



## Original Article

# Hypergravity enhances RBM4 expression in human bone marrow-derived mesenchymal stem cells and accelerates their differentiation into neurons



Masataka Teranishi <sup>a</sup>, Tomoyuki Kurose <sup>a</sup>, Kei Nakagawa <sup>a</sup>, Yumi Kawahara <sup>b</sup>,  
Louis Yuge <sup>a, b, \*</sup>

<sup>a</sup> Division of Bio-Environmental Adaptation Sciences, Graduate School of Biomedical and Health Sciences, Hiroshima University, Hiroshima, Japan

<sup>b</sup> Space Bio-Laboratories Co. Ltd. Hiroshima, Japan

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## ABSTRACT

**Introduction:** The regulation of stem cell differentiation is important in determining the quality of transplanted cells in regenerative medicine. Physical stimuli are involved in regulating stem cell differentiation, and in particular, research on the regulation of differentiation using gravity is an attractive choice. We have shown that microgravity is useful for maintaining undifferentiated mesenchymal stem cells (MSCs). However, the effects of hypergravity on the differentiation of MSCs, especially on neural differentiation related to neural regeneration, have not been elucidated.

**Methods:** We induced neural differentiation of human bone marrow-derived MSCs (hbMSCs) for 10 days under normal gravity (1G) or hypergravity (3G) conditions using a gravity controller, Gravite®. HbMSCs were collected, and cell number and viability were measured 3 and 10 days after induction. RNA was also extracted from the collected hbMSCs, and the expression of neuron-associated genes and regulator markers of neural differentiation was analyzed using real-time polymerase chain reaction (PCR). Additionally, we evaluated the NF-M-positive cell rate 10 days after induction using immunofluorescent staining.

**Results:** Neural gene expression and the NF-M-positive cell rate were increased in hbMSCs under the 3G condition 10 days after induction. mRNA expression of RNA binding motif protein 4 (RBM4) and pyruvate kinase M 1 (PKM1) in the 3G condition was also higher than that in the 1G group.

**Conclusions:** Hypergravity can enhance *RBM4* and *PKM1*, promoting the neural differentiation of hbMSCs. © 2023, The Japanese Society for Regenerative Medicine. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

## 1. Introduction

Regulation of stem cell differentiation, including mesenchymal stem cells (MSCs), is crucial in the field of regenerative medicine. The regulation of stem cell differentiation is vital in determining the quality of transplanted cells and affects the clinical efficacy of

cell therapy [1]. Therefore, research on regulating stem cell differentiation and its mechanisms has been conducted worldwide using various methods. In general, cytokines and gene transfection are used to control stem cell differentiation; however, it has been suggested that those methods may induce chemical side effects and tumorigenesis [2]. On the other hand, regulation of stem cell differentiation via physical stimuli can reduce those risks and may be a valuable method for improving the safety and therapeutic effect of transplanted cells.

Physical stimuli, including gravity, are an attractive choice as factors affecting biological events in living organisms. This reveals that physical stimuli trigger the biochemical signals related to molecular cascades that result in altered cell proliferation and differentiation and, thus, in variations in tissue structure and function [3–5]. We examined the effects of various physical stimuli

**Abbreviations:** MG, microgravity; MSCs, mesenchymal stem cells; PKM, pyruvate kinase M; RbBM4, RNA binding motif protein 4.

\* Corresponding author. Division of Bio-Environmental Adaptation Sciences, Graduate school of Biomedical and Health Sciences, Hiroshima University, Hiroshima 734-8551, Japan. Fax: +81 82 257 5344.

E-mail address: [ryuge@hiroshima-u.ac.jp](mailto:ryuge@hiroshima-u.ac.jp) (L. Yuge).

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on cell differentiation. The musculoskeletal system is susceptible to physical stimuli, as exemplified by muscle and bone atrophy in the space environment [6]. We reported that myoblast differentiation is suppressed under microgravity (MG) and enhanced with electrical stimulation [7–9]. Studies using osteoblasts also suggested that bone differentiation is suppressed in an MG environment and that magnetic fields and ultrasound stimulation promote bone differentiation [4,10,11]. In addition, other similar studies have demonstrated that these physical stimuli affect cell differentiation [12–15].

In the previous studies, we examined the effects of MG on MSCs. MSCs can secrete various neurotrophic factors and may differentiate into neurons, causing positive effects on neuroprotection and neurogenesis [16]. MSCs have therapeutic effects on central nervous system diseases [17]. We previously reported that MG effectively maintains the undifferentiated state of stem cells and affects the neural differentiation of MSCs [18–20]. We also demonstrated that the nuclear structure and cytoskeleton were altered in MSCs cultured with MG [21]. Conversely, it has been reported that hypergravity affects the differentiation of MSCs into osteoblasts [22–24]. Hypergravity also influences PC12 neuron-like cell differentiation [25]. However, these studies used short-term exposure to severe hypergravity (10–150G) as the experimental condition. The long-term effect of moderate hypergravity (2–3G) during the induction of differentiation was not considered and is yet to be investigated. In addition, no research has been conducted on the influence of hypergravity on the neural differentiation potential of MSCs.

Therefore, this study aimed to induce neural differentiation of MSCs under normal gravity (1G) or hypergravity (3G) conditions for 10 days and to investigate the effect of 3G on neural differentiation.

Furthermore, regarding the neuronal differentiation of MSCs, a previous study has reported that RNA binding motif protein 4 (RBM4) regulates MSC neural differentiation through intervention in pyruvate kinase M (PKM), switching from PKM2 to PKM1 [26]. Su et al. demonstrated that RBM4 expression is upregulated by exposure to hypoxic conditions [27]. Although it has been shown that RBM4 responds to hypoxia, its response to the gravity environment has not been reported.

Thus, we focused on whether RBM4 expression affects the neural differentiation of MSCs under the 3G condition.

## 2. Methods

### 2.1. Culturing of MSCs

Human bone marrow-derived MSCs (hbMSCs) were purchased from Lonza (Lonza, Switzerland). HbMSCs were seeded in culture dishes (Sumitomo Bakelite, Japan) and cultured to approximately 80% confluent. The cell growth medium for hbMSCs consisted of Dulbecco's Modified Eagle's Medium (DMEM)-low glucose (Sigma-Aldrich, USA) with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, USA), penicillin (pc; 100 U/mL), and streptomycin (sm; 100 µg/mL; both from Sigma-Aldrich). Cells adhered to the culture dish were used as hbMSCs. Cells were cultured under conditions of 37 °C and 5% CO<sub>2</sub>. The growth medium was changed once every 3 days. HbMSCs cultured at approximately 80% confluent were collected and seeded onto FALCON® 12.5-T culture flasks (Corning, USA) at a density of  $5.0 \times 10^3$  cells/cm<sup>2</sup>.

### 2.2. Neural differentiation

According to our reported methods [17], we used a neural conditioning medium and a neural differentiation medium for inducing neural differentiation. Specifically, cells were cultured

with the growth medium until 60%–80% confluent in culture flasks. Then, the medium was switched to the neural conditioning medium containing DMEM/Ham's F-12 (FUJIFILM Wako Pure Chemical, Japan) with 1% FBS, 1% pc/sm, and basic fibroblast growth factor (100 ng/mL; PeproTech, USA). After incubation in this neural conditioning medium for 3 days, the medium was changed to the neural differentiation medium composed of neural conditioning medium with forskolin (10 µM; Sigma-Aldrich), and cells were cultured in this neural differentiation medium for 7 days. The cultures were incubated at 37 °C and 5% CO<sub>2</sub>. Moreover, the induction of neural differentiation was performed using the flask filled with neural conditioning or differentiation medium. Cells were collected 3 and 10 days after the induction of neural differentiation when the medium was switched or the protocol was finished. The experimental protocol is shown in Fig. 1.

### 2.3. Experimental conditions

Cells were divided into 1G or 3G conditions to induce neural differentiation in altered gravity. The 3G condition was produced using Gravite® (Space Bio-Laboratories, Japan), which can establish 3G conditions via controlled centrifugation of one axis. As for the control group, cells under the 1G condition were placed statically within an incubator during neural differentiation. HbMSCs, in passages 3 or 4, were used for this experiment.

### 2.4. Cell viability assay

The cells collected 3 and 10 days after the induction of neural differentiation were stained with trypan blue stain (Thermo Fisher Scientific). Live and dead cells were counted using a WATSON® hemocytometer (FukaeKasei, Japan), and the number of cells was measured using the inverted phase contrast microscope (Eclipse TE300; Nikon, Japan). Cell viability was also estimated by dividing the number of live cells measured by the total number of cells.

### 2.5. RNA extraction and reverse transcription

After 3 and 10 days of neural differentiation under either 1G or 3G condition, hbMSCs were collected in phosphate buffer saline (PBS). Total RNA was extracted using NucleoSpin® RNA (MACHERY-NAGEL, Germany) according to the manufacturer's protocol. We used a NanoDrop spectrophotometer (Thermo Fisher Scientific) to measure RNA concentration. According to the manufacturer's instructions, complementary DNA was synthesized using the ReverTra Ace-α-(Toyobo, Japan) and T100™ Thermal Cycler (Bio-Rad Laboratories, USA).

### 2.6. Real-time PCR

A real-time polymerase chain reaction (PCR) was conducted with the CFX96 Touch Real-Time PCR Detection system (Bio-Rad Laboratories). Real-time PCR was performed using Fast Start Universal Probe Master (Roche, Switzerland). The oligonucleotide primers (Sigma-Aldrich) correspond to nestin (NES) as a neural progenitor cell marker, achaete-scute homolog 1 (ASCL1) as an intermediate progenitor cell marker, neuron-specific class III beta-tubulin (TUJ1) as an immature neuronal marker, microtubule-associated protein 2 (MAP2), neurofilament medium chain (NF-M), and neurofilament heavy chain (NEFH) as mature neuronal markers, and RNA binding motif protein 4 (RBM4). Additionally, TaqMan® probes (Thermo Fisher Scientific) corresponding to polypyrimidine tract binding protein 1 (PTB), pyruvate kinase M 1 (PKM1), and pyruvate kinase M 2 (PKM2) were also used.

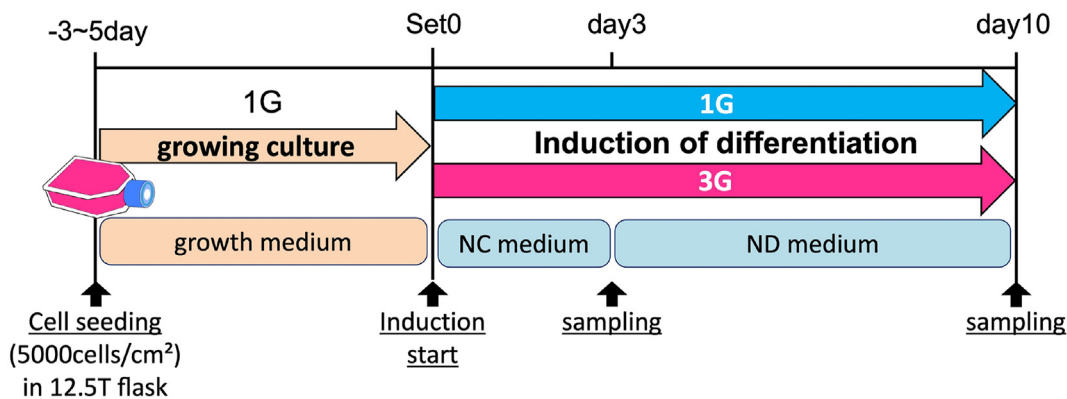


Fig. 1. The experimental protocol of neural differentiation induction. The schedule for the neural differentiation induction. NC: neural conditioning; ND: neural differentiation.

Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; Applied Biosystems, USA) was used as an internal control.

### 2.7. Immunofluorescent staining

The cells were washed twice with PBS and fixed with a 4% paraformaldehyde solution. The cells were washed with PBS three times. Blocking was performed with PBS containing 1% bovine serum albumin for 60 min. The primary antibody against NF-M was the mouse anti-Neurofilament-M antibody (RMO14.9; Cell Signaling Technology, USA), diluted 1:100. The primary antibody reaction was performed overnight at 4 °C. The secondary antibody was goat Alexa Fluor® 488 anti-mouse IgG antibody (Invitrogen, USA) diluted 1:500. The secondary antibody reaction was conducted for 60 min at room temperature (23°C–25 °C) in the shade. For nuclear staining, 4',6-diamidino-2-phenylindole dihydrochloride (Kirkegaard & Perry Laboratories, USA) was used at a dilution of 1:800. The reaction was conducted for 10 min at room temperature (23°C–25 °C) in the shade. The region excluding the primary antibody was also prepared as a negative control.

### 2.8. Evaluation of positive cell rates

The NF-M-positive cell rate 10 days after induction was evaluated using a multifunction microscope (BZ-9000; KEYENCE, Japan). Ten fields of view were taken at random with no overlapping points in each group. Image analysis software within the multifunction

microscope was used to count the number of NF-M-positive cells and cell nuclei.

### 2.9. Statistical analysis

For proliferation, cell viability, and gene expression analysis, data were analyzed statistically using a two-way analysis of variance with Student's t-test and expressed as mean ± SEM. Unpaired Student's t-test was used to evaluate the NF-M-positive cell rate 10 days after induction. A value of  $p < 0.05$  was considered statistically significant. All statistical analyses were performed using the JMP Pro 16 statistical software (SAS, USA).

## 3. Results

### 3.1. Collected cell number and cell viability

The number of collected cells and the percentage of cell viability in each group was calculated on days 3 and 10. There was no significant difference between 1G and 3G groups in cell number and viability at any time point (Fig. 2A and B).

### 3.2. mRNA expression of neural markers

The mRNA expression of MAP2, NF-M, and NEFH was significantly higher in the 3G group than in the 1G group on day 10 (Fig. 3A–C). There was no significant difference between 1G and 3G

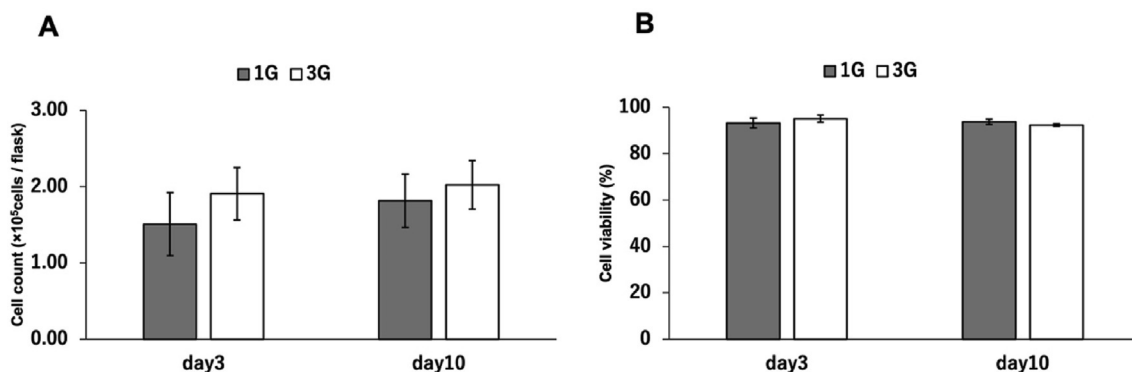
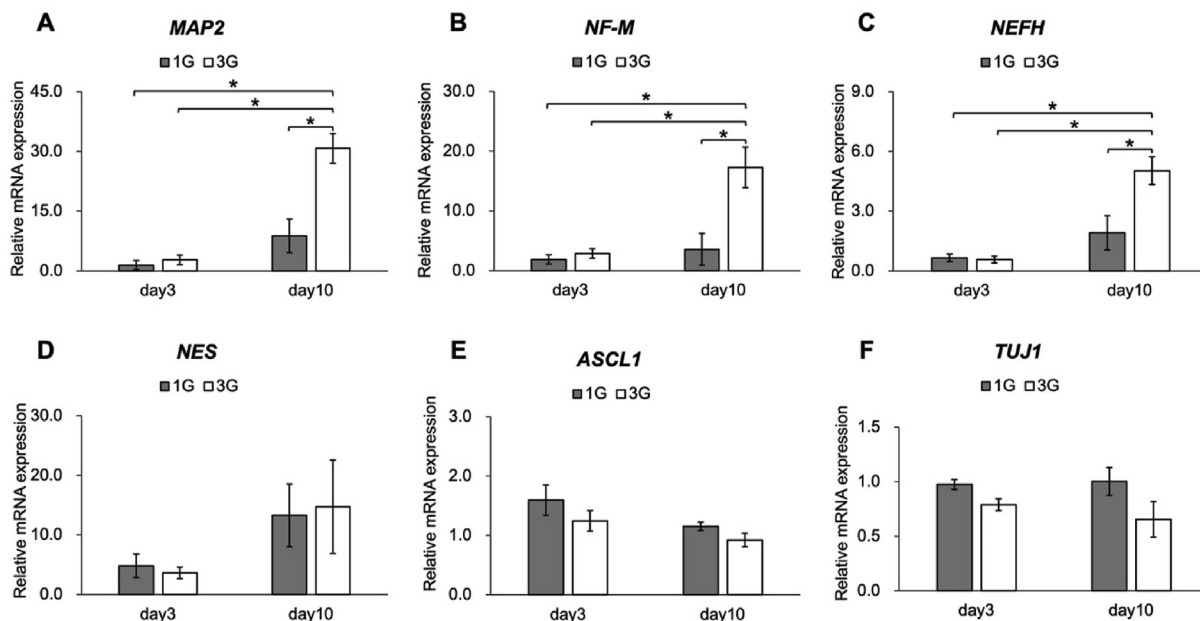


Fig. 2. Collected cell number and cell viability in hbMSCs. The number of collected cells and the percentage of cell viability in each group were calculated on days 3 and 10 (A) Cell count on days 3 and 10 in each group. (B) Cell viability on days 3 and 10 in each group. Data = mean ± SEM, n = 3 in each group.



**Fig. 3.** Gene expressions of neural markers in hbMSCs. mRNA expressions of MAP2 (A), NF-M (B), NEFH (C), NES (D), ASCL1 (E), and TUJ1 (F). Data = mean ± SEM, n = 3 in each group. \*p < 0.05 vs. 3G on day 10. MAP2: microtubule-associated protein 2; NF-M: neurofilament medium chain; NEFH: neurofilament heavy chain; NES: nestin; ASCL1: achaete-scute homolog 1; TUJ1: neuron-specific class III beta-tubulin; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

groups in the mRNA expression of NES, TUJ1, and ASCL1 at any time point (Fig. 3D–F).

### 3.3. Positive cell rates of NF-M protein

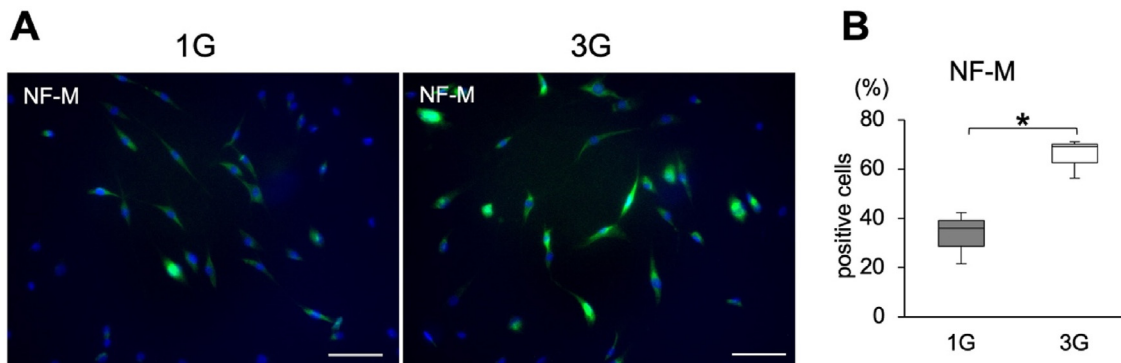
The expression of NF-M protein in each group was investigated via immunofluorescence staining. After induction of neuronal differentiation in 1G and 3G groups, the NF-M-positive cells were detected 10 days after induction (Fig. 4A). The percentage of NF-M-positive cells in the 3G group was significantly higher than that in the 1G group on day 10 (Fig. 4B).

### 3.4. mRNA expression of RBM4, PTB, and PKM

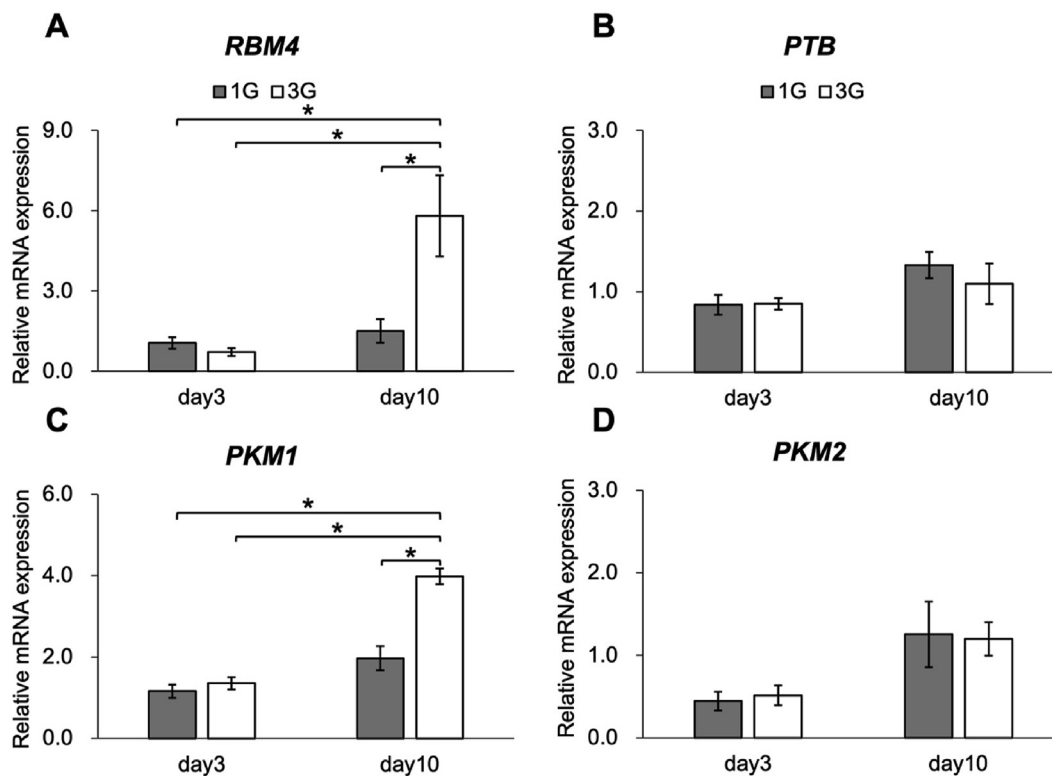
The mRNA expression of RBM4 and PKM1 was significantly higher in the 3G group than in the 1G group on day 10 (Fig. 5A and C). There was no significant difference between 1G and 3G groups in PTB and PKM2 expression at any time point (Fig. 5B and D).

## 4. Discussion

We investigated how 3G hypergravity affected hbMSCs during the induction of neuronal differentiation. It is proposed that MSCs have different characteristics depending on the tissue from which they are derived [28,29]. Because hbMSCs have a limited potential for neural differentiation, they were selected in this study to elucidate their effect on neuronal development. As a result, on day 10, the mRNA expression of MAP2, NF-M, and NEFH in hbMSCs was significantly higher in the 3G group than in the 1G group. Moreover, the 3G group showed a significant increase in a time-dependent manner. Alternatively, the 1G group showed an increase in the expression of neural differentiation markers during the induction of neural differentiation but no significant change. On day 10, the percentage of NF-M-positive cells was higher in the 3G group compared to the 1G group. These results suggest that the 3G condition with neural differentiation-inducing enhanced the neural differentiation of hbMSCs, which are difficult to differentiate. Previous research has shown that cultural environment alterations



**Fig. 4.** The percent of NF-M positive cells. (A) Immunofluorescent staining images of each group on day 10. NF-M (day 10) was dyed (green), and nuclear staining (blue) was performed. Scale bar = 100 μm. (B) The percent of NF-M-positive cells were counted on day 10. Data = mean ± SEM, n = 3 in each group. The boxes indicate the first quartile to the third quartile, and the lines in the boxes indicate the median. The error bars indicate the minimum and maximum values. \*p < 0.05 vs. 3G on day 10.



**Fig. 5.** Gene expressions of neural differentiation regulators in hbMSCs. mRNA expressions of RBM4 (A), PTB (B), PKM1 (C), and PKM2 (D). Data = mean  $\pm$  SEM,  $n = 3$  in each group. \* $p < 0.05$  vs. 3G on day 10. RBM4: RNA binding motif protein 4; PTB: polypyrimidine tract binding protein 1; PKM1: pyruvate kinase M 1; PKM2: pyruvate kinase M 2; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

significantly impact how MSCs differentiate into neurons [20,30,31]. Wang et al. demonstrated that hypoxia promotes dopaminergic neuronal differentiation of MSCs [30]. Our previous study reported that electrical stimulation effectively induced neuronal differentiation of MSCs [31]. Furthermore, we discovered that MSCs cultured in a microgravity environment effectively induced neuronal differentiation under 1G [20]. Conversely, hypergravity affects the differentiation of PC12 neuron-like cells, accelerating neurite emission and increasing neurite length [25]. As a result, we have further proof that changed gravity, particularly the 3G condition, can influence and accelerate the neural differentiation of MSCs.

We also looked into the mechanism underlying hbMSC neural differentiation in the 3G condition. RBM4 and PTB have vital roles in the neural differentiation of MSCs [26,27,32–34]. RBM4 promotes the differentiation of neuronal progenitor cells and neurite outgrowth of cultured neurons via its role in splicing regulation [33,34]. PTB is a negative regulator of neuronal development and stimulates PKM2 expression [27,35]. A previous study has demonstrated that RBM4 promotes neuronal differentiation of MSCs by suppressing the PTB expression and upregulating the expression of neuron-associated genes via PKM isoform switching from PKM2 to PKM1 under the hypoxia condition [27]. By regulating the alternative splicing of PKM pre-mRNA, RBM4 promotes the differentiation of MSCs into neural cells [27]. This suggests that the PKM isoform shift mediated by RBM4 plays a role in differentiating MSCs into neural cells. PKM2 expression is shown to be highly elevated in neural progenitor cells during proliferative culture and declines, while PKM1 expression increases with neural differentiation [36]. As a result, this study looked at the mRNA expression of RBM4, PTB, and PKM related to the neural

differentiation of MSCs. The outcomes demonstrated that on day 10, hbMSCs from the 3G group expressed considerably more RBM4 and PKM1 mRNA than those from the 1G group. Conversely, PTB and PKM2 expression of hbMSCs was not significantly reduced on day 10 after the induction. We showed that hypergravity could increase the expression of RBM4 and promote the neural differentiation of hbMSCs, increasing PKM1 expression.

HbMSCs are one of the stem cells that are applied clinically as a source of neuro-regenerative medicine because the collection method of hbMSCs is well established and a sufficient amount can be acquired, as typified by bone marrow transplantation for leukemia, although it is invasive [37]. Understanding the mechanism of MSC neural differentiation is essential to verify the degree and quality of stem cell differentiation and to accelerate the practical application of MSCs-based neural regeneration. Regenerative medicine is a developing field. According to the nerve regeneration process, appropriate cells must be transplanted at the right time to maximize the benefits of regenerative medicine.

## 5. Conclusions

We showed that the 3G condition effectively induced neural differentiation of hbMSCs. In fact, hbMSCs exposed to 3G differentiated earlier than those exposed to 1G. Additionally, our results demonstrated that 3G enhanced the gene expressions of *RBM4* and *PKM1* during neural differentiation in culture. We suggest that hypergravity may contribute to the differentiation of hbMSCs into neural cells during the induction of neural differentiation. This method of differentiation induction may contribute to shortening the induction period and improving the efficiency of regulation of stem cell differentiation.

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## Declaration of competing interest

L. Y. is a director of Space Bio-Laboratories Co.Ltd. (SBL) and Y. K. is the president of SBL. They are shareholder. They were involved in supervision and writing review & editing. They were not contributed to the measurement and analysis of the data. All other authors declare that no conflicts exist.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.reth.2022.12.010>.

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