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Review

Microgravity directs stem cell differentiation

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Summary. Stem cells are the cell of origin for organisms and their organs. These cells are critical for tissue regeneration, as well as regenerative medicine. Mechanical forces, such as gravity, have been demonstrated to provide important signals for stem cell fate. In fact, the absence of gravity, that is, microgravity, affects almost all aspects of human physiology, which has been partly attributed to changes in the biological behaviors of stem cells. In this review, we summarize the current understanding of the effects of microgravity on stem cell differentiation that control the fate of stem cells.

Key words: Microgravity, Stem Cells, Differentiation

Introduction

Gravity is constantly exerted on organisms and is necessary to maintain the biological process for cells to spread to tissues all over the body (Zayzafoon et al., 2005; Blaber et al., 2014). The impact of microgravity on organisms and cells is a fascinating scenario in biology and medicine. Stem cells have the capacity to self-renew and differentiate into multiple cellular lineages. Generally, stem cells range from totipotent stem cells that are capable of generating a complete organism, such as embryonic stem cells (ESCs), to pluripotent stem cells, such as hematopoietic stem cells (HSCs), that are able to differentiate into specific cells (Liu et al., 2012). Stem cells provide an excellent *in vitro* system to understand developmental processes, including cell differentiation and fate determination (Li et al., 2015). Moreover, stem cells hold great potentials as replenishable resources for cell therapies, and for use in drug discovery (Tevlin et al., 2016). In addition, the increased number of space flights and advances in devices for modeling simulated microgravity have led to recent findings that microgravity has a significant impact on stemness and differentiation of stem cells (Zhang et al., 2015a,b). These findings will be summarized and discussed below.

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Methods to simulate microgravity on earth

The best ways to achieve real microgravity are through parabolic flights, space crafts, or a space lab. Due to the limited frequency and high cost of space flights, the possibility to conduct experiments in real microgravity is limited. Thus, most of the experiments on the biological effects of microgravity are conducted in ground-based devices (Ulbrich et al., 2014; Maier et al., 2015). In simulated experiments, the magnitude of the earth gravity vector cannot be changed because the body force of gravity remains constant on the surface of earth, and only its influence can be changed (Briegleb, 1992). Consequently, microgravity cannot be achieved with a simulator. Nevertheless, certain devices may generate functional weightlessness from the perspective of organisms or cells, termed simulated microgravity (Herranz et al., 2013). For instance, various devices, such as a clinostat, a random positioning machine, a rotating cell culture system, levitating magnets, and hindlimb unloading, have been successfully used to model simulated microgravity (Ulbrich et al., 2014; Maier et al., 2015).

The clinostat is an effective, ground-based tool that

mimics the microgravity effect by nulling the gravitational vector through continuous averaging (Sarkar et al., 2000). Typically, the clinostat consists of chambers for cell culture and a motor for driving the chambers to revolve along the horizontal axis. A more widely used device is the random positioning machine (RPM), a three-dimensional (3-D) clinostat consisting of two frames, each driven by a dedicated motor. This allows both frames to rotate clockwise, anticlockwise, vertically, and horizontally, independent of each other (Wuest et al., 2015). The rotating cell culture system (RCCS)/rotating wall vessel (RWV) is a unique line of bioreactors designed for the creation and propagation of three dimensional tissue assemblies, as well as fragile, difficult-to-cultivate cells (Goodwin et al., 1992). Cells can be grown with or without solid supports (scaffolding microcarrier beads) in a slow rotating, relatively large liquid filled container (chamber). The RCCS rotates the chamber horizontally to suspend the inoculated cells in the culture medium and model simulated microgravity (Mellor et al., 2014). The rotation speed has to be adapted to the specific weight of the cells, the fluid density, and viscosity (Hammond and Hammond, 2001; Skardal et al., 2010).

Another ground-based method to simulate microgravity is to produce a magnetic force. Magnetic levitation occurs when the magnetic force counterbalances the gravitational force (Herranz et al., 2012). This type of system sustains the magnetic forces and models simulated microgravity for diamagnetic samples. The magnitude of this force depends upon the magnetic susceptibility of the material, which may vary greatly depending on the type of material (Liu et al., 2011).

Hindlimb unloading is also another well-established ground-based approach to model microgravity and musculoskeletal disuse and mimics many of the physiological changes associated with space flight (Niu et al., 2015). The hindlimbs of rodents are elevated to produce a 30° head-down tilt, which results in a cephalad fluid shift and prevents weight bearing on the hindquarters (Chowdhury et al., 2013). In particular, hindlimb unloading is an ideal model for studying the effect of microgravity on mesenchymal stem cells (MSCs) *in vivo*.

Microgravity affects the differentiation direction of ESCs

Embryonic stem cells (ESCs) are pluripotent cells that are capable of differentiating into all the terminal cell types of an organism (Noguchi et al., 2015). Investigation on the effect of microgravity on self-renewal and the differentiation of ESCs will provide greater insights into the understanding of embryonic development, as well as the ESC's culture system.

In a recent space flight experiment, it was shown that exposure to microgravity for 15 days maintained the expression of self-renewal and pluripotency markers (Sox1 and Sox2) in mouse ESCs, compared to the static condition (Blaber et al., 2015). Generally, leukemia inhibitory factor (LIF) acts as an indispensable factor to maintain the pluripotency of ESCs. Similarly, Kawahara et al. utilized a 3-D clinostat to simulate microgravity and maintain mouse ESCs in serum-free and feeder-free conditions without LIF, and they found that the expression of pluripotent markers (Sox2, Oct-4 and Nanog) was significant higher than cells in normal gravity after three days (Kawahara et al., 2009). However, Wang Y et al. found that there were no significant changes in the expression of Oct-4 and Nanog of mouse ESCs after exposure to simulated microgravity for 2 or 7 days using a 3-D clinostat (Wang et al., 2011). Further studies are required to detect the exact impact of microgravity on stemness of ESCs before it can be accepted as an effective method for ESC culture.

The results of the 15-day space flight experiment demonstrated that the expression of terminal differentiation markers for most lineages of the three primary germ layers (including pancreas, bone, immune system, muscle, liver, lung, and renal system) in mouse ESCs were inhibited, while the differentiation markers for mature neurons were found to be upregulated. The incomplete commitment of early stem cell progenitors to the path of differentiation, as well as the decreased calcium channel-mediated mechanotransduction signaling, may be attributed to an inhibition of the differentiation of ESCs (Blaber et al., 2015). Theses results highlight the negative effect of microgravity on the differentiation of ESCs. However, it has been reported that simulated microgravity can also promote the hepatic differentiation of ESCs. Specifically, Wang et al. found that mouse ESCs cultured in a 3-D clinostat with hepatic differentiation medium showed higher morphologic traits and biomarkers for liver cells than cells in a static condition, including albumin production, cytochrome P450 activity, and low-density lipoprotein uptake. Moreover, the cells that were exposed to simulated microgravity had the ability to further differentiate into hepatocyte-like cells in transplanted mice (Wang et al., 2012). Consistently, Zhang et al. also found that simulated microgravity generated by RCCS could significantly promote hepatic generation from mouse ESCs using hepatic differentiation medium, as indicated by the higher levels of liver-specific functions. In addition, an *in vivo* experiment further confirmed the ability of transplanted ESCs exposed to simulated microgravity for 14 days to be engrafted into the recipient livers (Zhang et al., 2013a,b).

Several possible factors may account for the discrepancies in the results of the differentiation directions of ESCs exposed to real or simulated microgravity, including the time of exposure to microgravity, the methods to simulate microgravity, and the culture parameters. For instance, Fridley et al. found that the RCCS bioreactor promoted mouse ESCs to differentiate into hematopoietic stem and progenitor

cells at high cell densities, while there was no significant difference in hematopoietic differentiation of ESCs at low cell densities between simulated microgravity and static condition. The rotation speed of the RCCS also had a vital effect on hematopoietic differentiation of ESCs (Fridley et al., 2010). Therefore, it would be important to further investigate the impact of microgravity or microgravity bioreactor on the differentiation directions of ESCs in order to provide a method for large-scale production of transplantable therapeutic cells.

Microgravity affects the differentiation direction of MSCs

Mesenchymal stem cells are pluripotent cells that mainly exist in bone marrow (Li et al., 2015). MSCs have low immunogenic response *in vivo* and have the capacity to self-renew and undergo multi-lineage differentiation, which endow them with great potential for regenerative medicine and tissue engineering (Ahmed and Hincke, 2014).

The effect of space flight on the stemness of MSCs has not been clearly identified to date. In one study, simulated microgravity modeled by a 2-D clinostat upregulated the expression of the pluripotency marker Oct-4 in rat bone marrow MSCs (BMSCs) at 72 hours (Wang et al., 2014). Moreover, Mitsuhara et al. also demonstrated that simulated microgravity generated by a 3-D clinostat significantly increased the expression of Oct-4 in rat BMSCs on day 7 (Mitsuhara et al., 2013). Consistently, it has been found that the RCCS microgravity bioreactor noticeably accelerated the expression of pluripotent markers (including Oct4, Nanog, and Sox2) in human adipose-derived MSCs at day 5 compared to static condition (Zhang et al., 2015). In addition, Cerwinka et al. showed that human BMSCs largely remained in an undifferentiated state after exposure to simulated microgravity (RCCS) for 3 days because stem cell markers CD44, CD133 and CD166 were robustly expressed (Cerwinka et al., 2012). These results suggest that simulated microgravity contributes to the maintenance of the stemness in BMSCs.

Similarly, simulated microgravity also exerts a remarkable influence on the differentiation fate of MSCs. Using the specific niche media, simulated microgravity was reported to enhance the differentiation of MSCs into endotheliocytes, neurocytes, adipocytes, and chondrocytes. However, abundant evidence has demonstrated that simulated microgravity significantly inhibits the osteogenic differentiation of MSCs.

Differentiation direction of MSCs is accelerated by microgravity

It has been reported that rat BMSCs under simulated microgravity (2-D clinostat) for 72 hours increased the expression of endothelial-specific markers (Flk-1 and vWF), compared to normal gravity conditions (Zhang et al., 2013). Consistently, using a 2-D clinostat Wang et al. demonstrated that after 72 hours of simulated microgravity there was an increase in the expression of the endothelium markers vWF and CD31 in rat BMSCs, relative to static condition. In addition, they also found that simulated microgravity increased the neurogenic and adipogenic differentiation of BMSCs, as demonstrated by higher expression of neurogenic markers (MAP2 and NF-H) and adipogenic markers (PPARγ2 and fat droplets) (Wang et al., 2014). Another report confirmed the stimulative effect of simulated microgravity on the neurogenic differentiation of rat BMSCs. BMSCs cultured in simulated microgravity with neuronal differentiation medium expressed higher neurogenic markers (MAP-2, TH and CHAT) while generating more mature and repetitive action potentials relative to cells from the normal gravity group (Chen et al., 2011). Emerging evidence has also suggested that there is a synergistic impact of simulated microgravity on the chondrogenesis of MSCs. For example, Wu et al. found that the expression of cartilage extracellular matrix protein (collagen II and aggrecan) in rat BMSCs cultured in a RCCS system was elevated, compared to the static culture group (Wu et al., 2013). Similarly, Yu et al. found that simulated microgravity using a RCCS promoted the expression of chondrocyte-specific genes, the matrix production of human adipose derived MSCs, and the activation of the p38 MAPK pathway. Moreover, treatment with a p38 MAPK inhibitor blocked the increased chondrogenesis of MSCs induced by simulated microgravity, suggesting that p38 MAPK signaling acts as an essential mediator in this process (Yu et al., 2011). Although the mechanism of simulated microgravity promoting MSCs differentiating into endotheliocytes, neurocytes, adipocytes, and chondrocytes is still unclear, the simulated microgravity culture system provides great potentials for the construction of tissue-engineered cells.

Differentiation direction of MSCs is impeded by microgravity

As one of the main sources of osteoblasts, BMSCs play an important role in bone formation (Biver et al., 2013). The effect of microgravity on the osteogenesis of MSCs and the underlying mechanisms has gained widespread attention because microgravity induces an observed bone loss in space flight (Smith et al., 2014). MSCs exposure to simulated microgravity modeled by the clinostat resulted in significant decreases in the expression of osteogenic markers (ALP, Runx2, Osx and RANKL), with reduced collagen content and calcium node formation (Dai et al., 2007; Yamazaki et al., 2011; Uddin and Qin, 2013). Similarly, using the RCCS system the results also showed that simulated microgravity remarkably inhibited the expression of osteogenic markers, such as ALP, OPN, OCN and Runx2, but promoted the expression of adipogenic markers, such as Adipsin, Leptin, Glut and PPARγ (Zayzafoon et al., 2004; Saxena et al., 2007; Zheng et al., 2007). Moreover, MSCs showed a similar differentiation fate for osteogenesis or adipogenesis in

the RPM system (Yuge et al., 2006; Gershovich et al., 2012). *In vivo*, hindlimb unloading decreased the osteogenic potential of MSCs, but increased the adipogenic potential of MSCs, as reflected by the decreased ALP activity, decreased expression of collagen I and OCN, increased expression of Adipsin and Leptin, and accumulation of lipid vacuoles (Pan et al., 2008; Ozcivici and Luu, 2010). These results indicate that simulated microgravity inhibits the osteogenesis of MSCs, whereas it promotes adipogenesis, which may explain the bone loss caused by space flights.

Numerous studies have investigated the underlying mechanisms of decreased osteogenic differentiation of MSCs caused by microgravity. Osteogenic differentiation of MSCs is regulated by several signaling pathways, and the extracellular matrix (ECM)-integrins/focal adhesion kinase (FAK)/extracellular signal-regulated kinase (ERK) pathway plays an important role in this process (Khatiwala et al., 2009). Collagen I is the most abundant protein in the ECM of bone and is necessary for osteogenic differentiation, but exposure to simulated microgravity significantly reduced the expression of collagen I (Zayzafoon et al., 2004; Zheng et al., 2007; Shi et al., 2010). Furthermore, gene ontology analysis showed that RCCS remarkably inhibited the gene expression of ECM and ECM-related proteins (Gershovich et al., 2012). Interestingly, it has been demonstrated that simulated microgravity significantly decreased autophosphorylation of FAK and PYK2 in MSCs (Meyers et al., 2005; Dai et al., 2007). Moreover, simulated microgravity inhibited the activation of ERK1/2, which mediates osteogenic differentiation through activating Runx2, a master osteogenic differentiation transcription factor (Zayzafoon et al., 2004). These results suggest that simulated microgravity perturbed the ECM-integrin/FAK/ERK pathway, which may mediate the inhibition of osteogenesis from MSCs under microgravity.

Another intracellular structure of MSCs that is dramatically disrupted by microgravity conditions is the actin cytoskeleton, which is a primary component of the cytoskeleton in eukaryotic cells. The actin cytoskeleton is comprised of individual actin proteins knows as globular actin (G-actin), as well as filamentous actin (F-actin). F-actin filaments consist of multiple G-actin subunits that interact with one another and are constantly assembled and disassembled (Lee et al., 2010). The dynamic remolding of actin cytoskeleton participates in most cellular behavior, including osteogenic differentiation (Kuo et al., 2015). After exposure to microgravity or simulated microgravity, F-actin stress fibers in MSCs became depolymerized and extenuated, and showed dispersed distribution while monomeric G-actin was increased (Meyers et al., 2005; Buravkova et al., 2008; Shi et al., 2010). The reorganization of the actin cytoskeleton is regulated by multiple factors, most notably the Rho family GTPases (Lee and Dominguez, 2010). Under simulated microgravity, the reorganization of the actin cytoskeleton can be attributed to Rho, which is reported to act as a sensor of microgravity (Louis et al., 2015). For the osteogenic process, increasing evidence has revealed a dramatic decrease of RhoA and Rock activity under simulated microgravity. In addition, simulated microgravity also significantly increased the dephosphorylation of cofilin, which can directly depolymerize F-actin (Meyers et al., 2005; Saxena et al., 2007; Gershovich et al; 2012). However, cytoskeleton integrity is essential for osteogenic differentiation of MSCs and the mature osteoblasts need to assemble a proper actin structure (Chen et al., 2015a,b). Intriguingly, introduction of an adenoviral vector containing constitutively active RhoA (RhoA-V14) led to the recovery of stress fibers, and expression of markers of osteogenic differentiation in MSCs cultured in simulated microgravity (RCCS) (Meyers et al., 2005). These results demonstrated that simulated microgravity disrupts the RhoA/F-actin pathway, which may partly mediate the decreased osteogenic differentiation of MSCs under simulated microgravity.

Recent studies indicated that several other signaling pathways may also mediate the inhibition of osteogenesis from MSCs under simulated microgravity. BMP2 is a member of the transforming growth factor-β (TGF-β) superfamily of proteins that is capable of inducing osteogenesis through activation of the intracellular receptor-regulated Smad protein (R-Smads, Smad1/5/8) and ERK (Bais and Wigner, 2009; Lee et al., 2010). As both gene and protein expression of BMP2 was inhibited by simulated microgravity, a possible mechanism by which simulated microgravity impeded MSCs osteogenic differentiation was proposed to be through the suppression of BMP2 expression (Sun et al., 2008; Qian and Zhang, 2014). Importantly, the SMG condition inhibited the ability of MSCs to differentiate into osteoblasts, and increased the expression of PPARγ2, a critical transcription factor involved in adipogenic differentiation. The expression and activation of PPARγ2 are mainly via the p38 MAPK pathway, which was dramatically activated through phosphorylation by simulated microgravity. A combination of BMP, FGF2, and a p38 MAPK inhibitor (SB203580) also significantly reversed the effect of simulated microgravity on osteogenic differentiation of MSCs (Zheng et al., 2007). These findings suggest that BMP2 and the p38 MAPK pathway can also partly decrease the osteogenesis of MSCs under simulated microgravity. In addition, we previously utilized a 2-D clinostat to model simulated microgravity, and demonstrated that simulated microgravity inhibits osteogenesis of BMSCs through downregulation of TAZ (transcriptional coactivator with a PDZ-binding motif), which acts as a vital regulator of osteogenesis (Chen et al., 2015a,b). Further study is required to clearly elucidate the mechanisms by which microgravity inhibits MSC osteogenesis, and identify efficient methods for intervention to prevent bone loss induced by space flight.

Microgravity and differentiation of other stem cells

Hematopoietic stem cells (HSCs) play an important role in maintaining hematopoietic homeostasis. This has led to interest in studying the effect of microgravity on HSCs. In one study, the effect of microgravity on HSCs differentiation was investigated after a spaceflight mission. The results showed that exposure to microgravity caused human HSCs to differentiate less into committed progenitor cells at day 11, but increased their differentiation into macrophages (Davis et al., 1996). In addition, Plett et al. also demonstrated that human bone marrow $CD34^+$ cells favored myeloid cell differentiation at the expense of erythroid development after exposure to simulated microgravity (RWV) for 48 hours, and this effect was reduced in the static group (Plett et al., 2004). These changes in the differentiation fate of HSCs may contribute to certain hematological abnormalities in humans during space flight, and the underlying mechanism needs to be elucidated further.

Liver stem cells have the capacity of self-renewal, and they display multipotent differentiation, which makes them an important source of cells (Michlopoulos and Khan, 2015). To explore the effect of microgravity on the differentiation of liver stem cells Talbot et al. cultured PICM-19 pig liver stem cells in space flight for 16 days and found that there was no significant difference in the expression of hepatogenic markers compared to the normal gravity group, including hepatocyte-enriched functional genes (i.e., HMOX1, hepatocyte growth factor receptor) and transcription factors for hepatocyte differentiation (i.e., NFKB, HNF1-α, HNF4-α) (Talbot et al., 2010). However, Majumder utilized a 3-D clinostat to simulate microgravity and found that murine oval liver stem cells expressed a higher level of HNF4-α after 2 hours of simulated microgravity treatment. These researchers also observed that simulated microgravity facilitated the downregulation of Notch 1 and the upregulation of BMP4 in liver stem cells and that by blocking BMP4, they were able to attenuate the increased hepatogenic differentiation of liver stem cells (Majumder et al., 2011). These findings mean that simulated microgravity may support hepatogenic differentiation of liver stem cells and provide a new strategy for large-scale generation of hepatocytes.

Currently, increasing evidence has revealed that tumors contain a variable number of cells that have the capacity for self-renewal and partial differentiation; these cells are known as cancer stem cells (CSCs) (Islam et al., 2015). CSCs play a vital role in tumorigenesis and drug resistance. It has been reported that the physical microenvironment and mechanical stimuli act as crucial regulators to preserve the function of CSCs (Cordenonsi et al., 2011). Interestingly, Pisanu et al. found that exposure to simulated microgravity (RPM) for 24 or 48 hours notably induced human lung CSCs to lose their stemness features, as reflected by a decrease in ALDH, and it inhibited the gene expression of the stemness markers Nanog and Oct-4. Nevertheless, CSCs stably acquired and preserved those traits after exposure to normal gravity (Pisanu et al., 2014). These findings provide a new understanding for the role of gravity in the maintenance of CSCs and cancer treatment.

Conclusions and perspectives

Gravity is essential to maintain human physiology. In this article, we reviewed the recently uncovered effects of microgravity on stemness and the differentiation fate of stem cells. Microgravity and simulated microgravity contribute to maintaining the

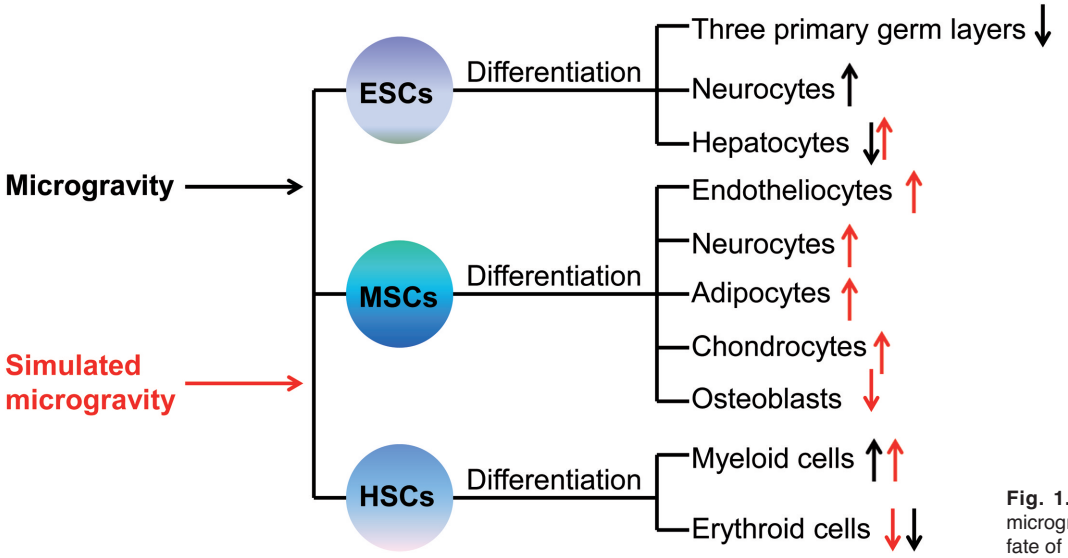

Fig. 1. Microgravity and simulated microgravity directs the differentiation fate of ESCs, MSCs and HSCs.

stemness of ESCs and MSCs, whereas it decreases the stemness of CSCs. For differentiation fate, the response of stem cells varies depending on the microgravity condition (e.g., space flights or simulated microgravity), and the types of stem cells (Fig 1). While the differentiation of stem cells under microgravity has generated significant interest, several issues remain unresolved: (1) What are the precise effects of microgravity on the differentiation fate of stem cells, and what is the underlying mechanism? (2) How could the negative effect of microgravity on the differentiation path of stem cells be intervened? (3) For tissue engineering, what are the optimal microgravity bioreactor and parameters for directing the differentiation of stem cells?

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