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Effects of altered gravity on the cell cycle, actin cytoskeleton and proteome in *Physarum polycephalum*

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Abstract

Some researchers suggest that the changes of cell cycle under the effect of microgravity may be associated with many serious adverse physiological changes. In the search for underlying mechanisms and possible new countermeasures, we used the slime mold *Physarum polycephalum* in which all the nuclei traverse the cell cycle in natural synchrony to study the effects of altered gravity on the cell cycle, actin cytoskeleton and proteome. In parallel, the cell cycle was analyzed in *Physarum* incubated (1) in altered gravity for 20 h, (2) in altered gravity for 40 h, (3) in altered gravity for 80 h, and (4) in ground controls. The cell cycle, the actin cytoskeleton, and proteome in the altered gravity and ground controls were examined. The results indicated that the duration of the G2 phase was lengthened 20 min in high aspect ratio vessel (HARV) for 20 h, and prolonged 2 h in altered gravity either for 40 h or for 80 h, whereas the duration of other phases in the cell cycle was unchanged with respect to the control. The microfilaments in G2 phase had a reduced number of fibers and a unique abnormal morphology in altered gravity for 40 h, whereas the microfilaments in other phases of cell cycle were unchanged when compared to controls. Employing classical two-dimensional electrophoresis (2-DE), we examined the effect of the altered gravity on *P. polycephalum* proteins. The increase in the duration of G2 phase in altered gravity for 40 h was accompanied by changes in the 2-DE protein profiles, over controls. Out of a total of 200 protein spots investigated in G2 phase, which were reproducible in repeated experiments, 72 protein spots were visually identified as specially expressed, and 11 proteins were up-regulated by 2-fold and 28 proteins were down-regulated by 2-fold over controls. Out of a total of three low-expressed proteins in G2 phase in altered gravity for 40 h, two proteins were unknown proteins, and one protein was spherulin 3b by MALDI-TOF mass spectrometry (MS). Our results suggest that a low level of spherulin 3b in G2 phase, which may lead to a reduction of Poly(b-L-malate) (PMLA), may contribute to the lengthened duration of G2 phase in altered gravity for 40 h. Present results indicate that altered gravity results in the prolongation of G2 phase with significantly altered actin cytoskeleton and proteome in *P. polycephalum*.

Keywords: Altered gravity; Cell cycle; Cytoskeleton; Proteome; *Physarum polycephalum*

Abbreviations: 2-DE, Two-dimensional electrophoresis; MS, Mass spectrometry; PMLA, Poly(b-L-malate); MSD, Murashige and Skoog medium; HARV, High aspect ratio vessel; FITC, Fluorescein isothiocyanate; BSA, Bovine serum albumin; PBS, Phosphate-buffered saline; TCA, Trichloroacetic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate; DTT, 1, 4-dithiothreitol; PAGE, Polyacrylamide gel electrophoresis; IPG, Immobilized pH gradient; IEF, Isoelectric focusing; SDS, Sodium dodecyl sulfate; UVP, Under voltage protection; MALDI-TOF-MS, matrix-assisted laser desorption ionization/time of flight/mass spectrometry; NCBI, National Center for Biotechnology Information.

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1. Introduction

During space fight, astronauts are constantly exposed to space radiation in a microgravity environment consisting of a field of electrons, protons and heavy ions [1]. The stress response pathways responding to microgravity exposure have many overlapping components including growth factors, cytokines, genes and proteins involved in cell cycle, apoptosis, and signaling pathways. The significant physiological changes occur responding to microgravity exposure, which include bone resorption, cardiovascular deconditioning, muscle atrophy, vestibular disturbances, orthostatic intolerance, fluid shifting, renal stones, and immune dysfunction [2,3]. These changes are therefore considered to be a challenge to life in space. This study aims to investigate the role of altered gravity across the cytoskeleton and proteins to the cell cycle.

Many results about the variation of the cell cycle under space or microgravity condition come from research of animal cells [4–6], while these studies have been limited. One main reason for this limitation is that the degree of mitotic synchronization in the great majority of animal cells is too low to carry out biochemical analysis of the cells, and chemical or physical treatments for synchronization may affect the cell cycle regulators. It has become a necessity, therefore, to provide evidence concerning the variation of cell cycle under naturally synchronized conditions. *Physarum polycephalum* may provide an alternative and suitable model for studying the molecular biology of the cell cycle. *P. polycephalum*, a member of the *Physarum* family of myxomycophyta, synchronizes under natural conditions. A macroplasmodium of *Physarum* about 5–6 cm in diameter contains a large number of nuclei (about 10^8–10^9), which go naturally synchronized into intranuclear mitosis. One can monitor the molecular details of the progress of a single cell throughout the mitotic cycle by repeated sampling from a single plasmodium. Therefore, *Physarum* is a perfect material to study the biochemical events in the cell cycle [7]. In the present paper, we used the slime mold *P. polycephalum* to study the effects of altered gravity on the cell cycle, actin cytoskeleton and proteome. An attempt was made to get some insights concerning cell cycles in the gravitational field and their interactions with the environment of altered gravity basing on the cytoskeleton behavior and proteins in altered gravity.

2. Materials and methods

2.1. P. polycephalum plasmodia

P. polycephalum strain TU291 was kindly provided to us by Dr. Philipe Albert (Cytobiology Laboratory of Reims University, France). *Physarum* was cultured in MSD medium (Solution A: 0.05% Hematin/1% NaOH; Solution B (pH 4.6): 0.354% citric acid; 0.006% $FeCl_2 \cdot 4H_2O$; 0.02% KH_2PO_4; 0.06% $MgSO_4$; 0.0084% $MnCl_2 \cdot 4H_2O$; 0.004% $ZnSO_4 \cdot 7H_2O$; 0.06% $CaCl_2 \cdot 2H_2O$; 1% Tryptone; 0.15% Yeast extract; 1% Glucose. A/B V/V = 1/66) in total darkness. *Physarum* was grown as an agitated suspension of microplasmodia for stock cultures, and microplasmodia were allowed to fuse and grow synchronously on sterile filter paper to a macroplasmodium as described previously [8,9]. The cell cycle lasted 9.5 ± 0.5 h at 26 °C, and metaphase of mitosis occurred with natural synchrony within 5 min throughout a whole macroplasmodium of 5 cm diameter.

Studies were performed to evaluate the effects of altered gravity on cell cycle. *P. polycephalum* was cultured in the NASA-approved high aspect ratio vessel (HARV) in total darkness. This system subjected cultured suspension cells to a time-averaged gravitational field (altered gravity) to simulate low gravity conditions.

2.2. Experiment details

Synchronous macroplasmodia of *Physarum* about 10 cm in diameter containing about 10^{16}–10^{18} nuclei were gently scraped from filter paper. Cells were evenly resuspended in MSD medium by passage through a Pasteur pipette to avoid cell clumping. Then the resuspended cells were divided into two groups in each experiment. One group was transferred to an HARV (15 rpm) in altered gravity and was allowed to grow as a suspension either for 20 h or 40 h or 80 h; after that, synchronous samples were incubated on filter paper for tracking one cell cycle (about 10 h) on the ground and *Physarum* of different phases was individually harvested by scraping gently from the same macroplasmodia. In parallel, the other group was processed under normal gravity conditions ("1*g*"). The mitotic stages and duration were investigated by observation in a light microscope. The specimens of S phase, G2 phase, prophase, metaphase, anaphase, or telophase were individually harvested from the same macroplasmodia by tracking one cell cycle for the following experiments of actin cytoskeleton or protein 2-DE. Ground controls were treated in parallel with samples

subjected to altered gravity. Three repeated experiments were evaluated.

2.3. The duration of different phases in cell cycle

The control and test synchronous specimens were individually fixed after every 5 min in 4% paraformaldehyde and were observed in a light microscope after Carbol fuchsia staining. The duration of different phases in a cell cycle was tested.

2.4. Microfilament staining

The control and test synchronous samples grown on filter paper were individually harvested by being gently scraped with toothpicks at each stage by tracking a cell cycle and spread onto air-dried coverslips for 1 min. Then coverslips were dipped in 4% paraformaldehyde for 15 min, washed twice in 0.1 M PBS. And then the detergent-resistant cytoskeleton of *P. polycephalum* was treated with buffers containing Triton X-100 according to Clayton et al. [10] using our slight modifications. *P. polycephalum* was preincubated in 0.5% TritonX-100 (0.1 M PBS) for 30 min. After that it was followed by 0.5% BSA (0.1M PBS) blocking buffer for 30 min. Cells were incubated in 1:50 FITC-conjugated phalloidin (Sigma) in 0.1 M PBS at room temperature for 1 h, briefly washed three times in 0.1 M PBS and mounted using 90% glycerol (0.1 M PBS). Microfilament morphology was visualized using a Zeiss laser scanning confocal microscopy system (excitation at 490 nm, emission at 520 nm). The morphology of the actin cytoskeleton was assessed from 10 visual fields in a microscopy with each of the three replicated experiments.

2.5. Protein preparation

The control and test synchronous samples in G2 phase were individually harvested and immediately frozen in liquid nitrogen. They were ground to a fine powder and suspended in 10% v/v TCA in acetone with 0.07% v/v β-mercaptoethanol at $-20\,^{\circ}C$ for 1 h, followed by centrifugation for 10 min at 4 °C 15,000g as described before [11]. The pellets were washed with cold acetone containing 0.07% β-mercaptoethanol and centrifuged for 10 min at 4 °C 15,000g. Then the pellets were suspended in 80% acetone with 0.07% β-mercaptoethanol and centrifuged for 10 min at 4 °C 15,000g. The pellets were dried by using a vacuum pump and were ground to a fine powder. The protein powder was solubilized in lysis buffer (8M urea, 4% v/v CHAPS, 65 mM DTT, 0.2% w/v Bio-Lyte). Finally, the sample was centrifuged 10 min at 20 °C 15,000g and concentration of the supernatant was determined by the Bradford assay (Bio-Rad) with BSA as the standard.

2.6. Two-dimensional PAGE

The lysis buffer with 0.05g of protein powder was loaded onto an IPG gel strip (17 cm, pH 3-10; Bio-Rad). IPG strips were rehydrated for 12 h prior to eletrophoresis. The first-dimensional isoelectric focusing (IEF) was conducted at 20 °C using a Protein IEF Cell (Bio-Rad). IEF conditions were: 250 V for 0.5 h, 1000 V for 1 h, 10,000 V for 4 h, and 10,000 V for a total of 60 kVh. The IPG gel strips were placed for 15 min in 500 μl of equilibration buffer (50 mM pH 8.8 Tris-HCl, 6 M urea, 20% v/v glycerol, 2% w/v SDS and 2% w/v DTT) and then for 15 min in 500 μl of equilibration buffer with 2.5% w/v iodoacetamide (50 mM pH 8.8 Tris-HCl, 6 M urea, 20% v/v glycerol, 2% w/v SDS, 2% w/v DTT, and 2.5% w/v iodoacetamide). The second dimension was run on vertical 13% polyacrylamide-SDS gels. Gels were stained with Coomassie Brilliant Blue (20% ethanol, 80% methanol, 0.25 g Coomassie Brilliant Blue).

2.7. Gel image analysis

Following staining, the 2-DE gel patterns were scanned using a UVP scanner and compared and analyzed using PDQuest software, Version 7.2.0 (Bio-Rad). Spots in all gels were detected using the same parameters and quantified by 2-D Gaussian modeling. A matchset was made to compare protein spots in the altered gravity and ground control. Landmarks were used to align and position all the members of the match set. Gels were then normalized: the intensity of every spot was expressed as the ratio of the total density in gel image 2-fold for evaluating the up-regulated or down-regulated proteins in the quantitative analysis. Then a scatter plot for all proteins detected from 2-D images of test samples and its control was drawn. pI was set as X-axis and the logarithm of intensity was set as Y-axis.

2.8. Protein identification

Protein spots by staining with Coomassie Brilliant Blue were excised from gels. Proteins were digested with trypsin and analyzed by MALDI-TOF-MS (performed by Genecore (Shanghai) Co. Ltd). MALDI-TOF-MS analysis was essentially performed as

described by Deshane et al. [12]. Peptide matching and protein searches from the NCBInr data base were performed using the MASCOT search engine as described in Deshane et al. [12] and Berndt et al. [13]. The peptide masses were compared with the theoretical peptide masses of all available proteins from all species. Unmatched peptides or miscleavage sites were not considered for protein identification.

3. Results

3.1. Cell cycle

The cell cycle of *Physarum* consists of S, G2, and M phases, and has no G1 [14]. S phase begins in telophase. The M/S boundary is supposed as 0 h, and S phase lasts about 3 h, from 0 to 3rd hour, G2 phase lasts about 6 h, from 3rd to 9th hour, M phase lasts about 1 h, from 9th to 10th hour [15,16]. In *Physarum* the nuclear membrane does not breakdown during mitosis as in yeast cells [17,18]. To investigate whether altered gravity affects the mitosis time of *Physarum*, we analyzed every stage of the cell cycle in both the altered gravity and 1-*g* samples. The results indicated that the duration of the G2 phase was lengthened 20 min, or 2 h or 2 h in altered gravity for 20 h or 40 h or 80 h, respectively, whereas the duration of other phases of the cell cycle was unchanged with respect to the control.

3.2. Actin cytoskeleton

In this paper, because of the significant prolongation (about 2 h) of G2 phase duration in altered gravity for 40 h and subsequent 1*g* for about 10 h, we analyzed the effects of altered gravity on the actin cytoskeleton in altered gravity for 40 h. *P. polycephalum* cells were stained with FITC-conjugated phalloidin to visualize the F-actin fibers. Optical sectioning of *P. polycephalum* was observed by confocal laser scanning microscope. As seen in Fig. 1a, the microfilaments in G2 phase appeared a less regular polygonal fibrillar network in the ground control. These networks were similar to the network of actin filaments seen by Naib-Majani et al. [19] and Clayton et al. [10] in *Physarum*. In Fig. 1b, the samples in G2 phase subjected to altered gravity for 40 h exhibited a distinctly different morphology with reduced fibers and became short, disordered, and depolymerized in some degree, whereas the microfilaments in other phases of the same cell cycle were unchanged with respect to the control.

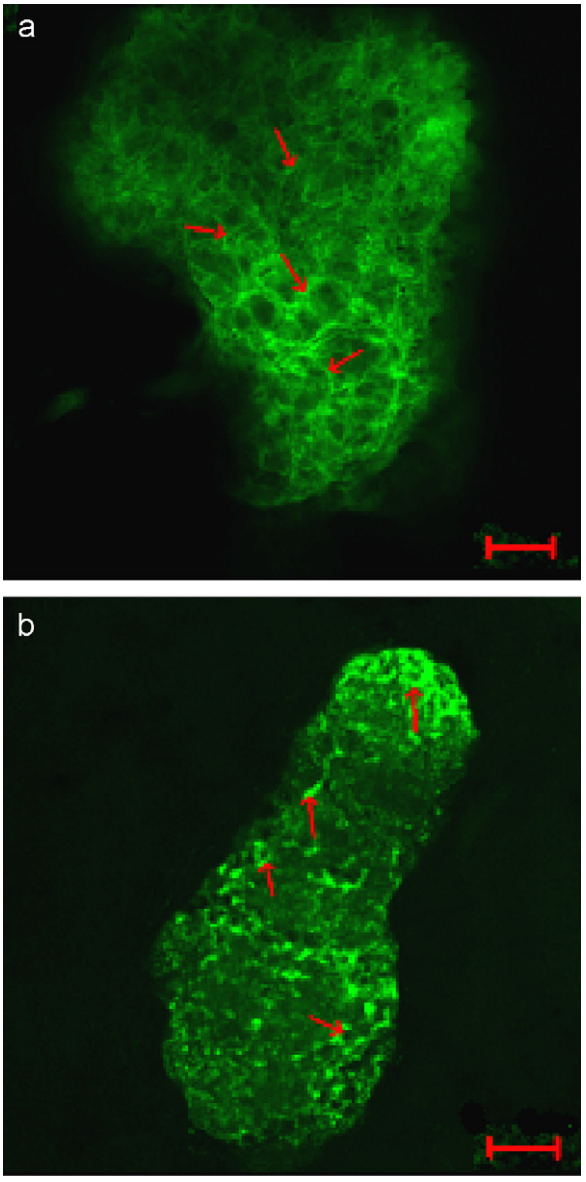

Fig. 1. The actin cytoskeleton of G2 phase in ground control and test samples in altered gravity for 40 h and subsequent 1*g* for about 10 h. The F-actin in *Physarum* spread on the coverslips was stained with FITC-phalloidin and the actin cytoskeleton was visualized by laser scanning confocal microscopy. Panel (a) showing the control group; panel (b) showing the test samples. Bar: 20 μm.

3.3. Proteomic analysis

In present study, the results that the G2 phase duration was significantly prolonged (about 2 h) and actin cytoskeleton in G2 phase was distinctly changed in altered gravity for 40 h and subsequent 1*g* for about 10 h prompted us to study the effect of altered gravity on

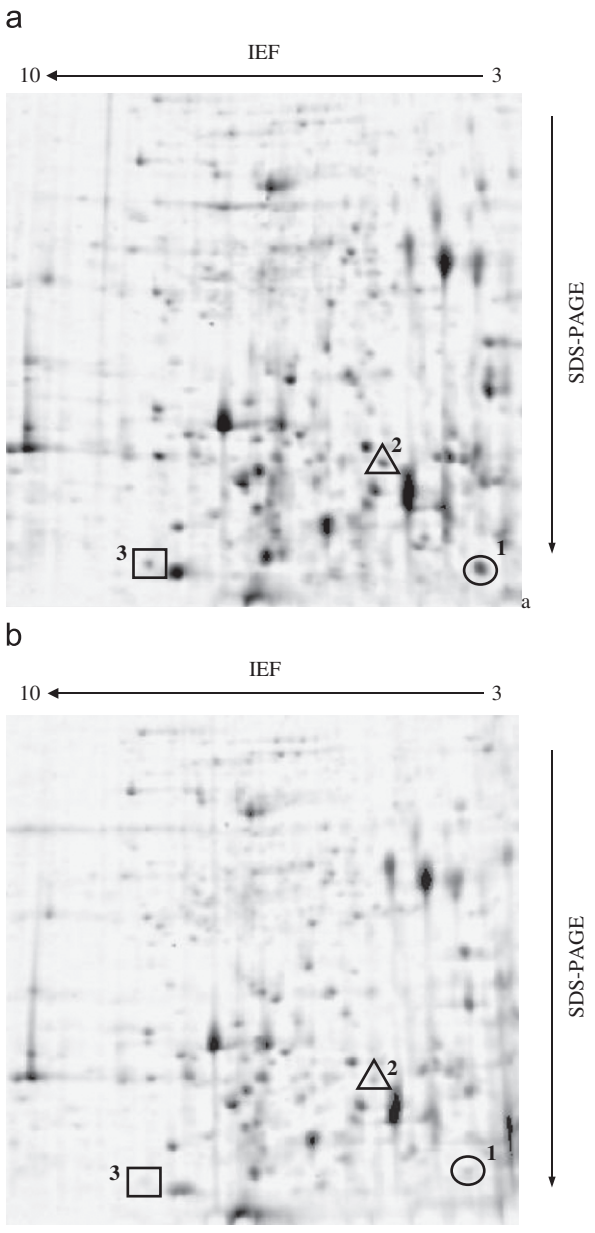

Fig. 2. 2-DE maps of G2-phase proteins in test *Physarum* in altered gravity for 40 h and subsequent 1*g* for about 10 h and its control. The proteins were visualized by Coomassie Brilliant Blue staining. The numbers indicate protein spots whose expressed level is different between the control and the test sample. Protein Spot 1 is spherulin 3b, which is expressed in a severe low level in test *Physarum*. Panel (a) showing the control group; panel (b) showing the altered gravity group.

the G2 phase proteome to further screen affected proteins in altered gravity for 40 h and subsequent 1*g* for about 10 h. The 2-DE method we adopted resolved and detected proteins with molecular masses between 250 and 10 kd and pIs between 3 and 10. Fig. 2 shows a pair of 2-DE gels of the *Physarum* proteins from control and altered gravity groups. We quantified the 2-DE protein patterns and mutually matched them by visual analysis across three repeated experiments. Three obvious protein spots that expressed in a severe low level in the altered gravity for 40 h were selected for MALDI-TOF-MS analysis to obtain the identity of these spots (Fig. 2). Out of the three spots, one was spherulin 3b (Fig. 2a, Spot 1, Table 1), another two were unknown proteins (Fig. 2a, Spots 2 and 3). After analyzing with the PDQuest software, we observed 231 spots in the ground control and 200 spots in altered gravity samples of G2 phase and 148 matched protein spots between control and altered gravity group (Table 2). Out of a total of 200 spots in altered gravity samples, 11 proteins were up-regulated by 2-fold and 28 proteins were down-regulated by 2-fold and 72 protein dots were specifically expressed as compared to control (Table 2). Out of a total of 231 protein spots in controls, 34 protein dots were not detected in samples exposed to altered gravity, but they were specifically expressed in controls (Table 2).

Using scatter plot analysis instrument of PDQuest, we analyzed the similarity of proteins in altered gravity and 1-*g* samples (Fig. 3). The coefficient of correlation of the match protein dots in them was 0.859787, which was obtained by the tropic in the Fig. 3.

4. Discussion

4.1. The effect of actin cytoskeleton on cell cycle in altered gravity

The cytoskeleton is highly sensitive to changes in the gravity environment. Hughes-Fulford and Lewis [20] described abnormalities in actin stress fibers for static cultures of anchorage-dependent mouse osteoblasts flown on the Shuttle. Meloni et al. [21] also indicated that the F-actin network of J-111 cells exposed to modeled low gravity showed a remarkable decrease in the filamentous biopolymer density. In previous studies, light and electron microscopy with antibodies against actin and two dimensional polyacrylamide gel electrophoresis results indicated the presence of actin in the myxamoebal cytoskeleton [10,19]. The cytoskeleton is known to participate in cytoplasmic streaming and cell organelle motion, mitosis, cytokinesis, endo- and exocytosis, as well as in intracellular transport of substances—all activities that are potentially gravity sensitive through the cytoskeleton [22]. If physical characteristics of the cell environment guide cell physiology (proliferation or differentiation),

Table 1
Differently expressed proteins in G2 phase from control and the test *Physarum* in altered gravity for 40 h and subsequent 1*g* for about 10 h[a]

Spot no.	Protein name	Accession no.	MOWSe score	Predicted M.W. (kd)	Predicted pI
1	spherulin 3b	AAY42820.1	82	11.2715	4.88

[a]Identified protein was obtained from MALDI-TOF-MS. It was expressed in a severe low level in the test group in comparison with the control. The accession number indicated was from National Center for Biotechnology Information (NCBI).

Table 2
Comparisons of G2 phase proteins in ground control and test *Physarum* in the altered gravity for 40 h and subsequent 1*g* for about 10 h

Total spots in control	Total spots in altered gravity	Matched spots	Quantity of up[a]	Quantity of down[b]	Special protein in altered gravity[c]	Special protein in control[d]
231	200	148	11	28	72	34

[a]The number of proteins up-regulated by 2-fold as compared to the control.
[b]The number of proteins down-regulated by 2-fold as compared to the control.
[c]The number of proteins expressed specially in test *Physarum* in G2 phase in the altered gravity for 40 h and subsequent 1*g* for about 10 h.
[d]The number of proteins expressed in controls, but not in test samples.

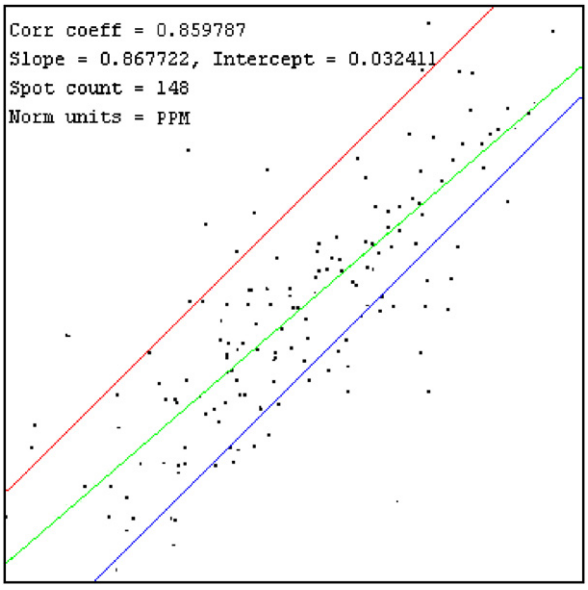

Fig. 3. The similarity analysis of G2-phase proteins in test samples in altered gravity for 40 h and subsequent 1*g* for about 10 h and its controls by PDQuest software. *X*-axis presents the protein intensity of controls, *Y*-axis presents the protein intensity of altered gravity samples, and dots in the image present the protein dots. The intensity of the protein dots in the area between the top line and the bottom line is between 2-fold and $\frac{1}{2}$-fold. The coefficient of correlation of the match protein dots in test samples and its controls is 0.859787.

microgravity could alter the relationships between cell structure and function. Actually, in this report, the 1-*g* samples in G2 phase had a less regular polygonal microfilament network, which was similar to the results observed by Naib-Majani et al. [19] in *Physarum* under a Zeiss microscope 110 and by Clayton et al. [10] in *Physarum* in electron micrograph. The duration of the G2 phase was prolonged 2 h and the microfilaments in the G2 phase had a reduced number of filaments and a unique abnormal morphology in altered gravity for 40 h and subsequent 1*g* for about 10 h as compared to the ground control. These findings suggest that microfilament dynamics is affected by altered gravity and the short microfilaments have lost their preferential orientation. The prolongation of G2 phase could be explained by the alteration of microfilaments in G2 phase of *Physarum* in altered gravity for 40 h and subsequent 1*g* for about 10 h. Our studies were different from *P. polycephalum* results obtained by Sobick and Briegleb [23], who indicated a significant shortening of mitosis under 0*g* simulation on the fast rotating clinostat compared to 1*g* controls. However, our observations were consistent with Jurkat cell line results obtained by Lewis et al. [4], who demonstrated a blockade of the cell cycle in G2M possibly because of the anomalies observed in the cytoskeleton in spaceflight, or in S and G2 + M phases of the cell cycle in microgravity, as demonstrated by Cogoli-Greuter et al. [5]. Our findings were also similar to the MCF-7 cell line results described by Vassy et al. [6], who suggested that the cell cycle was blocked in G2M and the longer MCF-7 mitosis duration could be interpreted by the alteration of cytoskeleton in weightlessness.

4.2. Changes of proteins

The orderly progression of eukaryotic cells from interphase to mitosis requires the close coordination

of various nuclear and cytoplasmic events. Although the mechanisms for the control of these events are not well understood, several lines of evidence suggest that there are factors (proteins) present in cells during mitosis that are absent during interphase [24,25]. While changes in transcription are common mediators of cellular response to microgravity, protein changes may be more relevant, longer lasting or more indicative of long-term cellular damage correlating with biological impact and risk. *Physarum* may undergo major changes in its protein levels in an altered gravity environment to counteract the stress. Identification of G2 phase proteins of *Physarum* in altered gravity different from that in control would improve our understanding of how G2 phase duration was lengthened in altered gravity. Toward such an effort, we screened G2 phase proteins of *Physarum* in altered gravity for 40 h and subsequent 1*g* for about 10 h and observed protein changes as compared to the control. The results show that 231 proteins in controls and 200 proteins in altered gravity groups were observed. Out of a total of 200 spots in altered gravity group, 11 proteins were up-regulated by 2-fold and 28 proteins were down-regulated by 2-fold as compared to control. The special expression of some proteins in altered gravity samples, not the ground control, confirmed microgravity-associated proteins. The present observations analyzed at the protein level show that some proteins are lost and the decrease in expression in *Physarum* subjected to altered gravity as compared to control thereby suggests either they are transcriptionally deregulated or may be an adaptive response to the new environment. These observations were in accordance with the previous results which showed that spaceflight altered protein metabolism and eventually decreased in total protein content [26] and simulated microgravity lead to a major loss of proteins in the hippocampus of mice [27]. It requires further study to testify whether changes of these proteins are related to the prolongation of G2 phase in *Physarum*. In our experiments, we found that the expression of spherulin 3b in G2 phase of test groups was down-regulated as compared to 1*g* control by using 2-DE and mass spectrometry system, which may contribute to the longer duration of G2 phase in altered gravity for 40 h and subsequent 1*g* for about 10 h. Pinchai et al. [28] showed that inhibitory RNA (RNAi)-induced knockdown of spherulin 3b-cDNA generated a severe reduction in the level of plasmodia to synthesize poly (malic acid) (PMLA), suggesting spherulin 3b functioned in regulating the level of PMLA in *P. polycephalum*. Karl et al. suggested that injection of PMLA into plasmodia increased the growth rate and shortened cell cycle duration, indicating that it could also be involved in molecular events concerned with growth and cell cycle duration in plasmodia [29,30]. PMLA might bind to histones and enhance the rates of transcription by facilitating chromatin remodeling [30]. We presume that similar pathway can be involved in the low expression of spherulin 3b in G2 phase in altered gravity for 40 h and subsequent 1*g* for about 10 h. The low level of spherulin 3b in G2 phase, which may result in a low level of PMLA, may contribute to the lengthened duration of G2 phase in altered gravity for 40 h and subsequent 1*g* for about 10 h.

Thus, we conclude that the changes of actin cytoskeleton and proteome in G2 phase of *Physarum* are concomitant with prolonged G2 phase in the cell cycle in altered gravity for 40 h and subsequent 1*g* for about 10 h. This should broaden our understanding of the biology effects of altered gravity.

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