Cell Growth and Differentiation Under Microgravity

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Abstract The mechano-biological coupling mechanism of cell response to altered gravity is crucial to understand physiological changes of astronauts in space microgravity environment and to develop relevant countermeasures. To address this issue, a novel space cell culture hardware mainly consisting of precisely controlled flow chamber and gas exchange unit is developed as an experimental payload in SJ-10 recoverable microgravity experimental satellite. Endothelial cells (ECs) and mesenchymal stem cells (MSCs) are cultured in the hardware during the SJ-10 mission, and recovered samples are analysed elaboratively. The results indicate that microgravity can suppress cellular metabolism. MSCs cultured with hepatic inducing medium are preferential in hepatic differentiation at long-term duration under microgravity. Both ECs and MSCs are regulated by microgravity and respond differentially in initiating cytoskeletal remodeling, or dysregulating signaling pathways relevant to cell adhesion, or directing hepatic differentiation.

Abbreviations

ALB	Albumin
ANOVA	Analysis of variance
CCD	Charge-coupled device
CCL5	C-C motif chemokine ligand 5
CYP450	Cytochrome P450
ECM	Extracellular matrix
ECs	Endothelial cells
eNOS	Endothelial nitric oxide synthase
F-actin	Actin filaments

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HUVEC	Human umbilical vein endothelial cells
ICAM-1	Intercellular adhesion molecule-1
IF	Immunofluorescence
IGFBP-2	Insulin-like growth factor binding protein 2
IL-1 R4	Interleukin 1 receptor 4
IL-8	Interleukin 8
mAbs	Monoclonal antibodies
MCP-1	Monocyte chemotactic protein 1
PDGF-AA	Platelet-derived growth factor AA
p-FAK	Phospho-focal adhesion kinase
PI3K	Phosphoinositide 3-kinase
rBMSCs	Rat bone marrow mesenchymal stem cells
SCCS	Space cell culture system
VCAM-1	Vascular cell adhesion molecule-1

1 Introduction

Physiological adaptation of astronauts to the microgravity environment is complicated, requiring an integrative perspective to fully understand the mechanisms involved. Responses of mammalian cells to gravity alteration remains fundamental to the issue, which also helps to elucidate the role that the gravity has played in the evolution of life on our planet. To date, it is still unclear if a single mammal cell could sense the gravity change or not, if the cell sensation is direct or indirect, how the gravity signals are transmitted or transduced into the cells, and what the underlying mechanisms are in regulating cell-cell or cell-surface interactions under microgravity (Bizzarri et al. 2014, 2015). The reasoning lies in the two aspects, at least. On one hand, the scarcity of flight opportunities in space makes it difficult to unravel these mechanisms under real microgravity condition. On the other hand, the experimental techniques in space cell biology are not well developed and standardized as those in laboratory on ground to collect the repeatable, reliable data. Thus, in the project of SJ-10 satellite (Hu et al. 2014; Li et al. 2018; Lü et al. 2019) we focus on both the scientific issues and technology upgrading to take full advantage of the limited and expensive space missions.

Various types of space cell culture devices have been developed since Russian scientists performed space cell biology experiments in 1960s (Krikorian 1996; Buravkova 2010; Freed and Vunjak-Novakovic 2002; Vandendriesche et al. 2004; Sun et al. 2008; Harada-Sukeno et al. 2009; Bizzarri et al. 2014, 2015; Kim et al. 2015). Those earlier space experiments have reported the inconsistent or even conflicting data mainly due to the technical difficulties in controlling cell culture conditions (Buravkova 2010; Bizzarri et al. 2015). Although the use of specialized devices with environmental control has improved the mission success rate of space cell growth, the absence of adequate analytical procedures for cell biology cannot ensure

the quality of data collected in spacecraft. Before the spacecraft possesses the capacity to do the on-site analyses for various behaviors of cellular responses in space, most of biological samples have to be analyzed off-line after recovering to laboratories on ground. At the current stage, the techniques of on-orbit environmental control for cell culture and sample preservation outweigh other considerations.

Technically, mammal cell growth in space requires the well-controlled nutrient supply, mass transport, and mechanical stimulation, as well as the defined temperature and pH value, since the disappearance of buoyant convection and sedimentation in space remarkably alters the processes of nutrient supply and mass transport as compared to those in conventional laboratories on ground. Thus, space cell biology experiments call for the specialized hardware to quantify the consistency between the two data sets from space mission and ground control, especially on the basis of very limited space mission opportunities. This work attempts to develop a novel space cell culture hardware mainly consisting of precisely controlled flow chamber and gas exchange unit and to investigate the cell growth and stem cell differentiation under microgravity. The specific aims are to collect the data on the metabolism, proliferation, apoptosis, differentiation, and cytoskeletal remodeling of human endothelial cells and rat bone mesenchymal stem cells. These new techniques and the related biological data are expected to reveal the effects of gravity on cell-cell interactions, to elucidate the underlying mechanisms of cell growth and differentiation in space, and to overcome the methodological bottlenecks of space cell biology research.

2 Scientific Issues

Cells may sense gravity change through different mechanisms. It is hypothesized that the cells possess the specialized structures or elements for gravity perception (*direct mechanism*) or are affected by the change in physical features of gravity-dependent microenvironment (*indirect mechanism*), or both mechanisms work cooperatively (Bizzarri et al. 2014, 2015). To address the issue, it is required to accumulate the ample data for characterizing the effects of nutrient supply, mass transport, and mechanical environment on the cells under microgravity. These types of data are very sparse and diverse at present, leading to the difficulty for comparing the data out of various labs.

Physiologically, the long-term exposure of astronauts to space condition induces typical alterations in bone loss, muscle atrophy, cardiovascular deconditioning, impairment of pulmonary function, and immune response (Bizzarri et al. 2014, 2015). Meanwhile, embryonic development and histogenesis could also be regulated by microgravity. At cellular level, their responses, as the basic element of life, are vital in space life sciences as well as space physiology and medicine. Thus, it is crucial to elucidate the mechano-biological coupling of the cells about how altered gravity regulates their biological responses. To do so, those cell types sensitive to mechanical stimulation are preferentially considered.

Endothelial cells (ECs) lining the inner surface of blood vessels play a critical role in maintaining vascular integrity, tissue homeostasis, and regulating local blood flow and other physiological processes. ECs are sensitive to mechanical forces including shear stress, tensile stretch, and mechanical compression (Byfield et al. 2009; Chancellor et al. 2010; Wang et al. 2014) as well as hypergravity and microgravity (Maier et al. 2015). For example, the altered EC morphology, cell membrane permeability and senescence are first documented by spaceflight experiments on cultured endothelium (Maier et al. 2015). Unfortunately, it is still unclear how the cells grow, line up, and vascularize in space. Here a human umbilical vein cell line, EA.hy926, is applied to investigate cell growth under microgravity.

Bone marrow mesenchymal stem cells (BMSCs), as a typical type of somatic stem cells, can differentiate into osteoblasts, chondrocytes, and adipocytes (Li et al. 2013). In the past decades, BMSCs have attracted much attention as a well-defined model in elucidating bone loss mechanism under microgravity and as a potential candidate in resisting bone loss since bone is a constantly renewed organ involving the interplay of various bone cells (Burger and Klein-Nulend 1998; Ulbrich et al. 2014). In fact, BMSCs possess wide plasticity to transdifferentiate into the cells of other germlayer. For instance, hepatocytes are derived from the endoderm, and the liver is an organ not bearing body weight specifically and not stimulated by mechanical forces frequently (Ayatollahi et al. 2010). It is unclear if the BMSC transdifferentiation to hepatocytes is also influenced by microgravity. Here the effects of microgravity on hepatocyte differentiation of rat BMSCs are investigated and the outcomes are significant for understanding the mechanism of stem cell responses to gravity change.

3 Hardware Development

The space experiment presented here is defined as one of ten life science payloads in SJ-10 recoverable satellite launched on 6th April and recovered on 18th April, 2016. The following supporting resource conditions are distributed from the satellite hub to this hardware: 365 × 285 × 250 mm in maximum geometry size, 17 W in average power, cabin vacuum environment, and heat conduction from installation plane with water circulation.

A space cell culture system (SCCS) is developed in-house upon the above supporting resources. All experimental modules are designed to be enclosed in an air-sealed box within an atmospheric pressure sensor used to monitor air leakage. To provide the basic conditions for mammal cell culture and the specific support for microgravity effect test, the SCCS system is composed of six modules of cell culture, liquid supply, temperature control, environmental monitoring, micro-image capture, and electrical control (Fig. 1).

The cell culture module (Fig. 2) is the core unit of the system. Six culture chambers are lined in parallel and connected with silicone tubes. For a single chamber, a cell culture plastic slide (Permanox® slide, 25 × 75 cm, Nunc) is used and sealed by a silicone gasket with an effective culture area of 12 cm^2 and the inner height of 1 mm

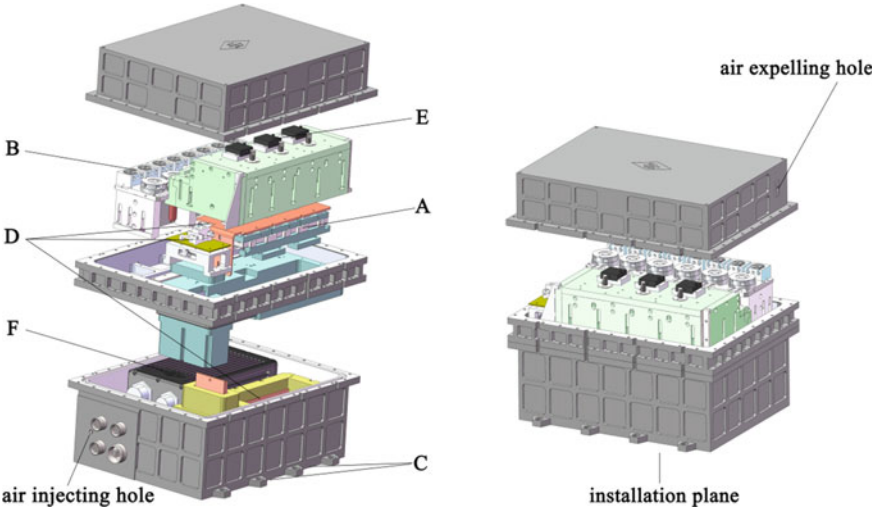

Fig. 1 Overview of the hardware mounted on SJ-10 satellite from the exploded (*left*) and assembled (*right*) view before the box is closed. It consists of six modules of cell culture (*A*), liquid supply (*B*), temperature control (*C*), environmental monitoring (*D*), micro-image capture (*E*), and electrical control (*F*)

(thereupon the medium volume of ~1.2 ml). The inlet of each chamber is coupled with a de-bubble unit that is specially designed to trap small bubbles in the liquid circuit (Sun et al. 2019). The silicone tube between the pump and chamber inlet is used as a gas exchanger for O_2 and CO_2 exchange in medium (Sun et al. 2019).

The liquid supply module (Fig. 2) is composed of a set of micro-peristaltic pumps and multi-channel pinch valves. The pumps and valves work upon manufacturers' instruction and in house-reprogramed codes. The module functions in supplying fresh medium, collecting culture supernatant, and fixing grown cells. To provide sufficient nutrient supply and mass transport and, meantime, avoid interference of flow shear when the medium flows through the chamber, the flow rate is estimated on the geometry of the chamber and the rate of cell glucose and oxygen consumption. Here we applied a flow rate of 0.3 ml/min and a wall shear stress of 1.05×10^{-3} Pa, which meets the nutrient demand of the cells but does not impair significantly the cell growth and behaviors.

The temperature control module (Fig. 2) governs two separated zones, *that is*, the culture zone that encloses all chambers and maintains the temperature at 36 ± 1 °C, and the medium storage zone that keeps the temperature at 4–10 °C. Semiconductor chilling plates fixed on the bottom of the box are used to manipulate the respective temperatures, in which the heating side faces to the former and the cooling side faces to the latter. Heat flux is conducted to the satellite platform through the installation plane wherein a circulating water cooling system is furnished (*cf.* Fig. 1). It takes about 2 h to reach from room temperature (20–25 °C) to the ones required.

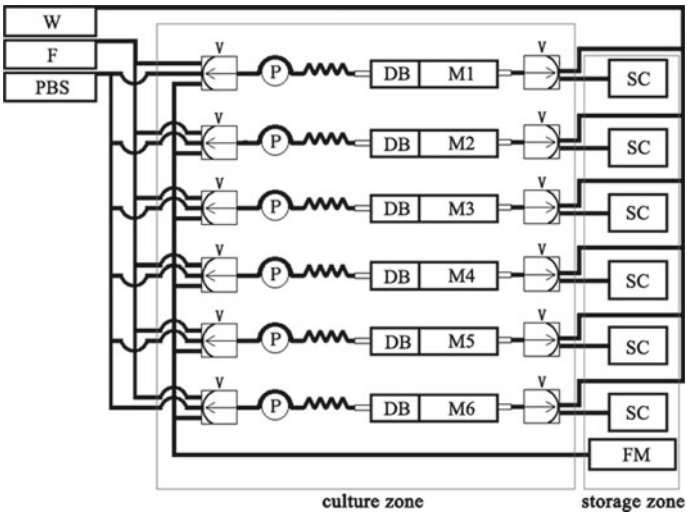

Fig. 2 Schematic of flow network of the SCCS. *M1–M6*, chambers of cell culture module; *DB*, de-bubble unit; *P*, micro-peristaltic pump; *V*, multi-channel pinch valve; *SC*, culture supernatant collection bag; *FM*, fresh medium storage bag; *PBS*, phosphate-buffered saline; *F*, fixing agent; *W*, waste collection bag. The *wave line* denotes the gas exchanger

The environmental monitoring module (Fig. 2) includes an atmospheric pressure sensor, two photoelectric pH sensors, and nine temperature sensors. The pressure sensor records pressure data within the box for judging if the vacuum seal fails. The pH sensors monitor the pH values of medium supplied for the cells. The temperature sensors monitor the temperatures of nine preset points in the box, in which three are set at the culture zone, two are placed at the medium storage zone, and the other four are located next to those pumps and valves of liquid supply module and to the chilling plates.

The micro-image capture module (Fig. 2) consists of three sets of CCD camera and microscopic lens, which are fixed on the top and bottom at three specific culture chambers, together with optical alignment. The images of cellular morphology in three chambers are captured automatically every 3 h using the image grabbing card integrated into the electrical control module.

The electrical control module (Fig. 2) is designed and constructed for implementing entire experimental procedure and operating all actuators with programmed software. It is also responsible for data transfer between the experimental device and the SJ-10 payload data manager center.

All the modules are assembled together into the box and the resulted hardware has been tested systematically to assure its reliability and robustness. These tests includes stress screening, vibration, impact, acceleration, thermal cycling, thermal vacuum, aging, reliability life, pressure leak, biocompatibility, comprehensive performance matching, and electromagnetic compatibility.

4 Materials and Methods

4.1 Cell-Culture Procedure

Human endothelial cell line EA.hy926 obtained from China Infrastructure of Cell Line Resource (Beijing, China) is cultured in Endothelial Cell Medium (Sciencell Research Laboratories, Carlsbad, CA) and incubated at 37 °C in a humidified incubator supplemented with supplemented with 5% carbon dioxide (CO_2).

Rat BMSCs or rBMSCs are isolated from 3- to 4-week-old male SpragueeDawley (SD) rats (Vital River Laboratory Animal Technology Company, Beijing, China). Briefly, the animal is sacrificed by cervical dislocation and the femur and tibia are collected. The bone marrow is flushed out, and the collected cell suspension is added into the MSC culture medium composed of DMEM/F12 medium (Gibco, USA) supplemented with 15% fetal bovine serum and 1% NEAA, 1% glutamine, 1% sodium pyruvate and 1% penicillin-streptomycin in a T-25 flask or 12-well plastic plate. Adherent cells are then maintained in a humidified, 95% air and 5% CO_2, 37 °C incubator by refreshing the medium every two or three days. When grown to 85–90% confluence, the cells are rinsed in Ca^{2+}- and Mg^{2+}-free PBS and then detached using 0.25% trypsin-EDTA for 1 min. This procedure is repeated three or four times to collect rBMSCs at ~90% purity. Collected rBMSCs are identified as described previously (Li et al. 2013).

Thirty hours prior to the launching, the two types of cells are detached using 0.25% trypsin-EDTA separately. Then the cell suspension is injected, respectively, into the chambers with 5×10^5 cells per chamber. Four chambers (M1–M4) are seeded with EA.hy926 cells, and two chambers (M5, M6) are seeded with rBMSCs. The chambers are placed incubator for 12 h to reach firm adhesion and then transferred into clean bench. The medium in the four chambers with EA.hy926 cells is removed and the individual chambers are re-filled with fresh medium to wash out the unattached cells and to eliminate air bubbles. The two chambers with rBMSCs undergo same washing procedure but replaced with hepatocyte induction medium, i.e., MSC culture medium supplemented with 20 ng/ml HGF and 10 ng/ml EGF.

After medium replacement and bubble elimination, the culture chambers are mounted in the culture zone of SCCS. Then the box is sealed and temperature control is initiated. Once these operations are completed, the air in the box is replaced by pre-mixed 95% air and 5% CO_2 through the air injecting and expelling holes embedded in the box wall (*cf.* Fig. 1).

4.2 Flight Mission Procedure

Eight hours before launching, the SCCS hardware is assembled onto the preset platform of the SJ-10 satellite and the temperature control module keeps running until taking off. At the moment the satellite enters orbit for half an hour, the programmed

procedure is initiated. This time point is defined as the starting time ($t = 0$) of space experiment in orbit. In brief, images of cultured cells are captured every 3 h. Cells in the chamber M1, M3 or M5 are fixed at $t = 72$ h (3 d) and meanwhile the supernatant in each chamber is collected individually and preserved in the medium storage zone. The medium in chambers M2, M4 and M6 is refreshed every 48 h and the supernatant in each chamber is also preserved as above. Cells in the chamber M2, M4 or M6 are fixed at $t = 240$ h (10 d), after which the temperature of culture zone is lowered to <20 °C till the satellite is recovered. Along the entire procedure in orbit, all captured images and environmental parameters are downloaded to the data acquisition system on ground and also backed up to an internal storage unit of the SJ-10 payload data manager center. These images and parameters are checked timely during the entire mission to make sure that the system works as designed. After running in orbit for 12 days, the satellite is recovered to the ground. All cell and medium samples are taken out and transported at 4–10 °C to the laboratory within 18 h.

The ground control experiments are performed four times independently following the same procedure by the same SCCS hardware in order to have identical preflight and postflight conditions.

4.2.1 Cell Metabolism Analysis

Once all samples are transported to the laboratory, the supernatants are stored at −70 °C immediately until test. Glucose consumption and L-lactate production are tested by commercially available glucose (Huaxingbio, Beijing, China) and L-lactate (BioAssay Systems, Hayward, CA) kits following the manufacturers' protocols, respectively.

4.2.2 Immunofluorescence (IF) Staining

Alexa Fluor® 594-conjugated rabbit anti-β-actin (13E5), Alexa Fluor® 555-conjugated rabbit anti-α-tubulin (11H10) and Alexa Fluor® 647-conjugated rabbit anti-vimentin (D21H3) monoclonal antibodies (mAbs) are purchased from Cell Signaling Technology (Danvers, MA). Alexa Fluor® 647-conjugated mouse anti-intercellular adhesion molecule-1 (ICAM-1, HCD54) mAbs are obtained from BioLegend (San Diego, CA). Alexa Fluor® 647-conjugated rabbit anti-vascular cell adhesion molecule-1 (VCAM-1, EPR5047) and anti-NF-κB p65 (clone E370), Alexa Fluor® 488-conjugated mouse anti-β1-integrin (12G10), phycoerythrin (PE)-conjugated mouse anti-CD44 (F10-44-2), mouse anti-Rac-1 (0.T.127), anti-phospho-focal adhesion kinase (p-FAK, Tyr397, M121) and anti-RhoA (1B12) mAbs, as well as mouse anti-Cdc42, rabbit anti-albumin (ALB) and anti-cytochrome P450 (CYP450) polyclonal antibodies (pAbs), DyLight® 594-conjugated donkey anti-rabbit and Alexa Fluor® 488-conjugated goat anti-rabbit and donkey anti-mouse secondary pAbs are all from Abcam (Cambridge, UK). For immunostaining, EA.hy926 or rBMSC cells are incubated successively in IF blocking buffer (1% BSA in DPBS),

primary Abs (10 μg/ml in blocking buffer) and secondary Abs (5 μg/ml in blocking buffer) for 1 h at 37 °C. The images of stained cells are collected using a confocal laser-scanning microscope (Zeiss LSM710, Germany) with a 63 × oil immersion objective.

5 Results and Discussions

Data hereinafter for space samples of EA.hy926 cells come from the culture chambers M1 and M4 and those for rBMSCs are derived from the chambers M5 and M6. Those samples from the chambers M2 and M3 are not included further analysis mainly due to incomplete cell fixation even though the related in-orbit images for cell growth are available.

5.1 Cell Metabolism

To test the possible energy metabolism deficiency caused by spaceflight, the glucose (Fig. 3a–b) and L-lactate (Fig. 3c–d) concentrations from the supernatants under different conditions are quantified. Here glucose concentration for the EA.hy926 cells and rBMSCs cultured for 3 days in space is increased additional 129% and 47%, respectively, compared with ground control (Fig. 3a–b). This is consistent with our previous findings in earlier space experiment where a distinct trophoblastic tumor cell line (JAR) yields slightly higher values in space on different flow condition (Long et al. 2009). By contrast, lactate production for the EA.hy926 cells and rBMSCs cultured for 3 days is respectively reduced to 16 and 0.4% in space compared to ground control (Fig. 3c–d). The significant differences of glucose and L-lactate metabolism are disappeared between EA.hy926 cells cultured for 10 days in space and on ground (Fig. 3a, c), while those for rBMSCs are reduced (Fig. 3b, d) possibly attributed to the slowed growth associated with long-term contact inhibition (Everding et al. 2000; Hung and Terman 2011). These first-hand results suggest that space microgravity can suppress energy metabolism with lower glucose consumption and lesser lactate production, which is in agreement with previous study (Chakraborty et al. 2018). Due to insufficient reduplicated space samples from these experiments with limited resources, it requires further replicated studies to reassure the hypothesis in the future.

5.2 Cell Morphology

The morphology of the two types of the cells exposed to microgravity is monitored and compared with those on ground. It is indicated that the shape of EA.hy926 cells

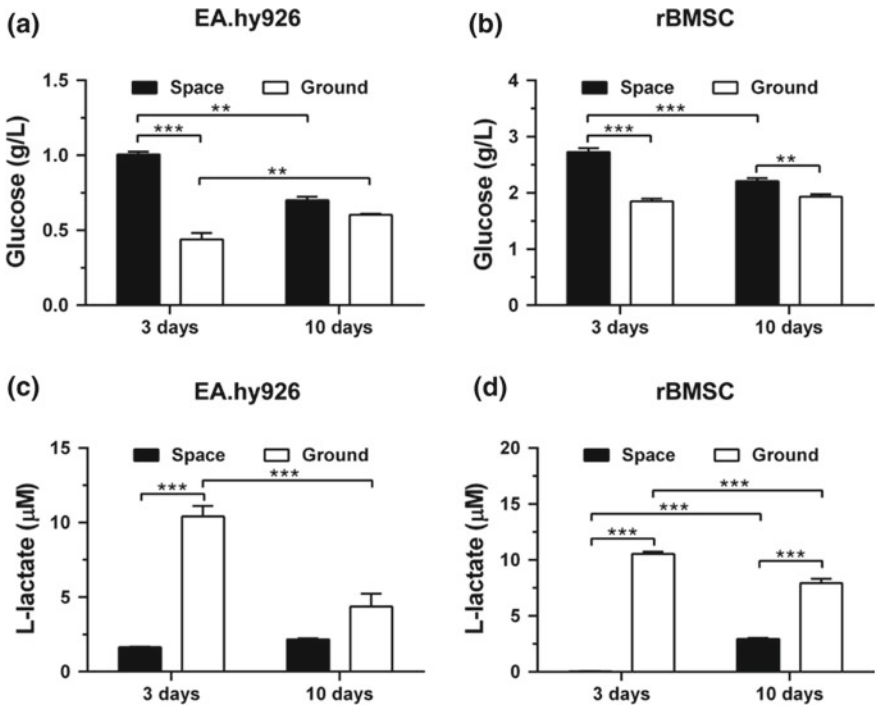

Fig. 3 Glucose (**a**–**b**) and L-lactate (**c**–**d**) concentrations of EA.hy926 cells (**a**, **c**) and rBMSCs (**b**, **d**) for 3 and 10 days cultured in space (*closed bars*) and on ground (*open bars*). Data are presented as the mean ± SEM of one experiment in space and 2–4 independent experiments on ground, both of which are performed in technical triplicate and analyzed with two-way ANOVA followed by Holm-Sidak test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (Reproduced from Li et al. 2018.)

cultured in space or on ground shows no significant difference. These endothelial cells present typical cobblestone shape at day 3 and are slighted elongated at day 10 (Fig. 4a–d). While the altered EC morphology is documented by previous spaceflight experiments on the cultured endothelium, it should also be noticed that those data is derived from human umbilical vein endothelial cells (HUVEC) cultured on microcarrier beads (Kapitonova et al. 2012, 2013). Regardless of different types of ECs used, other factors may also be involved in these differences between the observations here and those in the literatures, including the material and curvature of the carrier. We observe neither the grooved nor the tube-like structures formed by EA.hy926 cells on the entire culture substrate as those previous studies in microgravity effect simulation using clinostats (Grimm et al. 2009; Ma et al. 2014), showing that the ECs may respond differently to real space microgravity and clinostat culture.

On the other hand, the shape of rBMSCs cultured in space or on ground is also similar. In the hepatocyte induction medium, these mesenchymal stem cells present long and narrow shape at day 3 and are slighted elongated at day 10 (Fig. 4e–h). Intriguingly at day 10, the cells tend to form a visible plate-like structure (i.e., the hepato-

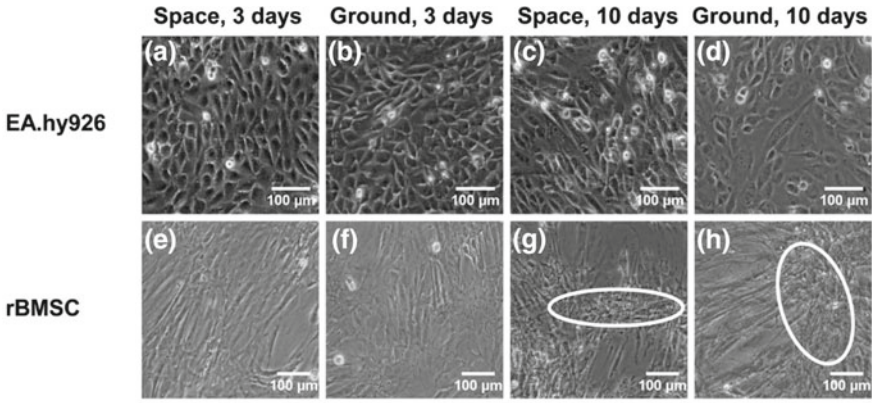

Fig. 4 Morphology of EA.hy926 cells and rBMSCs cultured in space or on ground. Presented are the optical images of EA.hy926 cells cultured for three (**a**–**b**) or ten (**c**–**d**) days in space (**a**, **c**) and ground (**b**, **d**) or rBMSCs cultured in hepatocyte induction medium for three (**e**–**f**) or ten (**g**–**h**) days in space (**e**, **g**) and ground (**f**, **h**), with a 10× objective. *Ellipses* indicate the plate-like structure for rBMSCs at day 10 (**g**–**h**)

cytes are arranged in a radial, plate-like pattern) in space than on ground (Fig. 4g–h), suggesting the possible effects of microgravity on directing the hepatocyte-like morphology from rBMSCs that has not been observed previously. Taken together, these results indicate that both EA.hy926 cells and rBMSCs grow normally under microgravity environment and no significant difference in cell morphology is observed using optical microscopy.

5.3 Cytoskeletal Remodeling

It is known that the cells in space usually undergo cytoskeletal remodeling (van Loon 2009; Long et al. 2015). Here we further test the observations by staining three key components, actin, tubulin and vimentin. Typical confocal analysis at day 3 indicates that the EA.hy926 cells cultured in space tend to disassemble their actin fibers and redistribute the disperse actin proteins at the periphery of the cells near the plasma membrane (Fig. 5a). By contrast, the actins are distributed over the entire cells on ground and no stress fibers are visible (Fig. 5b). The reorganization of actin including markedly reduced amount (Carlsson et al. 2003), depolymerization of actin filaments (F-actin) and clustering of the stress fibers at the cellular membrane (Infanger et al. 2007; Grenon et al. 2013) are known to be regulated by simulated microgravity effect on ground, but no direct evidences are reported for ECs under real space microgravity. Interestingly, HUVECs exposed to hypergravity of 3.5 × g for 96 h tend to accumulate actin fibers around the nucleus, but not at the periphery of the cells, although the total amount of actin proteins remains the same (Versari

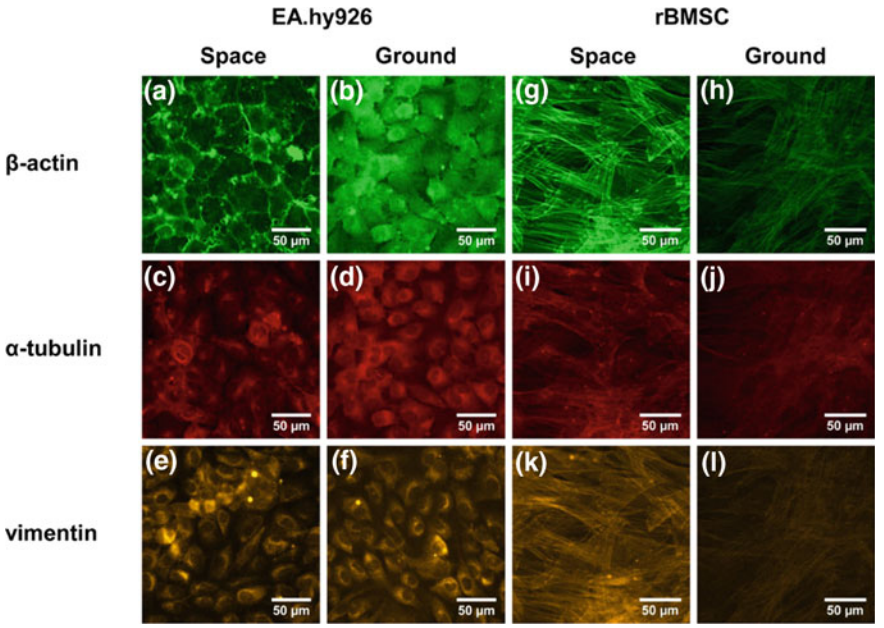

Fig. 5 Cytoskeletal remodeling of EA.hy926 cells and rBMSCs in space or on ground. Presented are the typical confocal images of β-actin (**a**–**b**, **g**–**h**), α-tubulin (**e**–**f**, **i**–**j**) and vimentin (**e**–**f**, **k**–**l**) in the two types of the cells cultured for three days in space (**a**, **c**, **e**, **g**, **i**, **k**) or on ground (**b**, **d**, **f**, **h**, **j**, **l**) for EA.hy926 cells (**a**–**f**) and rBMSCs (**g**–**l**). Bar = 50 μm

et al. 2007). Thus, our results corroborate the actin disassembly in space or clinostat culture for ECs and other types of cells (Maier et al. 2015).

Existing evidences indicate that those HUVECs re-adapted by subsequent passages after spaceflight exhibit the persisting changes in the organization of microtubules and form prominent bundles that occupies the peripheral cytoplasm (Kapitonova et al. 2012). The total amount of tubulin is also significantly reduced in the cytoplasm of these cells compared to ground control. Here we find that 3-day culture in space leads to a dramatic decrease in the amount of microtubules for EA.hy926 cells (Fig. 5c–d), which is consistent with the observations from previous spaceflight (Kapitonova et al. 2012) and clinostat culture studies (Buravkova et al. 2018) for HUVECs. More importantly, the enhanced mRNA-encoding actin and tubulin are observed in the exosomes collected from the supernatant of EA.hy926 cells cultured for 10 days in space, which may contribute to the disorganization of F-actin and microtubules in the cell body (Li et al. 2018).

Intermediate filaments such as vimentin contribute to maintain the structural and mechanical stability of cells (Zhang et al. 2017; Zhou et al. 2018). It is known that the expression of vimentin is enhanced in chondrocytes and papillary thyroid carcinoma cells cultured in clinostats (Infanger et al. 2006; Aleshcheva et al. 2013). While the expression of vimentin in EA.hy926 cells remains unchanged after 3-day culture in

space compared to the ground control (Fig. 5e–f), the extended exposure to space microgravity for 10 days leads to a significant increase of vimentin, which possibly compensates the loss of mechanical stability caused by the disorganization of F-actin and microtubules (Li et al. 2018).

On the other hand, cytoskeletal remodeling in rBMSCs cultured in hepatocyte induction medium presents a distinct pattern. The total expression of actin is higher in space than on ground, similar to those observations using parabolic flight at gene level (Aleshcheva et al. 2015). Here intracellular stress fibers are well formed at high intensity for the cells cultured in space (Fig. 5g). By contrast, only a few stress fibers are visible for the cells on ground (Fig. 5h). In particular, the actin cytoskeleton of rBMSCs exhibits significant redistribution and reorganization under real microgravity, presumably attributed to the coordinated regulation of biomechanical (microgravity) and biochemical (hepatocyte induction) signaling. Moreover, microtubules are also known to be gravisensitive. Here the total amount of tubulin in rBMSCs is increased significantly in space compared to ground control (Fig. 5i–j), also consistent with those observations at gene level using parabolic flight (Aleshcheva et al. 2015). In immunofluorescence analysis, tubulin filaments in space appears to present more visible bundles. While the expression of vimentin remains similar with that of tubulin (Fig. 5k–l), the exposure to space microgravity for 3 days leads to a significant increase of vimentin expression, which is consistent with previous studies via clinostat (Aleshcheva et al. 2015; Ebnerasuly et al. 2017).

To our knowledge, this is the first evidence, at protein level, of cytoskeletal alteration in hepatic induction of rBMSCs under real microgravity. More interesting is the finding of microgravity-induced enhancement of actin stress fibers, which could be associated with the plate-like structure (*cf.* Fig. 4g). Noting that actin proteins could either lose the stress fibers, present the perinuclear localization, or remain unchanged in different space flown experiments (Vorselen et al. 2014; Chen et al. 2016), these controversial observations are presumably attributed to different cell types, distinct space hardware, or differential quantification and standardization of mechanical environment such as mass transport.

5.4 Cell Adhesion

Energy metabolism deficiency in ECs exposed to space microgravity is cascaded into significant suppression of genes associated with host defense in previous spaceflight studies (Wang et al. 2015; Chakraborty et al. 2018). Decreased expression of cellular adhesive molecules (Grenon et al. 2013) and reduced release of pro-inflammatory cytokines (Grimm et al. 2010; Griffoni et al. 2011; Grenon et al. 2013) are also documented on ECs using clinostats. Here 3-day culture of EA.hy926 cells in space significantly decreases the presence of ICAM-1 (Fig. 6a–b) and VCAM-1 (Fig. 6c–d), two ligands for β2 and α4 integrins respectively, indicating the impairment of leukocyte recruitment and transmigration during inflammation. Exposure to space microgravity for 10 days further suppresses the release of a few pro-inflammatory (IL-8, MCP-

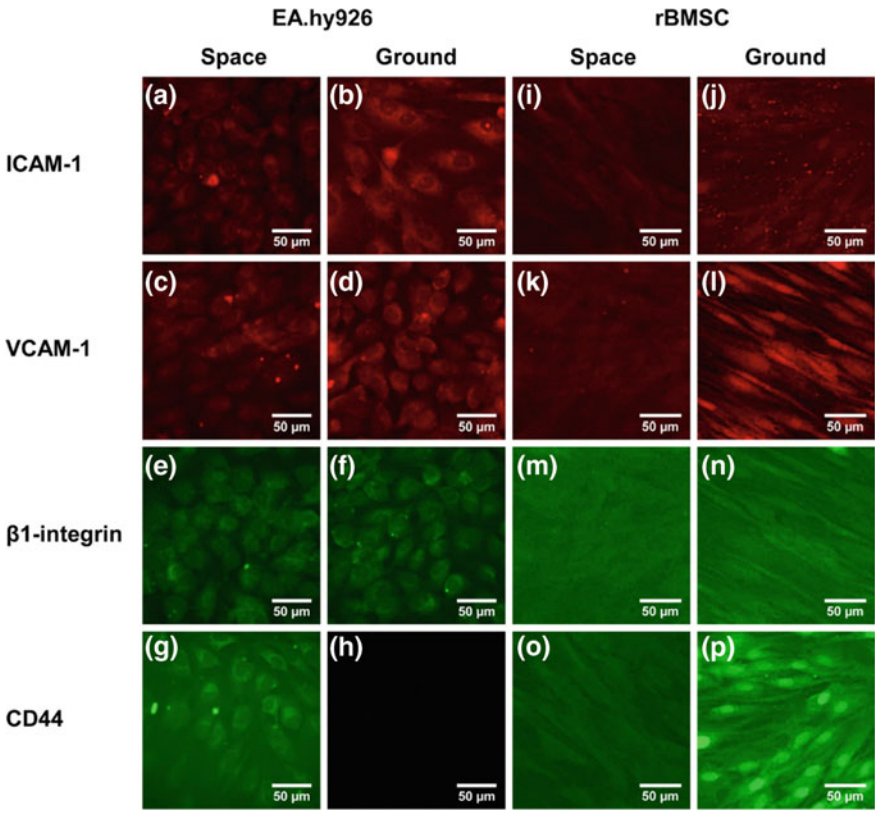

Fig. 6 Expression of adhesive molecules of EA.hy926 cells and rBMSCs in space or on ground. Presented are the typical confocal images of ICAM-1 (**a**–**b**, **i**–**j**), VCAM-1 (**c**–**d**, **k**–**l**), β1-integrin (**e**–**f**, **m**–**n**) and CD44 (**g**–**h**, **o**–**p**) on the surface of the two types of the cells cultured for three days in space (**a**, **c**, **e**, **g**, **i**, **k**, **m**, **o**) or on ground (**b**, **d**, **f**, **h**, **j**, **l**, **n**, **p**) for EA.hy926 cells (**a**–**h**) and rBMSCs (**i**–**p**). Bar = 50 μm

1, CCL5 and IL-1 R4) and pro-angiogenesis (Endoglin, IGFBP-2, PDGF-AA, and Pentraxin-3) cytokines in these ECs (Li et al. 2018). These data shed light on the suppressed host defenses and delayed wound healing faced by astronauts (Versari et al. 2013; Chakraborty et al. 2018).

Morphological alteration and cytoskeletal remodeling of cells in space are related to their anchorage onto the substrate via cellular adhesive molecules and extracellular matrix (ECM). β1-integrin and CD44 are two important adhesive molecules that interact with collagen I and hyaluronan (HA) in ECM, respectively, playing crucial roles in cell proliferation, angiogenesis and mechanotransduction (Savani et al. 2001; Provenzano and Keely 2011). Although the expression of β1-integrin on the surface of EA.hy926 cells after 3-day culture in space remains unchanged compared to the ground control (Fig. 6e–f), significantly reduction of collagen I is observed (Li et al.

2018), indicating the impairment of ECM/integrin/FAK pathway in space. Meanwhile, enhanced expression of CD44 is found in the presence of space microgravity (Fig. 6g–h), which is consistent with previous clinostat study in thyroid cancer cells (Grosse et al. 2012) and may lead to the augmented signals of Rho GTPases family (Bourguignon et al. 2006; Murray et al. 2014; Hyder et al. 2015).

Stem cell adhesion on substrate is also determined by the interactions between ECM proteins and transmembrane cellular adhesive molecules. Here it is indicated that, in the expression of several adhesive molecules for rBMSCs 3-day cultured in space, β1 integrin expression is significantly increased (Fig. 6m–n) but the expressions of ICAM-1 (Fig. 6i–j), VCAM-1 (Fig. 6k–l), and CD44 (Fig. 6o–p) are reduced at distinct degrees compared to those on ground. Thus, it is possible that the difference in ligand availability contributes to the differential regulation of distinct cell adhesion. For example, β1 integrin is the vital molecule in mesenchymal stem cells (Song et al. 2014) and hepatocytes (Speicher et al. 2014) and plays important roles in adhesion (Lee et al. 2004), mechanotransduction (Ode et al. 2011) and liver disease repairing (Aldridge et al. 2012). Here the enhanced expression of β1-integrin in space is found to be consistent with those observations derived from stimulated microgravity effect tests (Meyers et al. 2004). Since VCAM-1 expression in space is reduced and less β1-integrin-VCAM-1 pairs may be insufficient to support cell adhesion, one possible mechanism is that the extra β1-integrins could bind to other ligands to compensate the insufficiency. Collagen I is a candidate ligand since it binds to β1-integrin specifically and can be expressed in MSCs in ground-based stimulation (Meyers et al. 2004). This is critical especially for hepatic induction of rBMSCs since the reinforced β1-integrin signaling via VCAM-1 and/or collagen I binding could potentially favor hepatic differentiation (Popov et al. 2011; Bi et al. 2017).

5.5 Related Signaling Pathways

Cytoskeletal remodeling of cells in space is related to the regulation of underlying signaling molecules. Here the decreased collagen I expression (Li et al. 2018) leads to the impaired mechanotransduction through integrin-mediated signaling pathway and reduced tyrosine phosphorylation of FAK in EA.hy926 cells cultured in space (Fig. 7a–b), resulting in the depressed expression of phosphoinositide 3-kinase (PI3K) and endothelial nitric oxide synthase (eNOS) (Li et al. 2018) and down-regulation of the Rho GTPases family. RhoA, Rac-1 and Cdc42 are three Rho GTPases crucial for the rearrangement of cytoskeletal actin, tubulin and vimentin. Here spaceflight-induced increase of RhoA (Fig. 7c–d) is found in EA.hy926 cells cultured for 3 days, presumably associated with the enhanced expression of CD44 (*cf.* Fig. 6g–h). Meanwhile, 3-day culture in space does not affect Rac-1 expression in EA.hy926 cells (Fig. 7e–f) but reduces Cdc42 expression significantly (Fig. 7g–h). The decreased Cdc42 could result from the inhibition of ECM/integrin/FAK pathway (Li et al. 2018). The expression of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) is also quantified, and no significant difference is found between

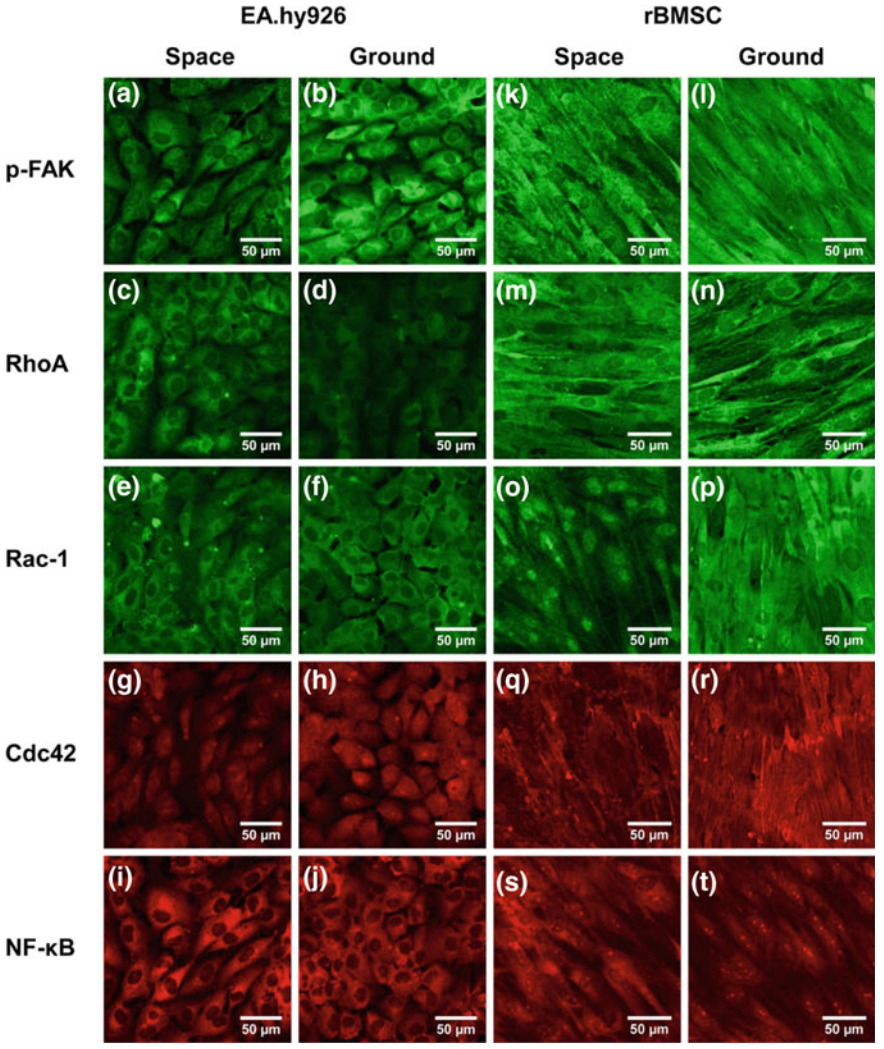

Fig. 7 Expression of signaling molecules of EA.hy926 cells and rBMSCs in space or on ground. Presented are the typical confocal images of p-FAK (**a**–**b**, **k**–**l**), RhoA (**c**–**d**, **m**–**n**), Rac-1 (**e**–**f**, **o**–**p**), Cdc42 (**g**–**h**, **q**–**r**) and NF-κB (**i**–**j**, **s**–**t**) in the two types of the cells cultured for three days in space (**a**, **c**, **e**, **g**, **i**, **k**, **m**, **o**, **q**, **s**) or on ground (**b**, **d**, **f**, **h**, **j**, **l**, **n**, **p**, **r**, **t**) for EA.hy926 cells (**a**–**j**) and rBMSCs (**k**–**t**). Bar = 50 μm

EA.hy926 cells cultured in space and on ground (Fig. 7i–j). These diverse regulations of distinct signaling molecules imply the complicated signaling network for ECs in space.

Abnormal regulations or functions of cellular cytoskeleton via cell adhesion alter cell phenotype. At early stage of the directed hepatic differentiation of rBMSCs for 3 days under microgravity, β1 integrin expression is upregulated, but autophosphorylation of adhesion-dependent kinase FAK (Fig. 7k–l) and expressions of Rho GTPases of Rho A (Fig. 7m–n) and Rac-1 (Fig. 7o–p) have no significant differences and Cdc42 has a slight decline (Fig. 7q–r), as compared to those on ground. At late stage of induced differentiation for 10 days, however, Rac-1 and Cdc 42 expressions are decreased and *p*-FAK and RhoA expressions are increased dramatically in space compared to ground control (*data not show*). NF-κB expression is significantly enhanced in space than that on ground at Day 3 but the difference between space and ground groups tends to reduce at Day 10 (Fig. 7s–t). Since hepatic differentiation of MSCs in space should result from the coupling of mechanical and chemical signaling, it is reasonably speculated that chemical induction plays major roles at early stage and mechanical factors work at late stage. To date, few studies are directed on hepatic differentiation of MSCs under space microgravity or ground-based simulation, and further studies are required to isolate the two distinct signaling mechanisms.

5.6 Hepatic Differentiation of RBMSCs

Directed differentiation of stem cells under microgravity is key for space reproductive biology and histogenesis. Here two typical biomarkers of hepatic differentiation are tested for rBMSCs cultured in hepatic induction medium. Interestingly, the cells yield fibroblast-like morphology and the expression of albumin (ALB) and cytochrome CYP450 enhances cell maturation and differentiation. ALB expression for the cells in space is higher at Day 10 than that at Day 3 (Fig. 8c and a) but is comparable between the two time points for the cells on ground (Fig. 8d and b). Specifically at the same point of Day 10, the expression is higher for the cells in space than that on ground (Fig. 8d). Similarly, CYP450 expression is higher in space at Day 10 (Fig. 8g and e) but comparable between Day 10 and Day 3 on ground (Fig. 8h and f). Also observed is the increased CYP450 expression at Day 3 in space compared to that on ground (Fig. 8e–f). These findings indicate that the differentiated cells present functional phenotype similar to those of hepatocytes (Choi et al. 2013). Collectively, these results suggest that rBMSCs are preferential in hepatic differentiation at long-term duration under microgravity. Actually, this is the first evidence for hepatic differentiation of rBMSCs under space microgravity. While liver is not a preferential candidate for gravity sensation, the observation that rBMSCs are more prone to hepatic differentiation under space microgravity than that under normal gravity is coincident with the theory of use and disuse. Elucidating the underlying mechanisms is helpful to understand the effects of gravity on stem cell differentiation.

In summary, we develop a novel SCCS system and elucidate the response of endothelial cells and mesenchymal stem cells to gravity alteration under real space microgravity environment in SJ-10 satellite. Our data indicate that space microgravity could suppress cellular metabolism. Both ECs and MSCs are regulated by

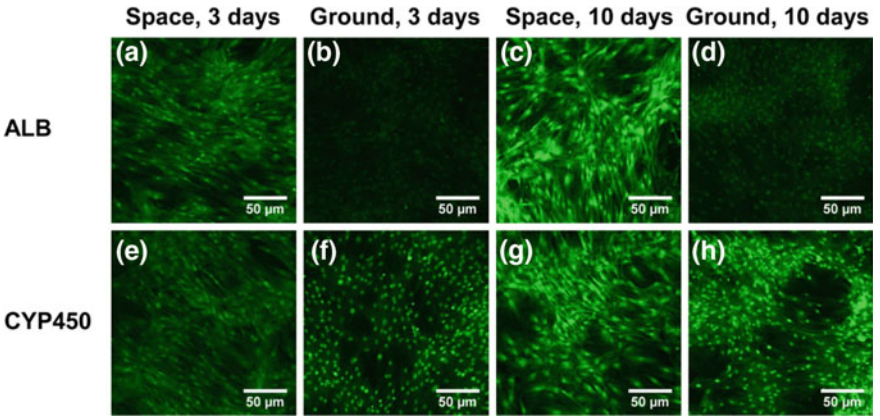

Fig. 8 Differentiation of rBMSCs to hepatocyte-like cells cultured in space or on ground. Hepatocyte biomarkers of ALB (**a**–**d**) and CYP450 (**e**–**h**) are stained in rBMSCs at day 3 (**a**, **b**, **e**, **f**) or 10 (**c**, **d**, **g**, **h**) in space (**a**, **c**, **e**, **g**) or on ground (**b**, **d**, **f**, **h**). Bar = 50 μm

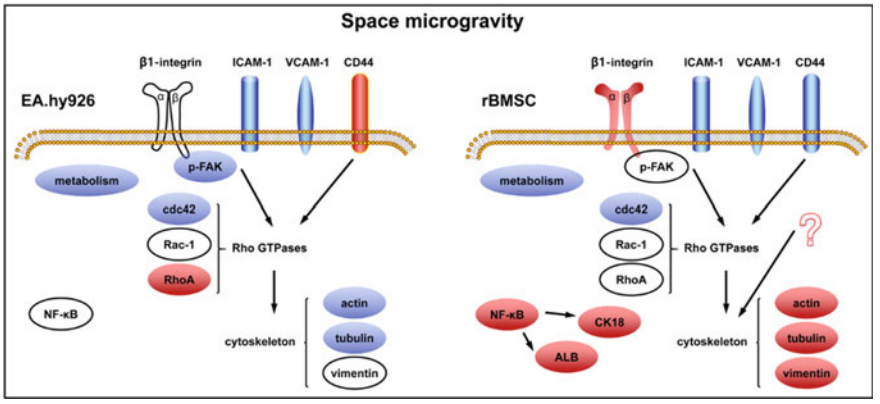

Fig. 9 Working model for the effects of space microgravity on EA.hy926 cells and rBMSCs at day 3. *Red*, *blue* and *white* icons represent up-, down-regulated and unchanged molecules, respectively

microgravity and respond differentially in initiating cytoskeletal remodeling, or dysregulating signaling pathways relevant to cell adhesion, or directing hepatic differentiation (Fig. 9). Since the space mission itself has inevitable limitations due to the well-known inconveniences such as the strictly-limited number of samples, the mechanical condition control, and the operational constrains, it is hard to unravel the underlying mechanism without more controllable or quantified mechanical and mass transport conditions. Nevertheless, this on-orbit experiment verifies the passive de-bubble technique and the effectiveness of designed medium supply patterns. These findings and techniques provide new potential bases for cell biology study under space microgravity.

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