered the role of isotropic gravity-induced epigenetic mechanisms in the maintenance of neurogenic differentiation potency, which has heritable effects on the regulation of gene expressions in that cells. Taken together, this chapter provided an insight into the fundamental epigenetic mechanism relating to directivity of neurogenic differentiation by exposure of isotropic gravity during hMSC expansion in addition to being a practical guide for device design, operation, and applications of the culture system *in vitro*.

Chapter 3

General Conclusion

3.1 Research summary

This study is the first investigation of dynamics hMSC behavior under isotropic gravity culture conditions and also presents the critical issues about cellular adaptation caused by altered gravitational culture environment. Figure 3-1 presents a schematic diagram showing the research summary of this study. In case the of cell behavior, cells cultured under unidirectional gravity conditions had stretched morphology and unidirectional migration with maintenance of their spindle-shape via fibronectin fibril formation in their bodies as well as focal adhesion stabilization with enrichment of apical actin stress fibers. However, cells cultured under isotropic gravity conditions showed multidirectional migration with active extension of leading edge due to paxillin phosphorylation. This cell migration was partially contracted with degraded fibronectin fibrils caused by inhibition of fibronectin fibrillogenesis though the up-regulation of MT1-MMP, which in turn causes the release of adhesions and immature stress fiber formation. As a result of these phenomena, it influences the mechanotransduction in the cells as cells cultured under unidirectional gravity conditions had apical stress fibers over the nucleus together with appearance of lamin A/C polarization subject to the compressive force by stress fiber tension. Nevertheless, cells cultured under isotropic gravity had few apical stress fibers with mostly non-polarization of lamin A/C in their nucleus.

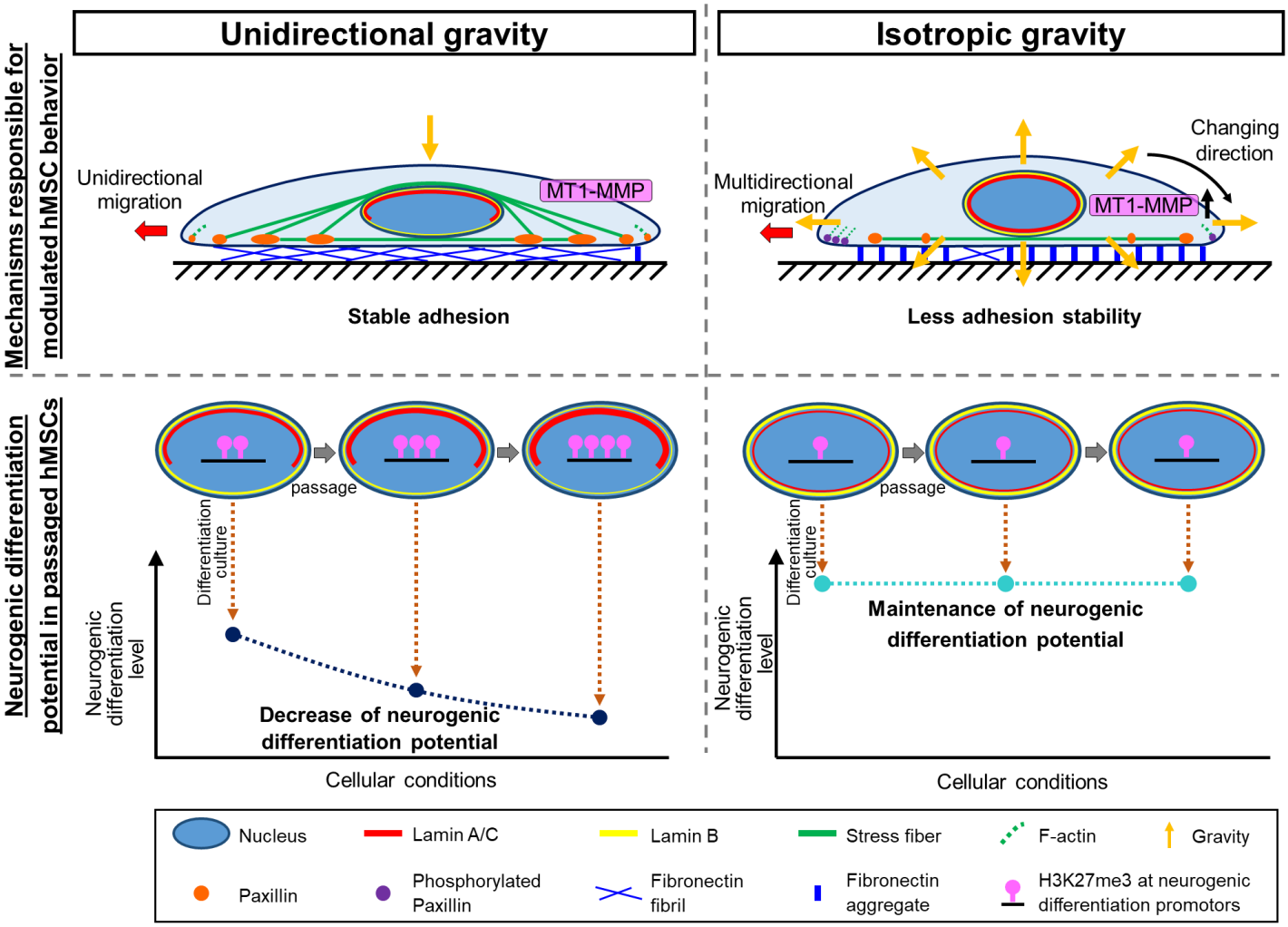

Fig. 3-1 A schematic diagram presenting the research summary of this study.

Next, this study was focusing on stem cells nucleus and differentiation potential in passaged hMSCs due to changes in mechanotransduction induced by altered gravitational culture conditions. This mechanical signaling has increased the lamin ratio between lamin A/C to lamin B in the cells cultured under unidirectional gravity conditions against an increase in their passage numbers. However, cells cultured under isotropic gravity conditions sustained the ratio between lamin A/C to lamin B in the cells, even with increasing passage numbers. Changes in nucleoskeleton organization have affected the chromatin-lamina attachments as well as genome organization. Cells grown under unidirectional gravity conditions had accumulation of H3K27me3 enrichment at neurogenic promoter sites, but cells under isotropic gravity

conditions had a repression of this specific methylation during the serial cultivation. As a result of these phenomena, cells grown under unidirectional gravity conditions showed a reduction in neurogenic levels against an increase in their passage numbers during growth culture. Nevertheless, cells cultured under isotropic gravity conditions retained their neurogenic differentiation potential during passage culture, which expressed significantly higher levels of NF-H and MAP2 proteins than those passaged under unidirectional gravity conditions. Therefore, isotropic gravity culture is a process that is capable of maintaining the neurogenic differentiation capacity in passaged hMSCs by suppression of specific epigenetic modification at the neurogenic promoters together with controlling organization of cytoskeleton and nuclear lamina during the passage culture. Thus, design of culture system, which could regulate the histone modification in hMSCs, is a great challenge for controlling the quality of differentiation ability through the epigenetic memory.

In conclusion, this present study shows the effect of during isotropic gravity culture conditions on hMSCs by the investigation of mechanisms responsible for modulating hMSC migration behaviors, which involves changes in cytoskeleton and nucleoskeleton via the alterations in fibronectin assembly and activity of MMPs. Moreover, the during isotropic gravity culture conditions also affect the mechanotransduction in the cells by altered cytoskeleton formation influencing on inhibition of mechanosensitive lamin A/C polarization and changes in composition of nuclear lamins in the nucleus. The modification of nuclear lamins induced epigenetic modification in passaged hMSCs by histone methylation at specific sites that is associated with the maintenance of neurogenic differentiation potential. Taken together, this study is the first time that successfully provides the critical insight into the influence of isotropic gravity on cell behavior, mechanotransduction, and epigenetics in hMSCs during expansion culture, which promote the directivity of neurogenic differentiation in

hMSCs. This pioneered information would be supportive to the utilization of isotropic gravity to develop the expansion culture system for the applications of cell-based therapy.

3.2 Future perspective

In this study presents the effects of isotropic gravity on neurogenic differentiation potential in hMSCs. This study also shows the investigation of cellular mechanism that influences on the capacity of differentiation by alterations in cytoskeleton formation, lamin organization, and histone modification illustrated in Fig.3-2A. To understand in detail of isotropic gravity effects on the cells relating to application in cell-based therapy, Fig.3-2B presents the flow chart of cellular mechanism that responses to isotropic gravity force. However, it is still many unknown information that should be clarified in the further studies. The following proposals are presenting the author's perspective on future investigations of isotropic gravity effects on the hMSCs in order to be fully understanding about the mechanism of isotropic gravity to the cells in details for supporting the applications of cell-based therapy.

Starting with investigation of cellular sensing components that response to isotropic gravity force. From author's opinion, they have three major parts that should be evaluated during rotating operation by a 3D-clinostat, which are deformation of cell membrane, characteristic of cell-matrix adhesion, and distribution-reaction of cellular components inside the cells. In case of cell membrane, the deformation of this phospholipid bilayers may be occurred in nanoscale. A stimulated-emission-depletion (STED) nanoscopy, which is super-resolution microscopy, is an optional tool for this investigation that has been proved to live-cell tracking for the dynamic of cell membrane in nanoscale (Sahl, S.J. *et al*., 2010). Therefore, this observation system should be installed into the 3D-clinostat system. Then, the cells can be live-fluorescent labeled for cell membrane or cholesterol molecules in the membrane. After that,

during the cell culture by rotations of 3D-clinostat, the effects of isotropic gravity on deformation of cell membrane can be clarified. This investigation would be served an important phenomenon in the cell influenced by isotropic gravity force.

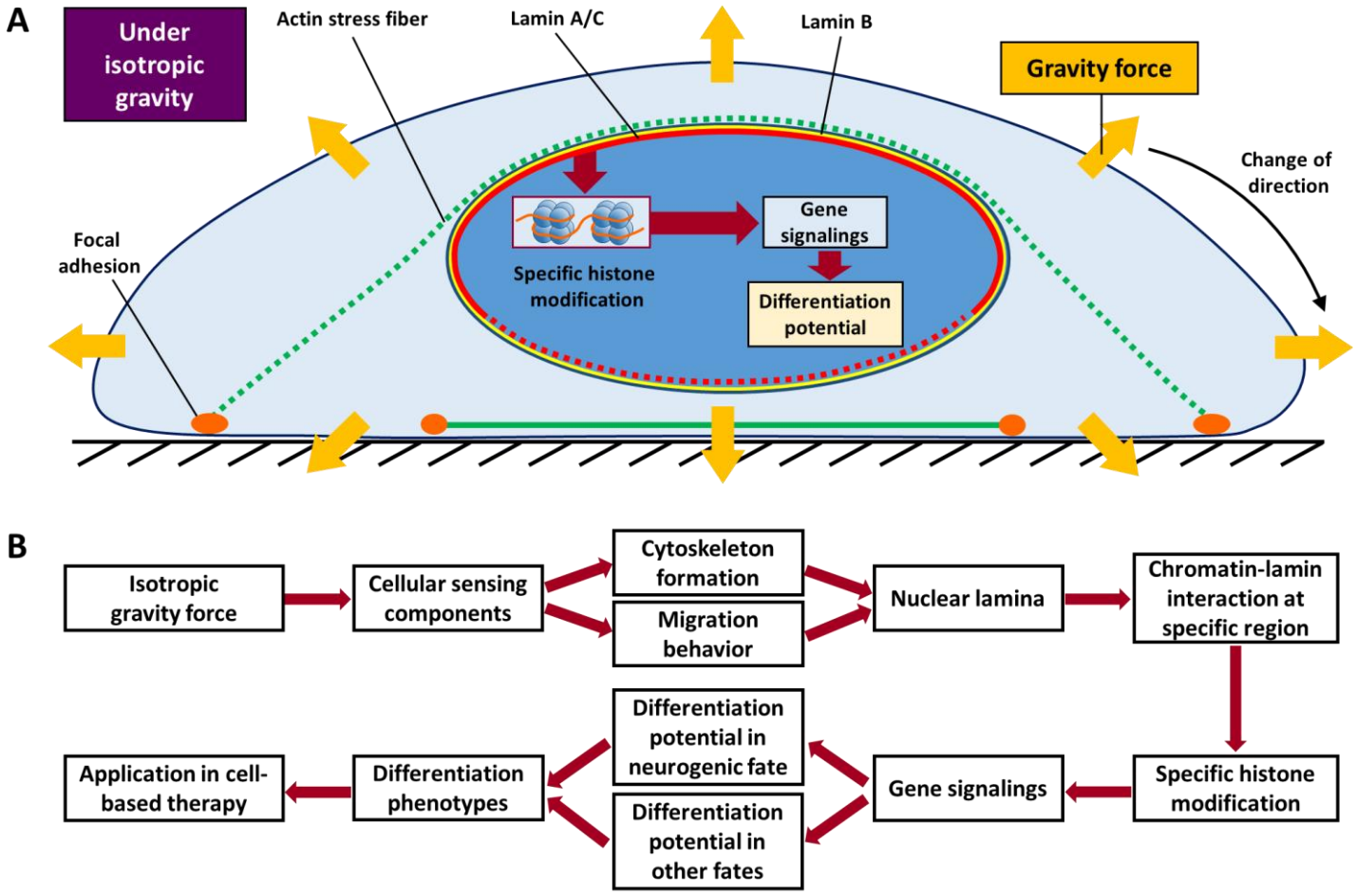

Fig. 3-2 A schematic diagram presenting the basic principle of isotropic gravity effects on differentiation potential in cells by changes in cytoskeleton formation, lamin organization, and specific histone modification that were discovered in this study (A), dash lines show the inhibition of that component formation. A flow chart of cellular responding mechanism to isotropic gravity force since cellular sensing components to differentiation phenotypes for applications in cell-based therapy (B).

In addition, Piezo channels have been reported that are mechanosensitive ion channels on the cell membrane, which regulates transportation of several ion molecules involving with many cascade biological roles including epigenetic modification and differentiation potentials (Coste, B. *et al.*, 2012). The super-resolution microscopy installed into a 3D-clinostat system can be used for tracking the fluorescent-label ions. Author hypothesized that isotropic gravity effects on the composition of the Piezo complex and the activity of these channels, which makes the different transportation rate of some specific ion in the particular area of the cells. This insight information would be great supports to understand the mechanism to other biological phenomena in the cells under isotropic gravity culture conditions.

To investigate the influence of isotropic gravity force on characteristic of cell-matrix adhesion, nanopillar platform should be constructed for being as a culture surface and a confocal laser scanning microscope should be installed into a 3D-clinostat for live cell observation during the rotating operation. These two systems allow to measure cellular tracking force occurring at the culture surface (Shiu, J.-Y. *et al.*, 2018). The live-fluorescent staining dyes for integrins, focal contact proteins, and ECM proteins can be used into this investigation in order to understand the characteristic cell-matrix adhesion in several parts of the cells responding to isotropic gravity force.

To understand the another trigger inside the cells that senses to the isotropic gravity, author hypothesized that the gravity influences on G-actin distribution and rate of F-actin polymerization, which in this study found the different of actin stress fiber formation in the cells cultured under altered gravitational conditions. Therefore, to clarify these phenomena, the fluorescence correlation spectroscopy is one of optional tools that can be used in this investigation during operation of 3D-clinostat. This confocal setup allows to track the fluorescent-labeled molecules such as GFP-actin and possible to measure their diffusion on short time at specific area during the culture (Engelke, H. *et al.*, 2010). This idea of developing

culture system would reveal the physical mechanism in molecular level that can support the understanding of gravitational effects on the sensing components and their responding in the cells.

Moreover, in this study presented that cells cultured under isotropic gravity have formation of basal stress fibers but have less stress fibers at apical site over the nucleus comparing with cells cultured under unidirectional gravity. Therefore, this phenomenon should also be more clarified. Actin depolymerizing factor (ADF)/cofilin is a family of actin-binding proteins which disassembles actin filaments (Wiggan, O.N. *et al.*, 2017). The gradient of ADF/coffilin has been found in the cells for regulating actin organization as well as cellular movement (Wioland, H. *et al*., 2019). Author hypothesized that isotropic gravity force influences on the distribution and localization of ADF/cofilin that effects on depolymerization of actin stress fiber at apical site. Therefore, in order to prove this event, the confocal laser scanning microscope needs to be installed into a 3D-clinostat system. Then, the cells should be fluorescent-stained for ADF/cofilin and F-actin. After that this investigation can be evaluated during the culture under isotropic gravity conditions.

As different of actin structure formation in the cells between the cultures under altered gravitational conditions, which the actin structure relates to migration behavior of the cells. Briefly, RhoA associate with stress fiber formation, Rac1 involves with lamellipodia construction and Cdc42 relates with filopodia protrusion. These three members of the Rho family of small GTPases, regulate a signal transduction pathway linking to that organization of the actin cytoskeleton and associated integrin adhesion complexes (Hall, A., 2005). Therefore, the active form of these Rho family of small GTPases should be investigated in order to clarify the effects on isotropic gravity on the activation of these signalings that can be useful for further implementation in other studies.

In case of mechanotransduction via actin cytoskeleton to nucleus, this study shows the different apical actin stress fibers formation in the cells cultured under altered gravitational conditions. Then, author refers to other publications to claim that apical actin stress fibers is a critical factor to make a compressive force to the nucleus. However, it would be great to prove the phenomenon of mechanotransduction into the nucleus in the cells culture under isotropic gravity conditions. Nesprin and LINC complex are essential components that connect between actin to nucleus membrane and nuclear lamina (Wang, N. *et al*., 2009). Therefore, the investigation of these connecting components together with F-actin in term of localization would be great supports of the phenomena relating to mechanical transduction from actin to nucleus. Moreover, to prove the effect of this mechanotransduction to the nucleus, some sensing events in the nucleus should be measured such as nuclear horizontal spreading ratio, nuclear roughness, and nuclear volume. Thus, this information would strengthen the information about the effects of isotropic gravity on the mechanotransduction between actin and nucleus.

In case of cell behavior, this study found the different in migration pattern of the cells between the cultures under unidirectional gravity and isotropic gravity. Author hypothesized that the change of migration behavior relates to the mechanotransduction to the nucleus presenting as alteration in lamin A/C polarization. To prove these phenomena, nanopillar platform and a confocal laser scanning microscope should be utilized in this study. During the rotating operation by a 3D-clinostat, the live fluorescent-labeled actin, focal contact proteins, and nucleus as well as deformation of the nanopillar module can present the level of tension force in the actin filament that influences to the nuclear deformation during cell migration. This information would greatly support the influence of migration behavior of the cells under both gravitational culture conditions on the mechanotransduction from ECM to the nucleus via actin cytoskeleton organization.

Moreover, the results of this study purposed that isotropic gravity affects the mechanotransduction in hMSCs. Recently, Hippo pathway-YAP/TAZ signaling has been reported that it relates to the mechanical signalings. The inactive phosphorylated YAP/TAZ located in cytoplasm can be transformed to active form of YAP/TAZ and translocate into the nucleus via the nuclear pore complex (Elosegui-Artola, A. *et al*., 2017). Localization of active YAP/TAZ are another indicator of mechanical memorization in the cells (Heo, S.J. *et al*., 2013). The activation of YAP/TAZ has been widely reported to involve with several differentiation potentials (Swift, J. *et al*., 2015). Moreover, nuclear pore composition has been reported that relates to the mechanical loadings (Donnaloja, F. et al., 2019) and regulates differentiation process in the cells (D'Angelo, Maximiliano A. et al., 2012). Hence, it would be worth to investigate these mechanosensitive signalings in the cells cultured under isotropic gravity conditions in term of YAP/TAZ activation and composition of the nuclear pore complex. These further information regarding the influence of isotropic gravity on YAP/TAZ signaling would be remarkable data for other scientists to implement the isotropic gravity culture on their targeted-applications.

As this study presents the difference of lamin A/C polarization in the cells cultured altered gravitational conditions. However, the information of relationship between nuclear lamins and histone modification in term of localization especially at apical and basal sites of the nucleus is still lack of understanding. Moreover, this study shows the histone modification only in case of H3K4me3 and H3K27me3. Therefore, acetylation and methylation at other lysine residues such as H3K9ac, H3K12ac, H3K16ac, H3K9me3, and H3K36me3 should also be clarified. Moreover, in the further study as investigation of histone modification by observation method should be performed in order to know the information about the influence of lamin A/C polarization on the distribution and localization of several histone modifications in the nucleus.

In addition, the mechanism of nuclear lamin organization to histone modification at specific site is still not fully understanding yet. The chromatin immunoprecipitation assays with sequencing (ChIP-seq), which is a recent powerful tool for defining DNA-protein transcriptomes and DNA adenine methyltransferase identification (DamID), which is a molecular biology tool used to map the binding sites of DNA proteins in nuclear lamina (Peric-Hupkes, D. *et al*., 2010). These two processes can be applied for this investigation that can allow to understand the whole interaction of nuclear lamin and chromatin region relating to histone modification at specific site. Moreover, changes in DNA methylation in the specific chromatin region is also another interesting point of epigenetic modification. The DNA methylation is a methylation process occurring on the DNA, this methylation inhibits the transcription of that gene regions and the DNA methylation is also another kind of epigenetic memory in the cells (Bird, A., 2002; Kim, K. *et al*., 2010). Therefore, it would be worth investigated by DNA methylation sequencing to obtain the data of DNA methylation in whole region. Therefore, this further information achieved by these recent technologies would be great supports for the critical data of isotropic gravity effects on whole epigenetic modification in the cells.

As the ability of hMSCs is able to differentiate into osteogenic, adipogenic, and chondrogenic fates. However, this study presents only the mechanism relating to differentiation potential into neurogenic fate. Therefore, the isotropic gravity effects on changes in histone acetylation and methylation at specific regions and DNA methylation at particular sites relating to other lineages of differentiation should be more clarified. Moreover, the differentiation potentials in passaged hMSCs into those lineages are also attractive information. Quantitative data of differentiation products produced by differentiated cells, which are widely used to quantify the differentiation potential found in the most international publications, should be evaluated. For example, in case of osteogenic fate, the calcium deposition is a product of bone

cells. This calcium mineralization can be measured by Alizarin Red S staining quantification assay. In case of adipogenic fate, lipid is a main product of the fat cells. These lipid droplets can be measured by Oil Red O staining quantification assay. About chondrogenic fate, glycosaminoglycan (GAG) and type II collagen are two main components produced by chondrocytes, which can be measured by Dimethylmethylene Blue assay and western blot analysis, respectively (Tsai, C.C. *et al*., 2012). These further data would be great supports for development of cell expansion process for wider range of therapeutic applications by using hMSCs.

In order to support the utilization of isotropic gravity in the applications of cell-based therapy, the additional experiments in the *in vivo* system are required. The *in vivo* system should be demonstrated the animal model involving with a target of the therapy. Then, the cells which cultured under isotropic gravity conditions should be used to clarified the characteristic and performance of therapy *in vivo*. The successful of this study would greatly support the important information relating to the applications of cell-based therapy.

Lastly, exosome technology is recently focused by many researchers caused by unique compositions within the exosome vesicle such as genetic materials (mRNA, mi-RNA), signaling molecules, and specific enzymes. Generally, exosome is produced and secreted from donor cells and then affects the particular functions in the receiving cells. Currently, applications of exosome have been widely studied in several clinical trials as “cell-free therapy”, and exosome could become a new powerful material with more efficiency in therapeutics applications than recent pharmaceutical treatments in some diseases. Many researchers have been reported that culture environment and mechanical signalings in the cells influence on the properties and secretion of the exosome (Kusuma, G.D. *et al*., 2017). As this study demonstrates that isotropic gravity influenced the dynamic hMSC behaviors and the mechanotransduction in the cells. Therefore, the author would like to propose the further study

in the influence of isotropic gravity on the properties and characteristics of secreted exosome. The co-culture system with other cell types as well as specific chemical induction are also worth considering parameters that need also be investigated. It is thought that these data will provide the cutting-edge information that could be applied for the production of exosome in biomedical industry, which can further improve the performance of regenerative medicine as well.

Nomenclature

α	Attachment efficiency	[-]
μ	Specific growth rate	[h^{-1}]
C_t	Cycle threshold value	[cycle]
X_0	Cell density at the seeding	[cells/cm^2]
X_{24}	Cell density after seeding for 24 h	[cells/cm^2]
X_{120}	Cell density after seeding for 120 h	[cells/cm^2]

Abbreviations

µm	Micrometer
3D	Three dimensional
ANOVA	Analysis of variance
BCA	Bicinchoninic acid
ChIP	Chromatin immunoprecipitation
ChIP-seq	Chromatin immunoprecipitation assays with sequencing
CHO	Chinese hamster ovary
CNS	Central nervous system
CO_2	Carbon dioxide
DamID	DNA adenine methyltransferase identification
DAPI	4′,6-diamidino-2-phenylindole
DMEM	Dulbecco’s modified Eagle’s medium
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
F-actin	Filamentous actin
FBS	Fetal bovine serum
G	Gravity
GAG	Glycosaminoglycan
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
h	Hour(s)
H3	Histone H3
H3K4me3	Histone H3 trimethylation at lysine 4
H3K27me3	Histone H3 trimethylation at lysine 27

hESCs	Human embryonic stem cells
hMSCs	Human mesenchymal stem cells
IgG	Immunoglobulin G
ISCT	The international Society for Cell and Gene Therapy
Iso	Isotropic gravity conditions
LINC	Linker of nucleoskeleton and cytoskeleton
MAP2	Microtubule-associated protein 2
miRNA	Micro ribonucleic acid
MMPs	Matrix metalloproteinases
mRNA	Messenger ribonucleic acid
MT1-MMP	Membrane-type 1 matrix metalloproteinase
NF-H	Neurofilament heavy
P	Passage number
PBS	Phosphate buffered saline
PVDF	Polyvinylidene fluoride
qRT-PCR	Quantitative real-time reverse transcription–polymerase chain reaction
RIPA	Radioimmunoprecipitation
RNA	Ribonucleic acid
RPM	Random positioning machine
RT-PCR	Real-time reverse transcription–polymerase chain reaction
RWV	Rotating wall vessel
SCI	Spinal cord injury
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Si-RNA	Small interfering RNA
TAZ	Transcriptional coactivator with PDZ-binding motif
TBI	Traumatic brain injury
TBS	Tris-buffered saline
Uni	Unidirectional gravity conditions
YAP	Yes-associated protein

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List of publications

- Koaykul C., Kim M.H., Kawahara Y., Yuge L., and Kino-oka M., (2019), "Alterations in Nuclear Lamina and the Cytoskeleton of Bone Marrow-derived Human Mesenchymal Stem Cells Cultured Under Simulated Microgravity Conditions", **Stem Cells and Developments**, Vol. 28, No. 17, pp. 1167-1176. (doi:10.1089/scd.2018. 0229).
- Koaykul C., Kim M.H., Kawahara Y., Yuge L., and Kino-oka M., (2019), "Maintenance of Neurogenic Differentiation Potential in Passaged Bone Marrow-derived Human Mesenchymal Stem Cells Under Simulated Microgravity Conditions". **Stem Cells and Developments**, Vol. 28, No. 23, pp. 1552-1561. (doi:10.1089/ scd.2019.0146).

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