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Stem Cell Culture Under Simulated Microgravity

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Abstract

Challenging environment of space causes several pivotal alterations in living systems, especially due to microgravity. The possibility of simulating microgravity by ground-based systems provides research opportunities that may lead to the understanding of *in vitro* biological effects of microgravity by eliminating the challenges inherent to spaceflight experiments. Stem cells are one of the most prominent cell types, due to their self-renewal and differentiation capabilities. Research on stem cells under simulated microgravity has generated many important findings, enlightening the impact of microgravity on molecular and cellular processes of stem cells with varying potencies. Simulation techniques including clinostat, random positioning machine, rotating wall vessel and magnetic levitation-based systems have improved our knowledge on the effects of microgravity on morphology, migration, proliferation and differentiation of stem cells. Clarification of the mechanisms underlying such changes offers exciting potential for various applications such as identification of putative therapeutic targets to modulate stem cell function and stem cell based regenerative medicine.

Keywords

In vitro model · Simulated microgravity · Stem cells

Abbreviations

Ad-hMSC	human adipose-derived mesenchymal stem cell
BDNF	brain derived-neurotrophic factor
bFGF	basic fibroblast growth factor
BMOL	bipotential murine oval liver
BM	bone marrow
cMSC	cranial bone derived-mesenchymal stem cell
CBSC	cord blood stem cells
GDNF	glial-cell derived-neurotrophic factor
GDNF	glial cell line-derived neurotrophic factor
hDPSC	human dental pulp stem cell
HSCs	hematopoetic stem cells
HGF	hepatocyte growth factor
hEpSCs	human epidermal stem cells
mBMSC	mouse bone marrow stem cell
MG	(simulated) microgravity
NG	normal gravity

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Rab-BMSC	rabbit bone marrow stem cell
RCCS	rotating cell culture system
RWV	rotating wall vessel
SSCs	spermatogonial stem cells
TGF-β	transforming growth factor
VEGF	vascular endothelial growth factor

1 Introduction

Space missions induce many changes in human health and physiology, and among many factors changes in gravitational loading is one of the major contributors to these changes (Garrett-Bakelman et al. 2019). Mechanical forces, including those governed by gravitational loading, act as regulatory signals that effect morphology and function of tissues (Benjamin and Hillen 2003; Buravkova et al. 2008; Ozcivici et al. 2010a). Therefore, lack of gravity causes several health problems such as bone loss (Vico et al. 2000), muscle atrophy (Fitts et al. 2001), cardiovascular deconditioning (Aubert et al. 2005) and immune system dysregulation (Crucian et al. 2008). Biomedical implications of gravitational alterations highlight the importance of microgravity research to ensure the possibility of extension of space flights duration for future missions and potential colonization efforts in space bodies with different gravity fields compared to that of earth.

Consistent with tissue-level changes, microgravity is known to induce structural and functional changes in various cell types (Buravkova et al. 2004; Kacena et al. 2003). For instance, even though immune cells in circulation were found to be increased in number during spaceflights (Mills et al. 2001), microgravity weakens host defense by decreasing phagocytic capacity of these immune cells (Kaur et al. 2005). As another example, in bone tissue microgravity reduces the number and function of osteoblasts involved in bone formation (Hughes-Fulford and Lewis 1996), reducing new bone formation as well as loss of regulation over bone resorption by osteoclasts (Nabavi et al. 2011), resulting in net bone loss. Although studies focusing on mature cells provide valuable biological information on adaptations to microgravity, tissue level implications of long-term spaceflight infer deterioration of different magnitudes (Buravkova 2010). An important aspect of long-term health considerations for space-flight focus on augmenting recovery through regeneration (Blaber et al. 2014). Stem cells with their vast capacity for self-renewal and differentiation carry a great regenerative potential for tissues (Roobrouck et al. 2008) and therefore they are considered as strong therapeutic candidates for space biomedicine.

Spaceflight technologies allow life sciences laboratory setups in orbit for the execution of *in situ* microgravity experiments, both *in vivo* (Sandonà et al. 2012) and *in vitro* (Tamma et al. 2009). However, opportunities on space-based experimentation are rare and expensive (Delikoyun et al. 2019a), highlighting the importance of earth-based models for microgravity research. It is possible to simulate reduced weight-bearing (Wagner et al. 2010) or complete lack of weight-bearing (Judex et al. 2013; Judex et al. 2016; Ozcivici et al. 2007; Ozcivici and Judex 2014; Ozcivici et al. 2014) in rodents however, these models are mostly specific to testing hypotheses on the musculoskeletal tissues (Morey-Holton and Globus 2002). Alternatively, several microgravity simulation devices have been developed to study molecular and cellular level adaptations *in vitro*. Here, we reviewed the available literature for the operating principals and findings of *in vitro* microgravity simulation devices with a specific emphasis on stem cells.

2 Earth-Based *In Vitro* Techniques That Simulate the Microgravity

In order to simulate effects of microgravity, that is also depicted as weightlessness to define the situation where a net sum of all forces is zero (cell surface binding, hydrostatic pressure etc.) (Anken 2013), several physical techniques were translated and developed into laboratory devices. These devices are (1) Clinostat; (2) Random

positioning machine (RPM); (3) Rotating wall vessel (RWV); and (4) Magnetic levitation-based systems.

2.1 Clinostat

Clinostat devices (Fig. 1) rotate samples (i.e. cells) perpendicular to the gravitational vector in order to counteract and negate gravitational acceleration on them (Briegleb 1967; Eiermann et al. 2013). These devices can reduce gravitational pull on samples to an average of 10^{-3} g (1 g = 9.81 m/s^2). Clinostat systems were initially designed with slow rotation frequencies (<10 rpm, rpm: revolutions per minute) to study the gravity-related effects on the growth of plant specimens (Dedolph and Dipert 1971; Hasenstein et al. 2015). The system was then upgraded to reach faster rotational velocities to nullify the gravity vector on smaller objects including cells (Briegleb 1992). Basically, cells in the fast-rotating clinostat system move on circular paths and the diameter of this path decreases as the rotation speed increases and finally relative liquid motion is canceled out in the system. This phenomenon occurs because the radially transferred velocity of the chamber wall and in turn cells in the device begin to rotate around their own center with their surrounding liquid boundary layers. In this condition, cell sedimentation stops and the gravitational pull becomes imperceptible by the cells (Dedolph and Dipert 1971; Klaus 2001).

Clinostats may be in different configurations depending on the number of rotation axes as 2D clinostats or 3D clinostats. Clinostats with the configuration providing rotation in one rotation axis are commonly referred as 2D clinostats (Eiermann et al. 2013; Shang et al. 2013) and sometimes as 1D clinostats (Uddin et al. 2013; Uddin and Qin 2013). 2D clinostats were mainly designed to expose cell suspensions to the simulated microgravity condition (Klaus et al. 1998), but then adapted for the culture of adherent cells (Eiermann et al. 2013). Most of the *in vitro* stem cell culture microgravity studies have been performed in adherent culture condition with varied designs of clinostats; such as a horizontally rotating sealed culture chamber with a medium chamber and a gas-permeable membrane (Chen et al. 2015) or horizontally rotating coverslips inserted into the fixture of chambers (Xue et al. 2017). Although clinostats offer simplicity in application, its rotating nature is an important limitation in design. Smaller centrifugal acceleration is generated on the cells located closer to the center of rotation and in order to minimize variations for accurate force balance, the diameter of the chamber should be kept minimal (~ a few mms), limiting clinostat working volume to accommodate samples of various sizes (Briegleb 1992; Grimm et al. 2014). Another clinostat design, referred as

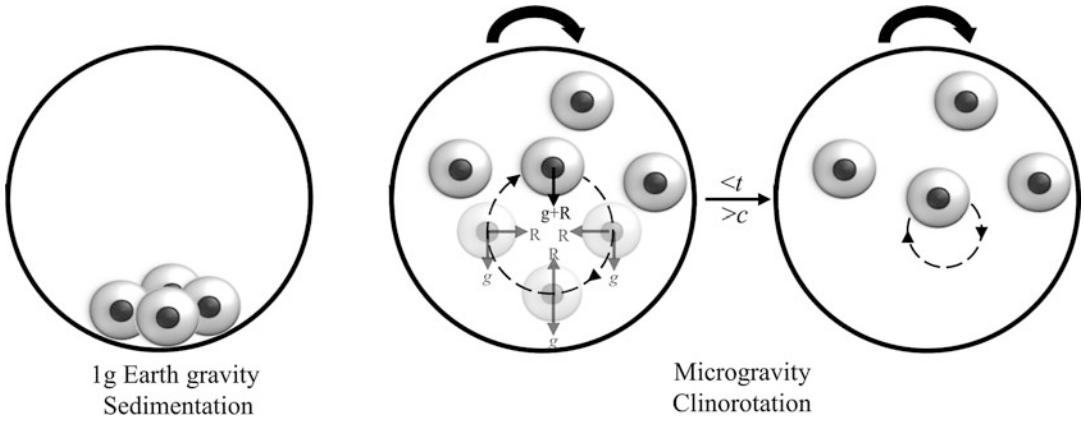

Fig. 1 Principle of the fast-rotating clinostat system. Cells in the clinostat follow a circular trajectory. Increase in the frequency of rotation (*t*) will be resulted in a decrease in perimeter of circular path (*c*) and microgravity is simulated when the cells eventually begin to rotate around its own axis. g: gravity vector, R: radial force

3D clinostat, has two rotation axes in a gimbal mount with an operation of constant direction of rotation (Hoson et al. 1997), offers a larger working capacity than single-axis clinostats (Hauslage et al. 2017). Despite the limited number of studies reporting stem cell culture in 3D clinostats, the effects of microgravity created with 3D clinostats on stem cells have been reported on different culture conditions; monolayer culture (Otsuka et al. 2018) and scaffold-based three-dimensional (3D) culture (Nishikawa et al. 2005). Approximately one third of the reports investigating the effect of microgravity on stem cells using Earth-based *in vitro* techniques were performed with various forms of clinostats.

2.2 Random Positioning Machines (RPM)

In addition to horizontal rotation, it is possible to add a vertical rotation component in clinorotation-based devices to create a different magnitude of the outward acceleration for enhancing the resultant mechanical field (Hoson et al. 1997). Random positioning machine (RPM) rotates samples around two rotational axes in a gimbal mount similar to 3D clinostats. Unlike 3D clinostats however, RPMs operate with changing not only the velocity but also the direction of rotation randomly for constant reorientation of the gravity vector (Wuest et al. 2015). The region for the most effective microgravity is the center of the two rotation axes, this limits the preferred sample volume for RPMs (Grimm et al. 2014). Centrifugal acceleration of RPMs depends not only on rotation velocity and distance from the center of rotation, but also position of the sample in space and time due to the resultant force of two axes (Fig. 2). Therefore, these devices provide a better microgravity environment than clinostats, especially for larger samples.

Originally introduced for plant-based studies (van Loon 2007), RPMs have been adapted for environmental requirements (i.e. temperature, humidity and CO_2) of mammalian cell cultures. Currently RPMs are available with desktop configurations (Borst and van Loon 2009) and majority of stem cell related RPM studies were conducted on such devices (Gershovich et al. 2012; Ratushnyy et al. 2018). In alternative RPM designs, it has been shown that it is possible to increase the working volume greatly up to 14 L (Wuest et al. 2014), or algorithm based controlling of the motion to reach partial gravity rather than complete weightlessness (Benavides Damm et al. 2014).

2.3 Rotating Wall Vessel (RWV)

The rotating wall vessel (RWV) platform was developed and commercialized by NASA

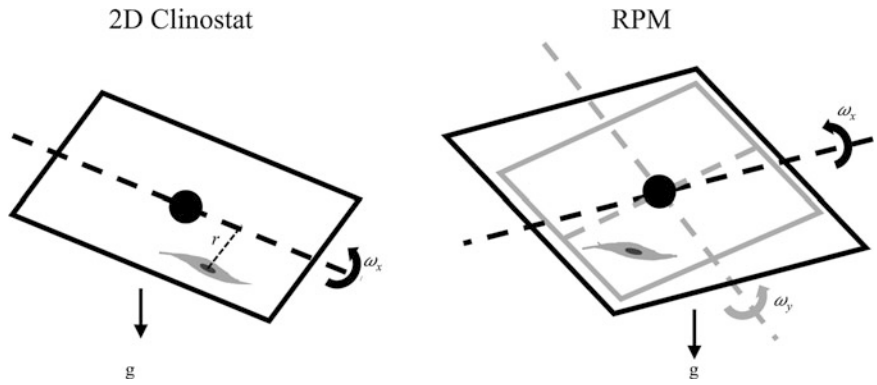

Fig. 2 Working principles of the 2D clinostat (left) and RPM (right). Acceleration in a clinostat rotating around one axis is time independent and determined by the rotation velocity (ω_x) and distance of the cell from the center of rotation (r), whereas acceleration in a RPM, that is rotating around two perpendicular axes, depends on time and position of the sample in space as well as the rotation velocities (ω_x, ω_y)

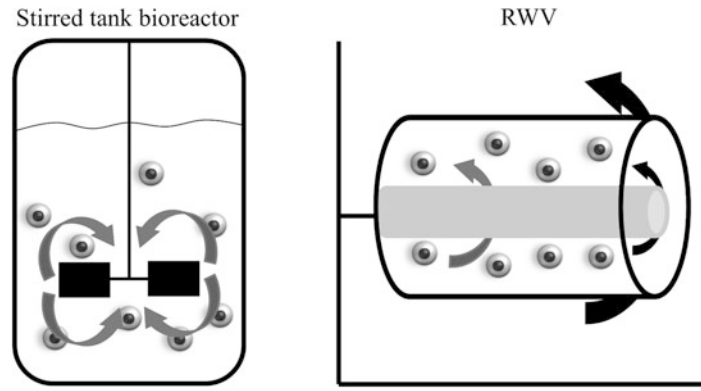

Fig. 3 Flow pattern in a bioreactor with internal mixer and a RWV (right). Grey arrows show flow pattern in the bioreactors. Unlike the stirred tank bioreactor, RWV minimizes turbulence owing to not requiring a mixer for transfer, and provides better formation and growth of 3D aggregates

(National Aeronautics and Space Administration) to simulate microgravity for cell culture applications (Schwarz et al. 1992). RWV is basically a specialized clinostat that transfer rotational velocity onto the cells in fully fluid-filled vessel, allowing them to fall through circular paths (Klaus 2001). Unlike clinostats however, RWVs provide oxygenation by a coaxial tubular oxygenator, that aims to provide sufficient cell movement for an efficient nutrient, oxygen and waste transport and an enhanced cell-cell contact (Schwarz et al. 1992). RWV system (Fig. 3) ensures minimized turbulence in the flow by virtue of eliminating the need to use the internal mixer for transport and it makes the system suitable for *in vivo*-like 3D aggregate formation and growth (Goodwin et al. 1993), however on this system cells cannot be cultured as monolayers on glass slides unlike the clinostat. Instead, for adherent cell culture polymer microcarriers should be used as solid support materials (Gao et al. 1997).

Almost half of the studies investigating the effect of microgravity on stem cells have been performed with RWVs. Commercial RWV systems allow tunable velocities and for oxygenation they can be equipped with different types of gas transfer membranes; high-aspect ratio vessels (HARV, volume up to 50 mL), and/or autoclavable slow turning lateral vessels (STLV, volume up to 500 mL). Stem cells can be cultured in RWVs in different forms, such as pellets (Luo et al. 2011), adherent on scaffolds (Koç et al. 2008) or on microcarriers (Sheyn et al. 2010), in encapsulated form (Hwang et al. 2009) or in organoid form (Mattei et al. 2018).

2.4 Levitation by Negative Magnetophoresis

Application of mechanical rotation to create microgravity inherently creates additional mechanical forces on cells, such as shear stress vectors based on fluid motion (Leguy et al. 2011) but the acceptable limits for rotational speed and thus these mechanical forces have still not been fully elucidated. As an alternative, microgravity of cells may also be achieved by compensating the gravitational vector by a counteracting external force.

Diamagnetic materials such as water, cells and proteins tend to move away from strong magnetic fields to weaker ones (Beaugnon and Tournier 1991b; Zhao et al. 2016). Using this principle, non-living (Beaugnon and Tournier 1991a) and living (Berry and Geim 1997) objects were successfully levitated under strong magnetic fields. Living structures show homogeneity in terms of diamagnetic property and therefore the magnetic force acts homogeneously on the entire structure at the molecular level, providing a proper microgravity (Schenck 1992).

In principle, the magnitude of the magnetic force acting on the object is directly proportional to the magnetic susceptibility difference between the object and the surrounding environment, and

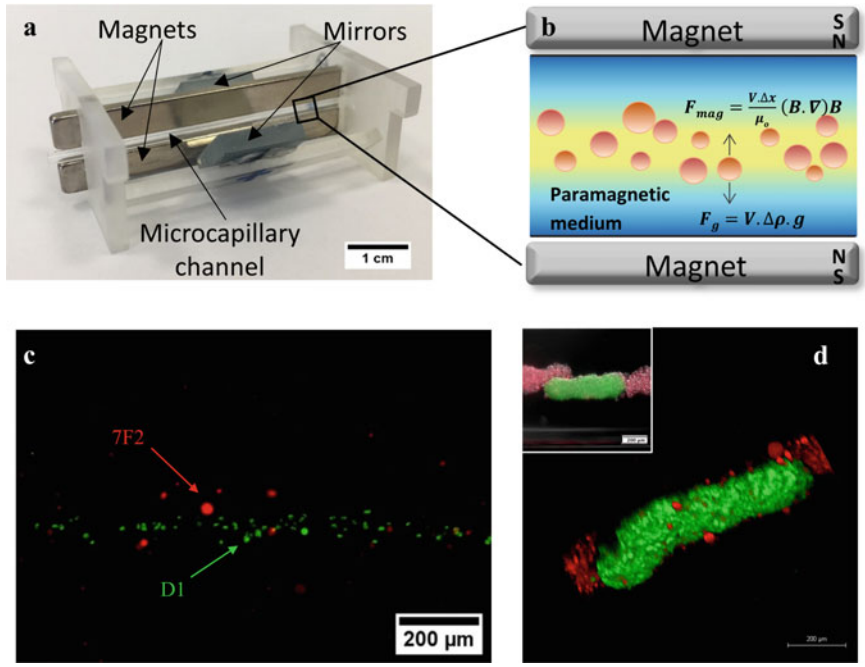

Fig. 4 Magnetic levitation of the cells in a paramagnetic medium. (**a**) Structure of the magnetic levitation device composed of two neodymium magnets, a microcapillary channel and two mirrors placed at 45°. (**b**) Forces acting on cells at the equilibrium position in the device, where F_{mag}: magnetic force, F_b: buoyancy force, V: cell volume, $\Delta\chi$: magnetic susceptibility difference between the paramagnetic medium and the cell, μ_0: permeability of free space, B: magnetic induction, $\Delta\rho$: density difference between the paramagnetic medium and the cell, g: gravitational acceleration. (**c**) Levitated quiescent D1 ORL UVA cells (mouse bone marrow mesenchymal stem cells, green) and adipogenic differentiated 7F2 cells (mouse bone marrow osteoblasts, red). Reprinted from Sarigil et al. (2019b). (**d**) Confocal and conventional fluorescence microscopy (upper left) image showing self-assembled coculture clusters formed with magnetic levitation (100 mM Gd-BT-DO3A). MDA-MB-231^{dsRed} (human breast cancer cells) cells were seeded onto D1 ORL UVA^{eGFP} clusters formed with magnetic levitation and (total 50,000 cells in the device). Reprinted from Anil-Inevi et al. (2018). Scale bars: 200 µm

magnitude of the magnetic field gradient (Yaman et al. 2018). In order to levitate diamagnetic objects, there is a need for stable substantially large magnetic field gradient; for example, superconducting magnets with large magnetic field intensity (i.e. ~ 12 Tesla) that were used to investigate the behavior of stem cells in microgravity (Meng et al. 2011; Shi et al. 2010). It is also possible to levitate objects with a moderate magnetic field gradient (<1 Tesla) in a medium that provides a high magnetic susceptibility difference (Mirica et al. 2009), and this principle can be applied to living cells in a microfluidic system consisting of two permanent magnets with the same poles facing each other (Tasoglu et al. 2015). This magnetic levitation system (Anil-Inevi et al. 2019b), that was initially used for cell separation based on single-cell density (Delikoyun et al. 2019b; Durmus et al. 2015; Sarigil et al. 2019a; Sarigil et al. 2019b), can also be used to apply microgravity condition on stem cell culture (Anil-Inevi et al. 2019a; Anil-Inevi et al. 2018) (Fig. 4). Although levitation approach is novel and at the initial stages in development, the system offers many advantages such as real time monitoring and rotation free application.

3 *In Vitro* Culture of Stem Cells in Simulated Microgravity

Studies on stem cell biology during microgravity exposure were conducted in various studies, which are reviewed below and summarized (Tables 1, 2, 3 and 4) based on the source of the stem cell used.

Table 1 Applications of microgravity simulation techniques for embryonic stem cell culture

Culture Method	Strategy	Cell type	Culture period in MG	Results	Reference
2D clinostat	Pipette method	Mouse ESCs	3 days (60 rpm)	Disruption of genes expression especially in cytoskeleton and changes in the cardimyogenesis process	Shinde et al. (2016)
3D clinostat	LIF-free cell culture	Mouse ESCs	7 days (10^{-3} G)	Cell number ↑	Kawahara et al. (2009).
3D clinostat	Cell culture	Mouse ESCs	up to 7 days (10^{-3} G)	Cell number ↓ (5 days)	Wang et al. (2011)
				Adhesion rate ↓ (8 h)	
				Apoptosis ↑ (2 days)	
				Delayed DNA repair	
Rotating microgravity bioreactor	Hepatic cell differentiation on biodegradable scaffolds	Mouse ESCs	14 days (35 rpm)	Hepatic differentiation: Albumin ↑ α-fetoprotein ↑ Cytokeratin 18 ↑ Transthyretin ↑ Glucose-6-phosphatase ↑	Wang et al. (2012)
Rotating microgravity bioreactor	Alginate encapsulation and cell culture	Murine ESCs	After undifferentiated 3 days, cultured up to 21 days (25 rpm)	More realistic 3D structure and functional bone tissues Metabolic profile during culture in RWV: Glucose ↓ Lactate ↑ Ammonia ↑ PH ↓ Oct4 ↓ Osteogenic differentiation: OSX ↑ Cbfa-1/RUNX2 ↑	Hwang et al. (2009)

Table 2 Applications of microgravity simulation techniques for hematopoietic stem cell culture

Culture method	Strategy	Cell type	Culture period in MG	Results	Reference
RWV	Cell culture	HSCs from human umbilical cord blood	8 days (6 rpm)	Total cell number ↑	Liu et al. (2006)
				$CD34^+$ cells ↑	
RWV	Cell culture	HSCs from bone marrow of male mice	7 days (30 rpm)	Number of progenitor cells ↓	McAuliffe et al. (1999)
				High-proliferative potential colony-forming cells ↑	
RWV	Cell culture	Human BM $CD34^+$ cells	4–6 days (8 rpm)	Rate to exit G_0/G_1 phases of cell cycle ↓	Plett et al. (2001)
				Degree of hematopoietic potential ↑	
RWV	Cell culture	Human BM $CD34^+$ cells	2 to 18 days (short term and long term), 10–12 rpm	F actin expression ↓	Plett et al. (2004)
				Migration to SDF-1α ↓	
				Cell-cycle progression ↓	
				Erythroid differentiation ↓	
RWV	Cell culture + growth factors and microcarrier beads	Human umbilical cord blood stem cells (CBSCs)	14 days (gradually increase from 8 to 20 rpm in 48 h)	Cellular proliferation with 3D tissue-like aggregates ↑	Chiu et al. (2005)

Table 3 Applications of microgravity simulation techniques for culture of other stem cell types

Culture method	Strategy	Cell type	Culture period in MG	Results	Reference
Rotary bioreactor	Cell culture on Cytodex-3 micro-carriers	Human epidermal stem cells (hEpSCs)	10–15 days (12 rpm-1 day, 22 rpm 14 day)	Proliferation ↑	Lei et al. (2011)
				Viability ↑	
				Higher percentage of ki67 positive cells	
				Low expression of involucrin	
Rotating cell culture system (RCCS)	Cell culture with glial cell line-derived neurotrophic factor (GDNF) growth factor and Sertoli cell feeder	Mouse spermatogonial stem cells (SSCs)	14 days (10–12 rpm)	Proliferation ↑	Zhang et al. (2014)
				Aggregates are similar to native in vivo cells	
				mRNA expression of Oct-4, GFRα1 and Bcl6b ↑	
3D clinostat	Cell culture	Bipotential Murine Oval Liver (BMOL) stem cells	2 h (10^{-3} G)	Proliferation ↑	Majumder et al. (2011)
				Induce hepatic differentiation	
				Downregulation of Notch1, upregulation of BMP4	

3.1 Embryonic Stem Cells

Studies investigating the effects of microgravity on embryonic stem cells (ESCs) are not as common compared to other types of stem cells. Most of the studies involving ESCs focused on using microgravity as a biotechnological tool for ESC expansion and differentiation. A study performed with 2D clinostats indicated that while microgravity had no effect on the distribution of ESCs in different cell cycle phases, it disrupted cytoskeletal gene expression and altered the process of cardiomyogenesis of ESCs (Shinde et al. 2016). Another study performed using 3D clinostats revealed that the need for feeder layer, serum and Leukemia-inhibitory factor (LIF) in the conventional method to prevent the mouse ESCs to spontaneously differentiate was eliminated by 3D clinostat culture (Kawahara et al. 2009). In another clinostat based study, ESC pluripotency markers Oct-4 and Nanog as well as ALP were found to be unaffected from 7 days of microgravity exposure (Wang et al. 2011). This study also suggested that, like 2D clinostats, with 3D clinostat-based microgravity exposure cells showed no significant alteration in the cell cycle. However, mouse ESCs showed sensitivity to microgravity with increased apoptosis and delayed DNA repair processes.

In addition to basic cell biology, effect of microgravity in tissue engineering applications was also examined with ESCs. Hepatic cell differentiation and expansion on polymeric (non-aligned poly-L-lactic acid – poly-glycolic acid) scaffolds was achieved from mouse ESCs under induced microgravity using RWV (Wang et al. 2012). Alginate encapsulated mouse ESCs were also exposed to microgravity during osteogenesis, showing enhanced osteogenic differentiation in the absence of embryoid body formation (Hwang et al. 2009). Though number of ESC based studies are limited for earth-based microgravity applications, generation of novel pluripotency inducing pipelines (Okita et al. 2007; Yu et al. 2007) that are free of ethical considerations are expected to help addressing hypotheses regarding developmental pathways during microgravity.

3.2 Adult Stem Cells

3.2.1 Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) are present in various tissues such as bone marrow, umbilical cord, adipose tissue, dental pulp and they have the capability of differentiation into cells of mesenchymal origin such as bone, fat, cartilage, muscle and tendons (Ding et al. 2011; Pittenger et al. 1999). With their ease of handling and high capacity for differentiation, MSCs were studied extensively under microgravity to model spaceflight. For example, the relationship between adipogenesis and osteoblastogenesis in bone marrow is crucial for bone health (Abdallah 2017; Muruganandan et al. 2009) and lack of mechanical loading such as spaceflight, bed-rest and immobilization can enhance adipogenic differentiation in bone marrow and inhibit bone formation (Arfat et al. 2014; Nishikawa et al. 2005; Ozcivici et al. 2010a, b; Uddin and Qin 2013). Furthermore, MSC derived osteoblasts are important regulators of bone resorbing osteoclasts (Zhang et al. 2017), implying that microgravity not only may have bone formation suppressive effects, but also in turn inhibitory control over bone resorption process performed by osteoclasts (Chatani et al. 2015).

Earlier studies on clinostat-based microgravity showed that proliferation rate of the human MSCs (hMSCs) decreased and more flattened cells were observed by exposure to microgravity during 10 days in comparison to normal gravity (Merzlikina et al. 2004). Consistent with hMSCs, proliferation rate of rat bone marrow stem cells (rBMSCs) was inhibited and the response to growth factors such as IGF-1, EGF and bFGF was decreased by simulated microgravity via clinostat (Dai et al. 2007). This study also demonstrated that microgravity disturbed gene expression by upregulating negative regulators of cell cycle and proliferation and down-regulating cytoskeletal and osteogenic genes. Similarly, proliferation of rBMSCs was also shown to be suppressed with microgravity applied with clinorotation for 7 days (Huang et al. 2009). Prolonged exposure of hBMSCs to microgravity for 20 days with clinostat revealed similar inhibition of cell proliferation as well as decreased expression of cell adhesion molecules with increased migration (Gershovich and Buravkova 2007). Moreover, it was reported that the changes of proliferation in MSCs of murine origin were related with the arrested cell cycle in G2/M phases and increased apoptosis after 48 h clinorotation treatment (Yan et al. 2015). Another study that applied simulated microgravity to primary mouse MSCs showed reduced cell proliferation and deteriorating cellular ultrastructure, which can be reversed by daily application of low intensity mechanical vibrations at 90 Hz (Touchstone et al. 2019), an exercise mimetic that was shown to be osteogenic (Demiray and Ozcivici 2015) and anti-adipogenic (Baskan et al. 2017) on mouse bone marrow MSCs previously.

Aside from cell proliferation and cell cycle, clinorotation systems were also used to investigate the effect of microgravity to osteogenic, adipogenic, cardiomyogenic, endothelial and neuronal commitment of rBMSCs (Chen et al. 2011, 2016; Chen et al. 2015; Huang et al. 2009; Uddin and Qin 2013; Xue et al. 2017; Yan et al. 2015; Zhang et al. 2013). As an example of effects of microgravity on osteogenic commitment, it was shown that expression of osteogenic markers such as Cbfa1, ALP and COL1A1 by rBMSCs cultured in induction medium was decreased via clinostat (Huang et al. 2009). In discordance, another study reported that microgravity exposure by clinostat decreased ALP expression at 3 days and increased ALP expression at 10 days for rBMSCs (Xue et al. 2017). Simulated microgravity also reduced expression of other osteogenic markers such as Runx2, OSX, OCN and OPN in MSCs derived from different origins (i.e. adipose tissue, bone marrow) (Chen et al. 2015; Uddin and Qin 2013). The suppressive effects of microgravity on osteogenic commitment can be reversed using external mechanical sources. For example, daily application (20 min/day for 5 days) of low intensity pulsed ultrasound to microgravity exposed adipose derived hMSCs increased ALP, Runx2, OSX, RANKL and decreased OPG gene

expression (Uddin and Qin 2013). Mechanistic studies linked the decrease in the expression of PDZ-binding motif (TAZ) gene to reduced expressions of Runx2 and ALP expression during microgravity and it was shown that TAZ activation by using lysophosphatidic acid (LPA) retained osteogenic differentiation of MSCs under microgravity (Chen et al. 2015). In a follow-up study, inhibition of osteogenic differentiation during microgravity was further linked to the depolymerization of F-actin related to F-actin-TAZ pathway (Chen et al. 2016). Adipogenic differentiation of stem cells was also addressed under simulated microgravity. For example, expression of adipogenic marker PPARγ was shown to be increased under microgravity (via clinostat) with or without adipogenic inducers in the culture media (Huang et al. 2009). Also, similar to that shown in ALP during osteogenesis, increased exposure time to microgravity exhibited reversed effect in adipogenic differentiation decreasing PPARγ expression at 10 days of exposure (Xue et al. 2017).

The effects of simulated microgravity were also tested on neuronal and endothelial differentiation of the MSCs by using clinostat system. Neuronal markers such as MAP-2, TH and CHAT were shown to be upregulated in rBMSCs under microgravity with an increase in secretion of select neurophins, such as BDNF, CNTF (Chen et al. 2011) remarking the therapeutic potential of simulated microgravity for neural tissue engineering applications. Increase in short-term (72 h) microgravity treatment and decrease in long-term (10 days) treatment were observed for ability of MSCs to differentiate into endothelial lineage based on endothelium-specific marker vWF (Xue et al. 2017). Furthermore, another study confirmed that vWF expression increased during culture with VEGF stimulation for 12 days when cells were pre-treated with a 72-h microgravity condition (Zhang et al. 2013). In that study, active level of RhoA marker was associated with lineage commitment during microgravity for adipogenic, endothelial, neural and osteogenic cell lines by regulating the cytoskeleton. In another study, it was demonstrated that exposure of rBMSCs to microgravity for 3 days inhibited cardiomyogenic differentiation evidenced by specific markers including cTnT, GATA4 and β-MHC (Huang et al. 2009).

Clinostat system based on seeding cells on coverslips enables detection of morphological changes in MSCs when they are exposed to microgravity. For example, hMSC had more flattened morphology once they were exposed to microgravity simulated by clinostat system (Merzlikina et al. 2004), and in contrast rBMSCs appeared to have more rounded morphology after exposure to microgravity for 3 days (Chen et al. 2011; Zhang et al. 2013). Cytoskeleton is responsible for cell morphology and structure in addition to other functions (Fletcher and Mullins 2010) and microgravity may induce changes in cytoskeletal components based on exposure time. For example, rBMSCs cultured for 3 days under microgravity revealed that gene expression of cytoskeletal components such as actin and Cofilin1 was downregulated (Dai et al. 2007). When microgravity exposure time was prolonged to 10 days, microtubules (Chen et al. 2011) and microfilaments (Zhang et al. 2013) were shown to be re-organised. RhoA activity that is associated with cytoskeleton regulation showed similar trend and the decreased activity of RhoA after a short microgravity exposure was increased when exposure time was prolonged to 10 days (Xue et al. 2017; Zhang et al. 2013). In addition to cytoskeletal components, migration capacity of rBMSC was inhibited during microgravity based on inhibition of actin reorganization (Mao et al. 2016).

Because of the limitation of 2D clinostats that cannot completely remove the gravity vector due to constant rotation in one direction, 3D clinostats are preferable for the proper simulation of microgravity based on multidirectional rotation. Human BMSCs that were cultured in 3D clinostat system showed increased cell proliferation during microgravity exposure (Yuge et al. 2006) in discordance with 2D clinostat (Gershovich and Buravkova 2007). The inconsistencies of these results may be caused by differences in mechanical environment caused by respective simulation techniques. 3D clinostats were also utilized to determine whether microgravity culture may

augment therapeutic potential of MSCs in cell-based therapy strategies. Exposure to microgravity condition of mouse (Yuge et al. 2010) and rat (Mitsuhara et al. 2013) BMSCs has been shown to increase CXCR4 expression, which is an important factor of cell migration. In human BMSCs (Otsuka et al. 2018), HGF and TGF-β expression associated with cell migration and proliferation have been increased. These studies demonstrated that functional recovery was enhanced by transplantation of MSCs after microgravity culture, suggesting that this technique can be advantageous for cell-based therapies, with increased potential of neuroprotective and anti-inflammatory gene expression.

Another alternative tool for microgravity simulation is RWV bioreactors, tested especially on osteogenic and adipogenic differentiation of MSCs. When the expression of osteogenic (ALP, COL1A1, Runx2, OC, COL1, ON, BMP-2) (Li et al. 2015; Meyers et al. 2005; Meyers et al. 2004; Sheyn et al. 2010; Sun et al. 2008; Zayzafoon et al. 2004; Zheng et al. 2007) and adipogenic (PPARɣ, Adipsin, Leptin, Glut4) (Meyers et al. 2005; Sheyn et al. 2010; Sun et al. 2008; Zayzafoon et al. 2004; Zheng et al. 2007) specific genes and proteins were tested, in general simulated microgravity suppressed the osteogenic differentiation and induced adipogenic differentiation. Similar to clinostat studies, in RWV cultures adipogenic differentiation potential and lipid accumulation of MSCs were increased even if the cell culture was performed without adipogenic inducers (Li et al. 2015; Sun et al. 2008). Moreover decreased expression of osteogenic markers such as Runx2, OC, COL1, ON in hMSCs was not reversible even when the cells were removed from microgravity condition (Zayzafoon et al. 2004). In contrast to the studies demonstrated the decrease in osteogenic differentiation under microgravity conditions, another study reported increased ALP activity of rBMSCs cultured on 3D bovine bones during microgravity (Jin et al. 2010). Furthermore, to investigate potential molecular mechanisms, it has been shown that overexpression of active RhoA, which is a positive regulator of osteogenesis and a negative regulator of adipogenesis, restored stress fibers and the expression of osteogenic markers and suppressed adipogenic gene expression in hMSCs cultured in microgravity (Meyers et al. 2005). On the other hand, the telomerase activity which is known to be related with lineage commitment of stem cells (He et al. 2018; Kang et al. 2004) was found to decrease during microgravity (Sun et al. 2008). Effects of microgravity on MSC commitment to other mesenchymal lineages such as chondrocytes, were also addressed with RWV on rabbit bone marrow stem cells (rabBMSCs) (Wu et al. 2013), human mesenchmal stem cells (hMSCs) (Mayer-Wagner et al. 2014) and human adipose derived stem cells (Ad-hMSCs) (Weiss et al. 2017; Yu et al. 2011). It was shown that rabBMSCs had upregulated the expression of chondrogenesis specific markers, such as collagen II, aggrecan and SOX-9 during pellet culture in RWV (Luo et al. 2011; Wu et al. 2013). In contrast, another study suggested an opposite effect showing that microgravity downregulated the expression of COL10A1, COL2A1 and aggrecan in hMSCs (Mayer-Wagner et al. 2014).

RPM is one of the most realistic simulators of microgravity and it was largely applied to test effects of microgravity on cytoskeleton rearrangements and differentiation of stem cells. It was shown that RPM culture affected dimensional structures, position and amount of actin filaments very quickly (30 min), and organization of actin cytoskeleton was partially or completely restored at 120th h of exposure in hMSCs (Gershovich et al. 2009). Besides, the number of detached cells, which increased in the first 48 h, decreased after 120 h, possibly due to alterations in proteins related with cell-cell and cell-matrix interactions such as vinculin and integrins (Carisey and Ballestrem 2011; Ratushnyy and Buravkova 2017). In accordance with these findings, expression of structural and regulatory genes interacting with actin cytoskeleton, α-actin, γ-actin, b-tubulin, cofilin, and small GTPase RhoA was altered after 48 h of microgravity exposure, and restoration of expression levels was observed after 120 h (Gershovich et al. 2012). Analysis of genes related to differentiation of stem cells revealed that prolonged RPM

exposure (10–20 days) showed an increased expression of adipogenic marker (PPARγ) and decreased osteogenic marker genes (ALPL, OMD). It was also reported that inhibition of osteogenic differentiation and induction of adipogenic differentiation were accompanied by the up-regulation of genes specific for tumorigenesis in the later stages of RPM exposure (14 days) (Li et al. 2019). Another study showed that as a result of the microgravity exposure on human ad-MSCs for 96 h, angiogenesis-related genes were up-regulated as well as genes associated with tumorigenesis (Ratushnyy et al. 2018). The effect of microgravity on stem cell morphology and viability was tested in rBMSC with RPM and it was seen that simulated microgravity (48 h) caused a decrease in cell area, an increase in cell aspect ratio and a reduction in viability (Monfaredi et al. 2017).

Environmental conditions in ground-based simulators can affect cellular properties. It was shown that oxygen concentration in RPM systems had a key role on cell adhesion, and signaling in hMSCs. (Versari et al. 2013). Besides, shear stress as a result of rotation in RWV bioreactors can affect osteogenic differentiation and mineralization (Grellier et al. 2009; Yeatts and Fisher 2011). The techniques working with rotation principle requires the adaptation of cells to the physical effects caused by rotation speed and fluid properties such as viscosity and density. A system that can cope with these challenges is magnetic levitation which is a novel technique for simulation of microgravity (Qian et al. 2009). Although the general interest towards this system has been increasing for the last decade, their use for testing stem cells is still rather limited. In a study that applied large gradient high magnetic field (LGHMF) to create a force against gravitational force on the cells and thus to simulate microgravity, it was shown that a 6-h treatment disrupted cytoskeletal filaments, and extending the treatment time (48 h) resulted in the death of almost all cells (Shi et al. 2010). In addition, by applying LGHMF-induced microgravity (6 h) at different stages of osteogenic induction process, postponement of treatment stage was shown to reduce the intensity of osteogenesis suppression. It was concluded that this treatment affects the initiation stage of osteogenesis. Levels of signaling molecules involved in osteogenic differentiation, such as ERK, FAK and their phosphorylated forms, except ERK, were decreased for treatments in all stages. Another result noted in the study is that the adipogenesis-related gene, PPARγ2, was not detected in any condition. Unlike studies reporting that clinorotation-induced microgravity had no effect on apoptosis of rat MSCs (Dai et al. 2007; Wang et al. 2014), a study examining the effect of LGHMF-induced microgravity on apoptosis showed that microgravity treatment (12 h) induced apoptosis associated with increased expression of p53 in hMSCs (Meng et al. 2011). Also, F-actin fibers disappeared mostly whereas α-tubulin was reorganized above nucleus, and nuclei shifted from center to one side of the cells under microgravity.

As 3D *in vitro* cultures play a key role in examining *in vivo*-like living structures (Anil-Inevi et al. 2020; Fatehullah et al. 2016; Yildiz-Ozturk et al. 2017), they can also be adapted to systems that simulate microgravity. As an example, it was observed that simulated microgravity by RWV enhanced chondro-induction and chondrogenesis in scaffold-based (type I collagen) coculture of MSCs and meniscus cells (Weiss et al. 2017). Another example is that RWV culture of the bone constructs resulted in better repair of the bone defects once transplanted compared to the static flask culture (Jin et al. 2010). In addition, magnetic levitation system can also be utilized for 3D cultures for tissue engineering studies. It was demonstrated that magnetic levitation could be used for microgravity culture and density measurement of MSCs (Anil-Inevi et al. 2019b). A recent study showed that magnetic levitation system enabled self-assembly of MSCs with label- and scaffold-free application under microgravity environment (Anil-Inevi et al. 2018). Also, this microgravity environment induced by diamagnetic levitation was capable of the detection of MSCs based on their densities (Sarigil et al. 2019b).

3.2.2 Hematopoietic Stem Cells

Hematopoietic stem cells (HSCs) are active regulators and participants of hematopoiesis and

they can be affected by microgravity conditions, causing anemia and hematopoietic disorders in astronauts exposed to the microgravity (Wang et al. 2019). These abnormalities related to spaceflight lead to research to understand the mechanosensitivity and mechanoresponse of HSCs to the microgravity condition in physiological and molecular basis. A study aimed to understand early hematopoiesis observed in space flights showed that treatment of mice HSCs with a 7-day RWV culture decreased the total number of progenitor cells, while increasing the high-proliferative potential colony-forming cells compared to standard flask culture (McAuliffe et al. 1999). For human HSCs, simulated microgravity via RWV bioreactor caused the cells to exit G_0/G_1 phases at a slower rate and maintain higher hematopoietic potential shown by the ability to initiate and maintain secondary long-term suspension, compared to cells cultured in 1 g (Plett et al. 2001). Another study on human HSCs showed that RWV culture resulted in a significant reduction of migration to stromal cell–derived factor 1 (SDF-1 α), accompanied by decreased levels of F-actin and cell cyclin proteins. It was also reported that RWV culture inhibited overall progenitor proliferation and erythroid differentiation (Plett et al. 2004). In contrast, long-term RWV culture (for more than 8 days) provided a more suitable environment for expansion of hematopoietic stem cells collected from human umbilical cord blood compared to standard flask culture (Liu et al. 2006). Besides, human umbilical cord blood stem cells (CBSCs) showed increased proliferation as 3D tissue-like aggregates and developed vascular tubular assemblies in RWV culture (Chiu et al. 2005).

3.2.3 Other Types of Stem Cells

In addition to stem cells types classified and mentioned above, other stem cell types were also studied under microgravity conditions for several tissue engineering applications. A study aimed at providing better culture conditions for skin tissue engineering applications revealed that rotating cell culture system provided enhanced proliferation and viability for human epidermal stem cells (hEpSCs) and they were able to form multilayer 3D epidermis structures (Lei et al. 2011). Another study examined effects of the simulated microgravity on differentiation of the bipotential murine oval liver (BMOL) stem cells (Majumder et al. 2011). It was reported that prolonged culture (240 min) of BMOL stem cells in simulated microgravity condition (via 3D clinostat) increased cell death, while short exposure time (up to 2 h) did not have a negative effect on cell viability. Moreover, exposure of BMOL stem cells to microgravity condition for 2 h was alone able to induce the differentiation of stem cells to hepatocytes within 2–3 days, interplaying with BMP4/Notch1 signaling. Finally, a study that applied simulated microgravity on spermatogonial stem cells which are a subset of primitive germ cells, also showed that cell culture in a rotating cell culture system enhanced proliferation of these cells (Zhang et al. 2014). Furthermore, after simulated microgravity treatment the cells maintained clone-forming capacity and differentiation ability, and were able to differentiate into round spermatids with flagella. These results have been suggesting that the technique may be used to restore fertility for cancer patients after chemotherapy or irradiation.

4 Future Outlook

Increasing interest in long-term space flights has led to growing interest in understanding the functional and structural changes in living systems under microgravity condition. Especially, microgravity-triggered changes in stem cells have gained remarkable attention due to their potency. Real spaceflight experiments, which provide wide opportunities to study *in vitro* and *in vivo* biological effects of microgravity, are challenging due to limited availability, high cost and complexity of experimental condition. Although there are available ground-based simulating systems to investigate microgravity-related *in vivo* effects such as rodent hindlimb unloading models, it is not possible to apply them for each type of tissue and to use for research at the cellular level. Ground-based simulated microgravity experiments for *in vitro*

Table 4 Applications of microgravity simulation techniques for mesenchymal stem cells

Culture method	Strategy	Cell type	Culture period in MG	Results	Reference
2D Clinostat	Cell culture	hBMSCs	1–10 h	Morphological changes: Flattened cells	Merzlikina et al. (2004)
				Proliferation rate ↓	
2D Clinostat	Stem cells seeding on coverslips transferred to polyethylene (PE) culture bag	rBMSCs	30 rpm, 1–4 days	Proliferation: p-ERK1/2 ↓ Akt ↓ Response to growth factors (IGF-I, EGF, bFGF) ↓ Apoptosis →	Dai et al. (2007)
				Cytoskeleton gene expression: Actin filaments ↓ Cofilin1 ↓	
				Osteogenic differentiaition: Cbfa1 ↓	
				Gene expression profile: Among 413 expressed genes; 207 genes were down-regulated 206 genes were up-regulated Mostly genes acting negative regulation of cell cycle and cell proliferation were up-regulated, osteoblast differentiation related-genes were down-regulated (look reference for detail)	
2D Clinostat	Cell culture	hBMSCs	6 rpm, 20 days	Proliferation rate ↓ (between 2.5 and 6.5 fold depending on passage)	Gershovich and Buravkova (2007)
				Migration activity ↑	
				Immunophenotypic analysis (CD34, CD45, CD54, CD106, CD105, CD90 and class 1 HLA) with or without osteogenic induction medium	
2D Clinostat	Coverslips transferred to PE culture bag	rBMSCs	30 rpm, 1, 3, 5 or 7 days	Reorganization of microfilaments Proliferation ↓	Huang et al. (2009)
				Osteogenic differentiation: With and without inducer ALP, Cbfa1, COL1A1↓	
				Cardiomyogenic differentiation: With and without inducer cTnT, β-MHC, GATA4↓	
				Adipogenic differentiation: With and without inducer PPARɣ↑	
				p-ERK1/2 ↓	

(continued)

Table 4 (continued)

Culture method	Strategy	Cell type	Culture period in MG	Results	Reference
2D Clinostat	Coverslips inserted into chamber	rBMSCs	30 rpm, 4 h, 72 h, 7 days, 10 days and 14 days	Cytoskeletal reorganization Ratio of length/width ↓ (round-shaped cell morphology)	Chen et al. (2011)
				Neuronal differentiation: MAP-2, TH, CHAT ↑	
				Neurotrophins: BDNF ↑ CNTF ↑ NGF ↓	
				Action potential: Membrane input resistance ↑ Membrane capacitance ↑	
2D Clinostat	Coverslips transferred into chamber	rBMSCs	30 rpm, 4 h, 72 h and 10 days	Morphological changes: Ratio of length/width ↓ (round-shaped cell)	Zhang et al. (2013)
				Cytoskeletal reorganization: Decreased microfilament and cytoskeletal tension in 4 h and 72 h Recovered microflament and cytoskeletal tension in 10 days Activation of RhoA ↓ in 72 h (80%, compared with 4 h) ↑ in 10 days (tenfold, compared with 72 h)	
				Differentiation capability: Flk-1 → for 4 h ↑ (1.71- fold) for 72 h – The most proper exposure time ↑ (1.28-fold) for 10 days	
				Endothelial differentiation: Removing MG after 72 h exposure and stimulation with VEGF for 12 days Flk-1 ↑ (1.46-fold compared with NG) vWF ↑ (1.31-fold compared with NG) Capillary formation ↑	
2D Clinostat[a]	Opticell	Ad-hMSCs	15 rpm, 5 days	Osteogenic differentiation: Runx2, ALP, OSX (osterix) ↓ RANKL ↓ OPG ↑ Collagen content (type I and III) ↓ Calcification ↓	Uddin and Qin (2013)
				Appliying low intensity pulsed ultrasound (LIPUS):	

(continued)

Table 4 (continued)

Culture method	Strategy	Cell type	Culture period in MG	Results	Reference
				ALP ↑ (compared with MG and NG-u) Runx2 ↑ (compared with MG and NG-u) OSX ↑ (compared with MG and NG-u) RNAKL ↑ (compared with MG and NG-u) OPG ↑ (compared with NG-u) OPG ↓ (compared with MG and NG) Collagen content ↓ (compared with NG-u) Collagen content ↑ (compared with MG and NG-u)	
2D Clinostat	Coverslips transferred into chamber	BMSCs	30 rpm, 48 h	Cell proportion in G1 phase ↓ (0.04-fold, similar between the two groups) in S phase ↓ (0.22-fold) in G2 phase ↑ (1.0-fold) in comparison with control Apoptosis ↑	Yan et al. (2015)
				Cell proliferation: PCNA ↓	
				Osteogenic differentiation with inducer (BMP2) ALP, Cbfa1, SATB2 ↓ Hoxa2 ↑	
				Overexpression of SATB2 SATB2 ↑ ALP ↑ (in comparison with that without SATB2 overexpression)	
2D Clinostat	Flat chamber	rBMSCs	10 rpm, 48 h	Osteogenic differentiation: ALP, OPN, OCN, Runx2 ↓	Chen et al. (2015)
				Adipogenic differentiation: PPARɣ ↑	
				TAZ ↓	
				TAZ ↑ with LPA (lysophosphatidic acid) correspondingly Runx2 ↑ with LPA	
2D Clinostat	Flat chamber	rBMSCs	10 rpm, 48 h	Morphological changes: F-actin reorganization (thinner and disordered) Fractal dimension ↓	Chen et al. (2016)
				ALP activity ↓ Runx2 ↓ TAZ nuclear aggregation (TAZ activation) ↓	
				Jasplakinolide or LPA induction in MG:	

(continued)

Table 4 (continued)

Culture method	Strategy	Cell type	Culture period in MG	Results	Reference
				ALP activity ↑ Runx2 ↑	
2D Clinostat	Flat chamber	rBMSCs	10 rpm, 24 h	Migration ↓ F-actin remodeling (thicker) Cell stiffness ↑ (Young's modulus↑) Rock-F-actin pathway	Mao et al. (2016)
				Restoring gravity: Cell stiffness and migration -could not recover totally	
2D	Coverslips transferred into chamber	rBMSCs	30 x g 72 h and 10 days	Decreased microtubule formation and α-actin filaments at 72 h and reestablished microtubeles and actin filaments at 10 days	Xue et al. (2017)
				Activation of RhoA ↓ in 72 h (83%, compared with 4 h) ↑ in 10 days (nine-fold, compared with 72 h)	
				Neuronal differentiation: MAP-2 ↑ at 72 h MAP-2 ↓ at 10 days (compared with 72 h)	
				Osteogenic differentiation: ALP ↓ at 72 h ALP ↑ at 10 days (compared with 72 h and NG)	
				Endothelial differentiation: vWF ↑ at 72 h vWF ↓ at 10 days (compared with 72 h and NG)	
				Adipogenic differentiation: PPARγ ↑ at 72 h PPARγ ↓ at 10 days (compared with 72 h and NG)	
3D Clinostat	Porous calcium hidroxyapatite ceramic blocks	rBMSCs	10^{-3} G, 2 weeks	ALP activity ↓	Nishikawa et al. (2005)
				ECM formation ↓	
				Total protein content →	
				Proliferation →	
3D Clinostat	Culture flask	hBMSCs	10^{-3} G, 5 rpm, 7 days	Proliferation rate ↑ (13-fold in a week) Cell proliferation in NG - 6.8% (d1), 8.3% (d3), 24.7% (d7) in MG – 23.5% (d1), 48.7% (d3), 85.5% (d7)	Yuge et al. (2006)
				Chondrogenic differentiation	

(continued)

Table 4 (continued)

Culture method	Strategy	Cell type	Culture period in MG	Results	Reference
				Aggregan: undetectable Collagen II: undetectable Telomere length → Telomerase activity: undetectable	
3D Clinostat	Opticell	mBMSCs	10^{-3} G, 7 days	Morphological changes in growth and neural induction medium →	Yuge et al. (2010)
				In growth medium: Oct-4 → NF-H no expression	
				In neural induction medium: Oct-4 ↑ (compared to NG in a week) NF-H no expression	
				Neural differentiation: MAP-2 ↓ NF-H ↓	
				CXCR4→ After transplantation CXCR4 ↑	
3D Clinostat	Opticell	rBMSCs	10^{-3} G, 7 days	Morphological changes: Round-shaped cell	Mitsuhara et al. (2013)
				Migration ↑ Oct-4, CXCR4 ↑	
				Functional recovery ↑ NGF, BDNF →	
3D Clinostat	Coverslips inserted into chamber	rBMSCs	30 rpm, 48 h, 72 h and 120 h	Ratio of width/length ↑ in all time duration (compared to NG) Ratio of width/length ↓ in 120 h (compared to 72 h) Apoptosis →	Wang et al. (2014)
				Pluripotency: Oct-4 ↑ in all time durations (compared to NG) Oct-4 → in 120 h (compared to 72 h)	
				Endothelial differentiation: vWF ↑ CD31 ↑	
3D Clinostat	Culture flask tranferred into Gravite®	h-cMSCs	10^{-3} G, 5 days	Differentiation potential (adipogenic, osteogenic and neural) →	Otsuka et al. (2018)
				Neurotrophic and anti-inflammatory factors: HGF, TGF-β ↑ BDNF, bFGF, GDNF, VEGF →	
				Motor functional improvements after transplantation ↑	

(continued)

Table 4 (continued)

Culture method	Strategy	Cell type	Culture period in MG	Results	Reference
RWV	Plastic microcarrier beads	hBMSCs	7 days +30 days (readaption) (MG-E)	During MG Actin→	Zayzafoon et al. (2004)
				Osteogenic differentiation: Runx2, ALP, OC, COL1, ON ↓	
				Adipogenic differentiation: PPARγ, Adipsin, Leptin, Glut4↑	
				Removing MG Runx2, OC, COL1, ON ↓ ALP ↑	
				PPARγ, Adipsin, Leptin, Glut4↑	
RWV	Polystyrene microcarrier beads	hMSCs	Readjusted rotation regarding to growing constructs, 7 days	Osteogenic differentiation: Runx2, ALP, COL1 ↓	Meyers et al. (2004)
				İntegrin↑ FAK↓ PYK2↓ GTP-Ras↓ p-ERK1/2 ↓ Akt →	
RWV	Polystyrene microcarrier beads	hMSCs Transduction of hMSCs by adenoviral vector, RhoA-V14	Readjusted rotation regarding to growing constructs, 7 days	F-actin↓ G-actin↑ GTP-RhoA↓ p-cofilin↓	Meyers et al. (2005)
				Osteogenic differentiation: Runx2, ALP, COL1 ↓	
				Adipogenic differentiation under MG: Glut4 ↑ Lipid accumulation ↑	
				Introduction of an adenoviral construct expressing constitutively active RhoA: Runx2, ALP, COL1 ↑ Leptin, Glut4↓	
RWV	Plastic microcarrier beads	hBMSCs	16 rpm, 14 days	ALP↓	Zheng et al. (2007)
				Adipogenic potential ↑	
RWV	Microcarrier beads	rBMSCs	15 rpm, 5 days/ 7 days	Osteogenic differentiation under MG: BMP-2 ↓	Sun et al. (2008)
				Osteogenic differentiation (by removing MG for 4 days): ALP activity and OC concentration↓	
				Adipogenic differentiation under MG: PPARγ2, adipocyte number ↑ (without inducer)	
				Telomerase activity ↓	

(continued)

Table 4 (continued)

Culture method	Strategy	Cell type	Culture period in MG	Results	Reference
RWV	Mineralized polymer foams	rBMSCs	20 rpm, 28 days	Feasibility of mineralized PLGA scaffolds for cell adhesion, cell viability and osteogenic differentiation	Koç et al. (2008)
RWV	Gelatin microcarrier	hBMSCs	16 rpm, 7 days	Cell viability → (compared with static condition) Adipogenic differentiation Lipid accumulation↑	Sheyn et al. (2010)
				Osteogenic differentiation ALP activity →	
				Gene expression (>1,250 genes) in MG: Adipogenesis-related genes mostly upregulated Osteogenesis- and chondrogenesis-related genes mostly downregulated (look reference for detail)	
RWV	Ceramic bovine bone blocks	rBMSC	Readjusted rotation regarding to growing constructs, 15 days	3D bone constructs Cellularity of blocks ↑ (compared to static culture) New bone formation; thicker in MG condition	Jin et al. (2010)
				ALP activity ↑	
RWV	Pellet culture	rabBMSC	6.5 rpm, 7 days	Size of pellets ↑ Cell proliferation ↑	Luo et al. (2011)
				Chondrogenic differentiation: S-GAG ↑ Collagen II ↑ Aggregan ↑ SOX-9 ↑ Aggregan/collagen ↑	
				Cell culture with TGF-β1 in MG (compared to condition without TGF-β1 in MG): Cell proliferation, S-GAG, Collagen II, Aggregan, SOX-9 ↑ Aggregan/collagen ↓	
RWV	Opticell	Ad-hMSC (hADSC)	Rotation speed varied between 11-25 rpm, 21 days	Size and weight of pellets ↑	Yu et al. (2011)
				Chondrogenic differentiation: COL2A1 ↑ COL10A1 → Aggrecan ↑ SOX-9 ↑	
				p-p38 ↑	
RWV	Pellete culture	Rab-BMSC	20 rpm, 2 weeks	Chondrogenic differentiation: Collagen II ↑ Aggrecan ↑	Wu et al. (2013)
				Proteoglycan ↑	

(continued)

Table 4 (continued)

Culture method	Strategy	Cell type	Culture period in MG	Results	Reference
RWV	Pellet culture	hMSC	Rotation speed varied between 4.2-7.2 rpm, last 7 days of 21 days differentiation	Chondrogenic differentiation: COL2A1 ↓ COL10A1 ↓ COL2A1/COL10A1 ↓ Aggrecan ↓ SOX-9 →	Mayer-Wagner et al. (2014)
RWV	Microcarrier beads	Mice-BMSC	10 rpm, 7 days	PPARɣ ↑ (without inducer) Runx2 ↓ TAZ mRNA → TAZ protein expression ↓ Localization of TAZ and Runx2: In nuclei in NG ↓ localization in nuclei in MG	Li et al. (2015)
RWV	PLGA scaffold	hDPSC	Rotation speed varied between 15-25 rpm, 3 days	Proliferation potential ↑ (for 72 h) Regulator proteins of the G1/S phase transition p27, p21 ↓ Cdk2, cyclin A, cyclin E ↑ Actin ↓ Tubulin ↓ Migration ↓ Cell adhesion: ITGA6, ITGAV, ITGB1, LAMB1, TNC ↑ MMP13 ↓	He et al. (2016)
RWV	Type I collagen scaffolds	Ad-hMSC	20–30 rpm, 21 days	Coculture study (mesenchymal stem cells and meniscus cells) Cartilaginous matrix formation ↑ Chondrogenesis: GAG ↑ Aggrecan ↑ COL10A1 ↑ COL2A1 → COL1A2 → SOX-9 → GREM1 ↓ MMP-13 ↑	Weiss et al. (2017)
RPM	Culture flask	hBMSC	30 min, 6 h, 24 h, 48 h, 120 h	Reorganization of actin cytoskeleton Redistribution of focal adhesion sites Expression of adhesion receptors: CD29 → CD49b, CD49d ↑ CD106 ↓ Effect of passage number to CD54 expression	Gershovich et al. (2009)

(continued)

Table 4 (continued)

Culture method	Strategy	Cell type	Culture period in MG	Results	Reference
				CD54 ↓ at passage 7 (compared with static condition, SC) CD54 ↑ at passage 8 (compared with SC) CD54 ↓ at passage 13 (compared with SC)	
RPM	Culture flask	hBMSC	10^{-3} G, 30 min, 6 h, 24 h, 48 h, 120 h	α-actin ↑ at 48 h β-actin → γ-actin ↓ at 48 h β-tubulin ↑ at 48 h and 120 h vinculin ↓ at 120h cofilin ↑ at 48h small GTP-ase RhoA (RhoA)↑ at 48h Rho-kinase(ROCK1) →	Gershovich et al. (2012)
				Expression of 84 genes: ↑ in 30 genes ↓ in 24 genes (look reference for detail)	
RPM	Opticell	Ad-hMSC	10^{-4} G, 14 days	Effect of oxygen concentration in SMG (look reference for detail)	Versari et al. (2013)
RPM	Culture flask	rBMSC	30 rpm, 12, 24 and 48 h	Cell morphology: Cell area: 12 h →, 24 h ↓, 48 h ↓ Cell aspect ratio: 12 h →, 24 h ↑, 48 h ↑ Cell rotation angle: 12 h →, 24 h →, 48 h →	Monfaredi et al. (2017)
				Cell viability: 12 h ↓, 24 h ↓, 48 h ↓	
RPM	Culture flask	Ad-hMSC	96 h	Expression of focal adhesion genes	Ratushnyy and Buravkova (2017)
				Changed expression levels of integrins, FAK-mediated signaling, G-protein-mediated signaling, integrin-associated signaling, AKT/PI3 signaling, caveolins, and actin binding proteins	
RPM	Culture flask	Ad-hMSC	10^{-4} G, 96 h	Angiogenesis-related gene and protein expression;	Ratushnyy et al. (2018)
				Genes: BDNF, CXCL1, VEGF-c, DKK1, FGF5, GDF10, VEGF-a ↑	
				Proteins: Serpin E1, Serpin F1, IGFBP-3, IL-8, VEGF ↑ TIMP-1 ↓ PTX3 and TSP-1 →	

(continued)

Table 4 (continued)

Culture method	Strategy	Cell type	Culture period in MG	Results	Reference
				Capillary-like tube formation ↑ Wound healing ↑	
RPM	Culture flask	hBMSC	0.1–10 rpm, 0 days, 2 days, 7 days, 14 days	Altered expression profiles of different genes:	Li et al. (2019)
				837 genes at day 2	
				399 genes at day 7	
				894 genes at day 14 (look reference for detail)	
RPM	Culture flask	Ad-hMSC	53–65 deg./s, 6, 24, 48, and 96 h	Cell viability →	Ratushnyy et al. (2019)
Levitation	Glass slide	hBMSC	0 g (magnetic field intensity: 12 tesla), 6, 12, 24 and 48 h	Actin filaments and tubulin ↓ Critically cell deaths at 48 h	Shi et al. (2010)
				6 h exposure in early stages of induction: Osteogenic differentiation: ALP, Runx2 ↓ Adipogenic differentiation: PPARɣ2 not detected Leptin not detected	
				Integrin, FAK → p-FAK, ERK, p-ERK ↓	
				6 h exposure in late stage of induction: ALP, Runx2 → Integrin, FAK, p-FAK, ERK, p-ERK →	
Levitation	Coverslip	hBMSC	0 g (magnetic field intensity: 12 T), 12 h	Proliferation ↓	Meng et al. (2011)
				Cell viability ↓	
				Apoptosis rate ↑	
				Caspase-3/7 activity ↑	
				p53 ↑	
Levitation	Contact-free	mBMSC	0 g (magnetic field intensity: < 1 T), 24 and 48 h	Self-assembly 3D cell culture	Anil-Inevi et al. (2018)
				Coculture of mesenchymal stem cells and cancer cells	
Levitation	Contact-free	mBMSC	0 g (magnetic field intensity: < 1 T), 10 min	Density and cell size measurement	Sarigil et al. (2019b)

↑ increasing
↓ decreasing
→ no significant difference
[a]This method was mentioned as 1D clinostat in the study
NG-u: ultrasound exposure under NG

investigations have been applied to overcome these limitations for more than 20 years. The existing systems are notably useful for researches at the cellular level, however, there is still a need for technological innovations and improvements to generate and monitor more complex structures with an *in vivo* microenvironment. These ground-based systems that are equipped with state-of-the-art devices for better monitoring and testing, may expand the knowledge in the field of gravitational biology. Technical evolution of devices to simulate microgravity and testing cells for new and partially unexplored scenarios may advance our understanding on molecular mechanisms underlying the alterations in stem cells under microgravity condition and on possibilities of managing them.

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