Microgravity Sci. Technol. (2015) 27:455–464
DOI 10.1007/s12217-015-9443-z

ORIGINAL ARTICLE

Responds of Bone Cells to Microgravity: Ground-Based Research

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Received: 25 Jan 2015 / Accepted: 10 Jun 2015 / Published online: 15 July 2015

Abstract Severe loss of bone occurs due to long-duration spaceflight. Mechanical loading stimulates bone formation, while bone degradation happens under mechanical unloading. Bone remodeling is a dynamic process in which bone formation and bone resorption are tightly coupled. Increased bone resorption and decreased bone formation caused by reduced mechanical loading, generally result in disrupted bone remodeling. Bone remodeling is orchestrated by multiple bone cells including osteoblast, osteocyte, osteoclast and mesenchymal stem cell. It is yet not clear that how these bone cells sense altered gravity, translate physical stimulus into biochemical signals, and then regulate themselves structurally and functionally. In this paper, studies elucidating the bioeffects of microgravity on bone cells (osteoblast, osteocyte, osteoclast, mesenchymal stem cell) using various platforms including spaceflight and ground-based simulated microgravity were summarized. Promising gravity-sensitive signaling pathways and protein molecules were proposed.

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Keywords Simulated microgravity · Clinostat · Diamagnetic levitation · Spaceflight osteopenia · Bone cells · Bone remodeling

Introduction

Gravity is one of the environmental physical factors necessary for life on Earth, as well as the fundamental prerequisite for origin and evolution of living beings. With the rapid development of space technology, chances for human exposed to microgravity in outer space have increased dramatically. Microgravity, or weightlessness, is mostly produced due to the orbit around the Earth. During long-term spaceflight, microgravity can lead to certain irreversible physiological alterations in astronauts. The significantly altered cellular behaviors of organ/tissue may be a possible mechanism.

Previous studies in space stations including Salyut program, Skylab, and MIR, suggest that bone loss is a continuous and progressive course for astronauts during long-term living in the microgravity environment of space. It has been revealed that the most dramatic loss of bone occurs at weight bearing bone such as calcaneus, femoral neck, lumbar spine, pelvis etc. Astronauts monthly lose 1-1.5 % of bone mineral density in hip and 1 % in spine (LeBlanc et al. 2000). Intensive daily exercises in space can help slow bone loss, but are not effective enough as a countermeasure for bone loss during long-term spaceflight (Cavanagh et al. 2005). Severe loss of bone increases the risk of fracture and renal stone due to the release of calcium from skeleton. The fully recovery of bone loss in astronauts is expected in less than 3 years even after their return to Earth (Sibonga et al. 2007).

Bone is a constantly renewed tissue, whose remodeling requires collaborations of various bone cells, including bone

mesenchymal stem cells (BMSCs), osteoblasts (bone formation cells), osteoclasts (bone resorption cells) and osteocytes (Raggatt and Partridge 2010). The coupled balance between bone formation and bone resorption is essential to maintain skeleton health (Feng and McDonald 2011). However, bone is such vulnerable that numerous chemical and physical factors, such as mechanical unloading under microgravity, can influence the balance of bone remodeling. Previous studies from human, animals and cells indicate that microgravity can directly affect morphology, proliferation, cell cycle, viability, apoptosis, cytoskeleton, gene and protein expression, and differentiation of bone cells (Shang et al. 2013). Nevertheless, the exact mechanisms still need to be elucidated.

In this review, several frequently used simulated microgravity (SM) methods, especially random position machine (RPM) and diamagnetic levitation (DL), are firstly introduced. Then, the available data on how bone cells are affected by spaceflight and SM were summarized and analyzed. Based on these studies, some possible underlying mechanisms were proposed.

Ground-Based Research Platforms for Simulated Microgravity

It is difficult to carry out scientific experiments under real microgravity conditions because it is not only costly but also limited to rare opportunities. Establishing ground-based platforms for simulated microgravity conditions is very necessary and useful. At present, various methods and facilities have been developed and applied for space life sciences, such as, clinostat, rotary cell culture system (RCCS), random positioning machine (RPM), and diamagnetic levitation (DL) for cell and tissue; hind-limb unloading for rat and mouse; 6° head-down bed rest and parabolic flights for human (Hu et al. 2014a; Arfat et al. 2014). These platforms can simulate the biological effects of microgravity to a certain extent, but do have some limits regarding their principles and practices. Numerous studies using these multiple platforms have examined the biological behaviors of bone cells in response to altered gravity environment to clarify the underlying mechanism. In order to get the same responses as those during spaceflight, researchers have carefully selected test parameters and cell types in the experiments using the SM platforms. Based on the results, some signaling pathways and mechanical-sensitive molecules have been proposed while still need to be verified in real microgravity. However, it is challenging to draw consistent conclusions due to the heterogeneous of the adopted SM platforms, cell types, and parameters for clinostat. To consistently and systematically find the most promising mechanisms at both cell and molecule levels, both the SM platform and cell line should consider certain criterion in the future studies.

Clinostat including 2D, 3D, and RPM is based on the principle of "gravity-vector-averaging" (Borst and van Loon 2009). When the changes of orientation of organism relative to the gravity's vector are faster than the response time, the biological system experience a status comparable to microgravity. The rotation parameters including radius and speed should be modified to avoid the influence of centrifugal force. At 2D/3D mode, the rotation route is a fixed circuit. For RPM, the mutually perpendicular axes rotated at random speed and random direction mode, by which the rotation route is irregular in 3-dimensional distribution. Then, organisms are more likely to tend to lose the sense of direction. For this reason, RPM is used more and more frequently (Wuest et al. 2014).

Diamagnetic materials in a large gradient high magnetic field (LGHMF) are subjected to a magnetic force. Reduced gravity or even real microgravity can be achieved when the subjected magnetic force acting on the test samples counteracts gravity (Qian et al. 2013a). Hyper-gravity can be also achieved when the magnetic force is homodromous to gravity. It should be noted that high magneto gravitational environment (HMGE) is a combined environment of microgravity and high magnetic field (HiMF). Generally, four groups are set in our studies, namely control (gravity, geomagnetic field), μ g (microgravity, 12 T), 1 g (gravity, 16 T) and 2 g (2-fold gravity, 12 T). We can get magnetic effects by comparing results of the control and 1 g group, and gravitational effects by comparing results of the μ g and 2 g group. This method is fundamentally different from RPM in essence. Like gravity, magnetic force is a body force that acts throughout the entire volume of objects. Each part of diamagnetic samples in LGHMF can be subjected to magnetic force.

SM, including DL and RPM, is practical, reliable and convincible. Through literature research in "Web of Science" and "PubMed", we found that clinostat and DL are the most popularly used SM for cellular studies. Both RPM and DL have the ability to stably sustain a long-term experiment and have been widely applied in bioscience research. In order to draw a more convincing conclusion on the response of bone cells to microgravity, we analyzed the literature using the most widely-studied SM and bone cells, and compared the results between SM and spaceflight.

Response of Bone Cells to Microgravity During Spaceflight

Microgravity during spaceflight is detrimental for bone metabolism, directly acting on osteoblasts and osteoclasts.

Osteoblast growth is attenuated with decreased glucose utilization (Hughes-Fulford and Lewis 1996). Microgravity also reduces expression of some anabolic signals such as TGFβ, cox2, OC, bcl2, bax etc. (Hughes-Fulford et al. 2006; Hughes-Fulford 2001). Changes in nuclear structure are evident (Hughes-Fulford et al. 2006). Nuclei are small and demonstrably fragmented or condensed (Nabavi et al. 2011). Microgravity also causes reorganized cytoskeleton. Stress fibers formed by actin become thinner (Nabavi et al. 2011), and the number is reduced at the same time (Hughes-Fulford and Lewis 1996). Microtubules become shorter and wavier. Focal adhesions are smaller and less (Nabavi et al. 2011). Furthermore, cytoskeleton disorganization is associated with disassembling vinculin, and markedly affected integrin-mediated cell adhesion (Guignandon et al. 2001). Microgravity exposure significantly decreases gene expression of osteogenic differentiation such as Col I, ALP, and OC, and thereby inhibites osteoblast differentiation (Carmeliet et al. 1997; Carmeliet et al. 1998; Landis et al. 2000).

During spaceflight, more mature osteoclasts are formed by their precursors and increase the ability of bone resorption. Osteoclasts and their precursors are directly affected by microgravity (Tamma et al. 2009; Nabavi et al. 2011). Osteoblasts are originated from MSCs, while osteoclasts are from hematopoietic stem cells (HSCs). Spaceflight reduces mesenchymal differentiation into osteoblasts and hematopoietic differentiation into osteoclasts (Blaber et al. 2014). These negative effects are not affected by the accelerations, noise and vibration of launch (Kacena et al. 2003). However, these preliminary and limited results could not provide a clear picture on how bone cells responses to microgravity. More extensive studies using ground-based SM are required.

Response of Bone Cells to Simulated Microgravity

Osteoblast

Osteoblast functions as bone formation. As mentioned above, spaceflight suppresses cell growth, disorganizes cytoskeleton, and inhibits osteogenic differentiation. Similar results have been obtained using SM (Table 1), which shed new lights on the possible mechanisms.

SM by RPM causes an inhibited effect on cellular proliferation and migration of osteoblastic MG63 cells. Furthermore, RPM reduces cell size and reorganizes actin filaments

Table 1 Effects of simulated microgravity on osteoblasts

Simulated microgravity	Indicator	Cell type	Main findings	Reference
RPM	Viability	MG63	Cell size, proliferation and migration were all reduced after exposed to short-term RPM.	(Luo et al. 2013)
	Cytoskeleton	MG63	Calcium influx through stretch-activated channels mediates microfilament reorganization.	(Luo et al. 2013)
		MC3T3-E1	Actin filaments were rearranged with decreased complexity and expression.	(Qian et al. 2012a)
	Differentiation	MC3T3-E1	The differentiation was inhibited at mineralization stage.	(Hu et al. 2014b)
		2T3	The differentiation was inhibited.	(Hu et al. 2013b)
DL	Morphology	MG63 and MC3T3-E1	Cell area was decreased. MC3T3-E1 exhibited a flat and polygonal shape. The nucleus was enlarged.	(Qian et al. 2008)
	Viability	MC3T3-E1	Osteoblast proliferation was enhanced in HMGE regardless of gravity level from micro (μ g) to hyper gravity (2 g).	(Qian et al. 2013b)
	Cytoskeleton	MG63	Cytoskeletons including actin and microtubule were reorganized with less complexity. The cell height and surface roughness were decreased.	(Qian et al. 2010)
		MC3T3-E1	Cytoskeletons including actin and microtubule were reorganized with less complexity. The cell height were decreased.	
	Ultrastructure	MG63 and MC3T3-E1	Osteoblast ultrastructure was affected by DL with an increased number of lysosomes, expansion of endoplasmic reticulum and mitochondria, distorted microvilli and aggregated actin filaments.	(Qian et al. 2013b)
	Differentiation	MC3T3-E1	The accelerated effects under DL is due to the HiMF.	(Hu et al. 2013a)

with dot pattern around the nucleus (Luo et al. 2013). Fractal dimensions (D) analysis is used to quantify the irregularity and complexity of cytoskeleton. SM by 3D or 2D clinorotation dramatically decreases the D value of actin filament. It indicates that the stress fiber formed by actin may be reorganized and has a significant reduction in complexity or chaos. Meanwhile, β-actin expression is decreased, which is consistent with the reduced fluorescence intensity of F-actin stained by rhodamine labeled phalloidin (Qian et al. 2012a). However, gadolinium chloride, an inhibitor for Ca^{2+} influx through stretch-activated channels, attenuates actin disruption. It indicates that SM by clinostat first leads to Ca^{2+} influx through stretch-activated channels, and then causes actin reorganization afterwards (Luo et al. 2013).

Like RPM, DL also causes a decreased cell area in both MG63 and MC3T3-E1 cells. MC3T3-E1 exhibits a flat and polygonal shape. The nucleus is enlarged (Qian et al. 2008). Osteoblast ultrastructure is affected by DL with an increased number of lysosomes, expansion of endoplasmic reticulum and mitochondria, distorted microvilli and aggregated actin filaments. Interestingly, osteoblast proliferation is enhanced in HMGE no matter which kind of gravity from micro (μ g) to hyper gravity (2 g) is adopted. It is indicated that HiMF may interfere with the effects of altered gravity by HMGE (Qian et al. 2013b).

Cytoskeleton rearrangement of osteoblast is induced by DL. Under DL, actin fibers become thin, discontinuous and irregular, while microtubules are disperse and sparse. The D value of cytoskeleton under DL is significantly decreased. In order to determine the effects of modifications of both actin and microtubule on cell architecture, atomic force microscopy (AFM) is used to analyze the height and roughness. The results show that both the average height and roughness of cytoskeleton are all decreased. It therefore suggest that osteoblast may respond to microgravity by adjusting its cytoskeleton architecture (Qian et al. 2010).

Bone formation highly depends on osteoblasts differentiation. The effects of DL and RPM on osteoblast differentiation at different differentiating stages are investigated. 12 h treatment of DL dramatically increases the ALP activity and mineralized nodule formation of 7 d differentiating MC3T3-E1 cells. The expressions of osteogenic genes including OC, Col Iα1, DMP1 and Runx2 are all up-regulated by 12 h treated with DL in both 4 d and 7 d differentiating cells. Furthermore, the combined environment of 1g and 16 T in HMGE also promotes osteoblast differentiation (Hu et al. 2013a). It is suggested that the promotion effect of DL is due to the high magnetic field (Zhang et al. 2014a). In addition, RPM is employed to investigate the acute effect of microgravity on osteoblast differentiation. At mineralization stage, if osteoblastic MC3T3-E1 cells is treated with RPM for 24 h, SM would suppress the nodules formation. The expressions of osteogenic genes are all down-regulated. (Hu et al. 2014b). After normally cultured for 7 d, preosteoblast 2T3 cells are subjected to SM for 24 h. Both ALP activity and osteogenic genes are significantly down-regulated. Extracellular signal-regulated kinase (ERK) pathway is involved in not only mechanotransduction (Jessop et al. 2002; Katz et al. 2006) but also osteoblast differentiation (Matsushita et al. 2009). In 2T3 cells, ERK is activated with increased phosphorylated ERK (p-ERK) level (Hu et al. 2013b). However, p-ERK level is reduced in MC3T3-E1 cells. The discrepancy may be associated with different stages in osteoblast differentiation.

As mentioned above, DL effects are composed of HiMF and microgravity. DL promotes both proliferation and differentiation in osteoblast (Qian et al. 2013b; Hu et al. 2013a). HiMF, as shown by control vs. 1g group, also has accelerated effects, while RPM has negative effects. The positive effects of HiMF on osteoblast differentiation are subsequently validated in our lab (Zhang et al. 2014b). Furthermore, analysis of gene expression profile demonstrates that many genes are regulated by HiMF than gravity. 2612 genes are regulated by gravity effects of μ g vs. 2 $g$group, while 1415 genes are altered by HiMF effects of control vs. 1 g group (Qian et al. 2009a). Therefore, it can be concluded that under DL, HiMF may have a greater influence on osteoblast more than microgravity. DL is not suitable for osteoblast in studying SM effects.

Osteocyte

Osteocytes are conceived to be the mechano-sensors of bone tissue (Xu et al. 2012b). Osteocytes can respond to mechanical stimulus by synthesizing and secreting bioactive molecules. Then bone homeostasis is orchestrated by regulating osteocyte itself and effective cells, e.g. BMSC, osteoblast and osteoclast (Bonewald 2011). But the effects of deprived gravity are not systematically clear yet. In this section, we reviewed how osteocytic MLO-Y4 responds to SM of parabola flight, DL, and clinostat. The results were summarized in Table 2, which indicate that osteocytes are sensitive and can respond to microgravity beyond the mechanical loading.

Osteocytes are stellate-shaped cells with dendritic processes connecting each other. SM can alter the cell morphology and reorganize cytoskeleton. DL reduces the number of dendritic processes and alters the cell morphology with both thin and long shape and nucleus. Furthermore, actin filaments are predominantly distributed to the cell periphery. Microtubule organizing center depolymerizes and microtubule becomes disorganized. Vimentin filaments predominantly localize in the polar regions of cell. Focal adhesion (FA) proteins are links between cytoskeleton and ECM, and exhibit mechanosensitive features. FAs including vinculin,

Table 2 Effects of SM on osteocytic MLO-Y4 cells

Results	Ground-based Simulated Microgravity Platforms		
	Parabolic flight	Diamagnetic levitation	2D-clinostat
Morphology	There are no obvious alterations.	Cells become slender. Both cell area and the number of dendrites are reduced.	Both cell area and the number of dendrites are reduced.
Cytoskeleton	The height was not changed. Cytoskeleton is reorganized including actin and microtubule.	Cytoskeleton is reorganized including actin and microtubule.	Cytoskeleton is reorganized including actin and microtubule.
apoptosis	Apoptosis is not induced.	Apoptosis is not induced.	Apoptosis is not induced.
Gene expression	Cx43 expression is down-regulated.	Cytoskeleton-associated proteins including vinculin, paxillin and talin are down-regulated.	Osteocyte secrets less nitric oxide, but more PGE2 and M-CSF.
Reference	(Di et al. 2011)	(Qian et al. 2012b)	(Xu et al. 2012a)

paxillin and talin, express less under DL compared with control (Qian et al. 2012b). Parabolic flight provides altered gravity from hyper (1.8 g) to hypo-gravity (μ g). Altered gravity produced by parabolic fights does not change the osteocyte morphology including cell area and height, but cytoskeleton is reorganized. As in DL, F-actin accumulates more at cell periphery, while microtubule organizing center disappears and α-tubulin compactly assembles around the nucleus (Di et al. 2011). SM provided by 2D-clinostat also alters cell morphology of MLO-Y4 by reducing the number of process. F-actin is destroyed and formed punctate spots across the cell. In spite of that, the apoptosis is not induced (Xu et al. 2012a).

Osteocytes are resided in the lacuna-canalicular system of bone matrix, where the interstitial fluid flow can be induced when bone is loaded. Osteocytes sense the flow shear stress and translate into biochemical signals such as nitric oxide (NO), PGE2, M-CSF etc. (Dallas et al. 2013). MLO-Y4 experienced 2D-clinostat treatment secrets less NO, but more PGE2 and M-CSF (Xu et al. 2012a). Interestingly, if osteocytes are pretreated with SM by 2D-Clinastat, the response of osteocytes to fluid flow stress is decreased with reduced production of NO and PGE2 (Yang et al. 2013).

Osteoclast

Osteoclasts function as bone resorption. During osteoclast differentiation, pre-osteoclasts first fuse into tartrate-resistant acid phosphatase (TRAP) positive multinucleated cells and then are activated to have the ability to resorb bone matrix. Osteoclast formation is promoted under microgravity by both spaceflight and SM. RPM increases proliferation and viability of human preosteoclast FLG29.1 cells. The differentiation is promoted with increased TRAP positive cells and intracellular TRAP activity (Di et al. 2012b). Under SM, osteoclast precursors negatively affect osteoblasts. Conditioned mediums collected from Raw264.7 treated by RPM inhibit viability of osteoblastic MC3T3-E1 cells but fail to change its morphology. Besides, the ALP activity and expressions of osteogenic genes are down-regulated (Di et al. 2012a).

Like RPM, DL also promotes cell viability of FLG29.1. Both the induced TRAP positive cells and TRAP activity are enhanced under DL than 2 g. It is suggested that reduced apparent gravity accelerates osteoclastogenesis. Interestingly, HMGE inhibits osteoclast differentiation regardless of altered gravity from μ g to 2 g (Di et al. 2012c). By using pre-osteoclast Raw264.7 cells, HMGE also inhibits osteoclast formation by reducing TRAP expression at the initial stage. But when the cells are continued to be cultured under normal environment after HMGE treatment, the suppression is reversed. The results indicate that HiMF has a negative effect on osteoclast differentiation (Sun et al. 2014). The studies regarding osteoclast in the response to microgravity are limited. Extensive studies on the mechanisms are needed to clarify the underlying mechanisms.

Bone Mesenchymal Stem Cells

Mesenchymal stem cells are multipotent cells that can differentiate into various types of cell, including osteoblasts, adipocytes and chondrocytes. MSCs are sensitive to microgravity, which causes apoptosis, cytoskeleton disruption and osteogenic differentiation inhibition. Microgravity simulation by diamagnetic levitation caused an inhibited effect on the growth of hMSCs with decreased cell viability. hMSCs morphology shows typical apoptotic phenotype such as shrinkage, rounded morphology membrane blebbing and nuclear condensation. Caspase-3/7 activity, a characteristic feature of an early stage in apoptosis, also increased. Cytoskeleton including F-actin stress fibers and

microtubules disorganizes and forms an actin ring around the periphery of nucleus. The apoptosis of hMSCs is regulated by p53, which is normally kept at a low level. When treated with DL, p53 accumulates. Pifithrin-α, an inhibitor for p53, reverses DL induced apoptosis and disorganized cytoskeleton (Meng et al. 2011). Osteogenic differentiation of hMSCs under DL is also suppressed. Interestingly, the degree of inhibition is dependent on the differentiation stages and reached maximum at the early stage. The decreased phosphorylation of FAK and ERK are involved in the sensation to the microgravity (Shi et al. 2010).

Bone Lining Cells

Bone lining cells are derived from mature osteoblasts and cover all the surface of bone, connecting with osteocyte network (Dierkes et al. 2009). They are thought to play a pivotal role for the initial process of bone remodeling. Bone lining cells first digest nonmineralized matrix via collagenases/matrix metalloproteinases, and then attract preosteoclast to these sites to resorb bone (Everts et al. 2002). Since bone resorption is activated immediately under microgravity, we speculate that bone lining cells may directly perceive loss of gravity and transduce these signals to preosteoclast. In addition, under some circumstances such as intermittent administration of parathyroid hormone (PTH), lining cells can differentiate into mature osteoblast to increase the number of osteoblasts (Kim et al. 2012). In spite of that, we still have limited description and understanding of the role of bone lining cells in bone remolding. The biological effects of microgravity on bone lining cells is also unclear.

The Promising Pathways Recommended to the Further Study

Extracellular Matrix (ECM)-Integrin-Cytoskeleton

ECM-integrin-cytoskeleton axis is generally considered to be essential for cellular mechanosensing and mechanotransduction (Alenghat and Ingber 2002). Cytoskeleton is the load-bearing architecture and plays pivotal roles in translating mechanical stresses into a chemical response and then causes cell adaptation by regulating integrin and ECM (Maniotis et al. 1997). Also, the "inside-out" regulation system is believed to be critical to sense gravity (Ingber 1999). Under microgravity, cytoskeleton may not organize the same way as they usually do on Earth. Either DL or RPM leads to cytoskeleton reorganization or disruption in bone cells. Under DL, disrupted cytoskeleton may directly lead to apoptosis in BMSC (Meng et al. 2011). Abnormal gravity depolymerizes microtubule organizing center, and actin filaments predominantly are distributed to the cell periphery in osteocytes (Di et al. 2011). For osteoblast, both actin and microtubule are reorganized with reduced complexity, height, and expression (Qian et al. 2010). Moreover, cDNA microarray in $\mu\ g$ vs. 2 g revealed that 13 cytoskeleton-related genes of osteoblast are all up-regulated (Qian et al. 2009a). These cytoskeleton-related proteins may further influence cytoskeleton arrangement in responds to gravity alteration by adjusting their expression or distribution. Under DL, both distribution and expression of vinculin, paxillin and talin is significantly reduced (Qian et al. 2012b). The results indicate these actin-associated proteins may be involved in osteocyte mechanosensation.

Fibronectin (FN)

As an extracellular matrix (ECM) protein, fibronectin (FN) plays important roles in various cellular behaviors, such as adhesion, migration, growth and differentiation (Singh et al. 2010). Microarray analysis shows that FN expression of MG63 is increased markedly under RPM (Qian et al. 2008). To further investigate the role of FN, both 3D-clinostat and DL are used to detect the alteration of FN in human osteoblast hFOB1.19 cells. All the SM platforms stimulates osteoblast to secret more soluble FN. When blocking the integrin binding site of FN by RGD peptide (Arg-Gly-Asp) or integrin function by its antibody, soluble FN is significantly increased (Li et al. 2011). It is suggested that microgravity may weaken the interaction between FN and integrin. Then, the activated downstream signaling pathway of integrin is influenced. As a feedback, osteoblast needs to secret more FN to compensate extracellular FN (Inside-Out). The enhanced FN strengthens the interaction with integrin, and stabilizes cytoskeleton (Baneyx et al. 2002). By these means, osteoblasts response and adapt to microgravity. The results show the extracellular up-regulation of soluble FN after mechanical unloading, an essential chemical signal in bone remodeling.

Microtubule Actin Cross-Linking Factor 1 (MACF1)

MACF1, also called ACF7 (actin cross-linking family 7), is an essential cytoskeletal linker protein belonging to spectraplakin family (Suozzi et al. 2012). MACF1 strengthen the cytoskeleton by simultaneously connecting actin and microtubule to each other (Bernier et al. 1996; Kodama et al. 2003; Wu et al. 2008). In osteoblast, widely expressed MACF1 protein is distributed cross the cytoplasm and partly co-localized with cytoskeleton. This shows that MACF1 may take pivotal functions in mechanotransduction. Altered gravity causes cytoskeleton reorganization in osteoblast. Based on this, we raise the hypothesis that the adaptive response of cytoskeleton to gravity may lead to the expression and distribution changes of MACF1. Under DL,

the expression of MACF1 is influenced, and its distribution concentrates at perinuclear region. Besides, the colocalization of MACF1 with actin and microtubule cytoskeleton is not apparent. However, there is no obvious alterations under 1 *g* vs. control-geomagnetic field group (Qian et al. 2009b). These findings indicate that MACF1 may be involved in the response to microgravity by mediating the cytoskeleton organization. cDNA microarray analysis also shows that MACF1 expression increases a lot under μ *g* vs. 2 *g* group, but not under 1 *g* vs. control-GMF (Qian et al. 2009a). These results suggest that MACF1 is more sensitive to gravity than HiMF.

Cx43

As the most abundant connexin (Cx) in bone cells, Cx43 forms gap junction channels and hemichannels to transmit and secret signaling molecules regulating bone remodeling (Batra et al. 2012; Plotkin and Bellido 2013). Previously, we found that Cx43 formed hemichannels are involved in maintaining bone mass, structure, strength and osteocyte viability, while gap junctions in regulating the rate of bone remodeling (Xu et al. 2014). Lloyd et al. also demonstrates that Cx43 deficiency decreases the effects of mechanical unloading on bone loss (Lloyd et al. 2012). Unlike osteoblast and osteoclast, osteocytes are sensitive to fluid shear stress in the canaliculi and lacunae, and translate physical factors into other bone cells located on the bone surface or marrow (Loiselle et al. 2013). Meanwhile, altered gravity produced by parabolic flight down-regulates Cx43 expression in osteocytic MLO-Y4 cells (Di et al. 2011). It is suggested that Cx43 plays a critical role in response to microgravity and needs to be further investigated.

Nitric Oxide

As a short-lived free radical gas, nitric oxide (NO) is synthesized by nitric oxide synthase (NOS) enzymes from oxygen and L-arginine, and acts as a regulator of bone formation and resorption (Wimalawansa 2010). Mice lacking endothelial NO synthase (eNOS) exhibits significant abnormalities in bone development (Aguirre et al. 2001) and inducible NO synthase (iNOS) null mice revealed imbalances in bone remodeling (van't Hof et al. 2000) and fracture healing (Baldik et al. 2005; Arasapam et al. 2006). During osteoclast differentiation, the iNOS-derived NO firstly acts like a negative feedback in an early phase (Zheng et al. 2006) and then enhances osteoclastogenesis during the fusion of mononuclear pre-osteoclast (Nilforoushan et al. 2009). Physical factors like mechanical stresses or some chemicals such as cytokines, estrogen, and growth factors, can affect bone metabolism via NOS stimulation and NO production at different degrees. Nevertheless, the involvement of NO signaling during the response of bone cells to SMFs has not yet been well studied. After treated by HMGE, NO concentration of the supernatant is slightly decreased in differentiated FLG29.1 cells (Di et al. 2012c). However, when differentiated Raw264.7 cells at an early stage are treated by DL and subsequently cultured back to normal condition, NO level is not attenuated significantly. On the other hand, NO production in 1 *g* group is significantly higher than the control group (Sun et al. 2014). In summary, these observations suggest that the stimulatory effects of osteoclast formation under DL are complex and need to be further studied to clarify the gravitational and HiMF effects.

Calcium Associated Hormones

Multiple biochemical (e.g. growth factors, cytokines, hormones, transcription factors etc.) and physical factors (e.g. mechanical stimulation, gravity, magnetic field etc.) have been identified contributing to maintain skeleton health. Microgravity not only directly influences skeleton system, but also causes hormonal, mineral and nervous challenges, which may indirectly affect bone homeostasis.

It has been found that hormones related with calcium regulation and stress have alterations under microgravity, which may indirectly influence bone homeostasis (Vico et al. 1998). The net bone loss by osteoclast-mediated resorption leads to a significant increase of calcium concentration in serum under microgravity. Parathyroid gland then gets negative feedback from high calcium levels, decreasing the release of PTH. Subsequently, both calcium excretion and phosphorus retention are enhanced, causing an increased ration of calcium/phosphate. The production of activated vitamin D is also decreased, impairing the intestinal calcium absorption. In turn, the negative calcium balance is exacerbated, because the body will mobilize calcium stores of bone to maintain calcium homoeostasis (Holick 2000). However, the role of calcium regulating hormones is not fully understood in the response of bone to microgravity. Stress related hormones, such as glucocorticoids and adrenocorticotropic hormone found to regulate bone homeostasis, are initially increased during the first few days of spaceflight, but return to normal values thereafter (Macho et al. 2001). This suggests that variations of stress related hormones may play a role in the bone changes observed in microgravity.

Sympathetic nervous system

Analysis of plasma level of noradrenaline and muscle sympathetic nerve activity in astronauts returning from outer space indicates that SNS is enhanced under microgravity (Mano et al. 2010). Since sympathetic nervous system

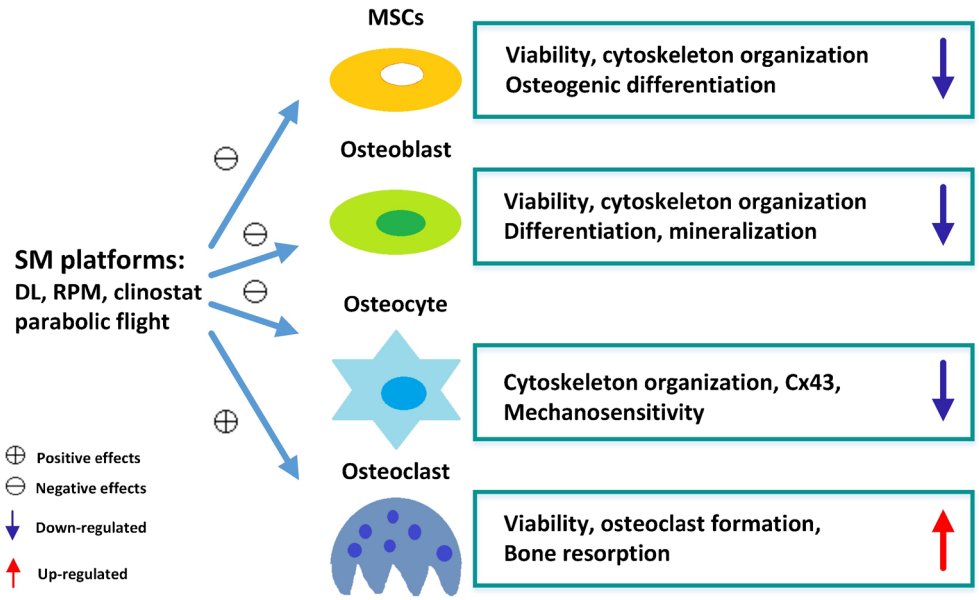

Fig. 1 SM modulates multiple behaviors of bone cells. Microgravity negatively influences mesenchymal lineages including MSCs, osteoblast and osteocyte. On the contrary, osteoclast function was promoted. Some regulatory molecules may be involved in the responses

(SNS) regulates bone metabolism through leptin released from adipocytes (Takeda et al. 2002), researchers investigate the role of SNS in regulating unloading-induced bone loss. They finds that treatment with β-adrenergic blockade suppresses the bone loss in hind-limb unloading mice through decreased cell activities of osteoblast and enhanced osteoclastic activities (Kondo et al. 2005). Further investigation shows that these effects may be indirectly due to the attenuated leptin in serum (Baek and Bloomfield 2009). The results indicate that SNS also mediates disuse-induced bone loss.

Conclusion

Bone loss induced by long-term spaceflight is partially due to the altered behaviors of bone cells. Spaceflight research demonstrates that microgravity has detrimental effects on cell growth, morphology, cytoskeleton organization and differentiation in osteoblast. Meanwhile, osteoclast differentiation is promoted. SM including RPM and DL is practical, reliable and convincible. Consistently, SM results are similar with spaceflight. The details regarding the responses of bone cells to SM were summarized in Fig. 1. However, DL is not a suitable SM platform for osteoblast research due to the dominant HiMF effects. Beyond that, further studies have been carried out and suggest some promising gravity-sensitive pathways including ECM-integrin-CSK axis, FN, MACF1, Cx43 and NO. In spite of that, these possible mechanisms should be validated in the future spaceflight studies.

Acknowledgments This research was supported by the National Basic Research Program of China (2011CB710903), National Natural Science Foundation of China (51477141, 81472090, 31100667), and Specialized Research Fund for the Doctoral Program of Higher Education of China (20126102110055), and the Fundamental Research Funds for the Central Universities of China (3102014KYJD020, 3102014ZD0045).

Conflict of interests The authors declare that they have no conflict of interest.

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