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SPACE AND OSTEOPOROSIS:

HOW GRAVITY AFFECTS BONE MICROENVIRONMENT

[MED/04]

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"Many times an obstacle is only a message given to you by life. You have to find another way, but that doesn't mean that you won't arrive at your destination."

Samantha Cristoforetti

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ABSTRACT

The aim of this study is to get insights into space-associated osteoporosis and cardiovascular deconditioning, which are important adverse effects of spaceflight. These disorders are strikingly similar to common diseases caused by sedentary life, senescence and degenerative diseases on Earth. Therefore, investigating the alterations occurring in microgravity will significantly improve our knowledge about the mechanisms leading to disease, thus fostering the development of novel approaches and countermeasures to improve the quality of life of millions of people on Earth and of a few hundreds in space.

Studies at the cellular and molecular level are necessary to understand the mechanisms underlying osteoporosis and cardiovascular deconditioning. Two main questions were asked: 1. how microgravity affects cultured human endothelial cells and 2. how microgravity impacts on bone cells. Different microgravity simulators were used for cell culture experiments.

Evidence has been provided about the marked sensitivity of endothelial cells to real and simulated microgravity. On the basis of previous experiments in space and on Earth, Human Umbilical Vein Endothelial Cells (HUVEC), widely used as a model of macrovascular endothelial cells, were studied and shown to dynamically adapt to simulated microgravity. Indeed, HUVEC rapidly upregulate heat shock protein (HSP)70 and this increase is maintained up to day 4 from exposure to microgravity. At later time points, HSP70 returns to basal level, while an overexpression of paraoxonase (PON)2, sirtuin (SIRT)2, HSP27 and P-HSP27 is detected. This late adaptive response counterbalances the increase of Thioredoxin-Interacting Protein (TXNIP) and prevents the accumulation of reactive oxygen species. Thanks to this adaptive response, no dysfunction occurs in HUVEC in simulated microgravity. This is an important finding, since endothelial cells are responsible for the integrity of the vascular wall.

Because of the heterogeneity of the endothelium, some experiments were also performed on human microvascular endothelial cells (HMEC). Like HUVEC, HMEC rapidly upregulate HSP70, indicating the activation of an adaptive response, and do not undergo apoptosis. Differently from HUVEC, HMEC are growth retarded in microgravity. Accordingly, p21 increases in a p53 independent fashion. Moreover, they secrete higher amounts of tissue inhibitor of matrix metalloprotease (TIMP)-2 and interleukin (IL)-6 than controls in 1G-conditions. The data obtained in HMEC were a pre-requisite for the following experiments. Since signals from endothelial cells condition the behavior of osteoblasts and are fundamental for healthy bone, I utilized media collected form HMEC exposed to simulated microgravity to culture human

osteoblasts. My studies demonstrate that conditioned media collected from microvascular endothelial cells exposed to microgravity inhibit osteoblast function by impairing alkaline phosphatase activity and calcium deposition in the extracellular matrix. This inhibitory effect might be due to the increased secretion by HMEC of IL-6 and TIMP-2. On these bases, it is feasible to propose that microgravity impairs osteoblast activity both directly -as demonstrated in real and simulated microgravity- and indirectly by altering endothelial/osteoblast communication.

Some studies have shown not only an impairment of osteoblast but also an increase of osteoclast activity in real and simulated microgravity. However, very little is known about the effect of microgravity on the osteogenic potential of human mesenchymal stem cells (MSC). To this purpose, MSC were cultured in simulated microgravity in the presence or in the absence of an osteogenic cocktail. No alterations in osteogenic differentiation of MSC occur in simulated microgravity as demonstrated by the modulation of osteogenic markers and by the deposition of calcium, suggesting that these cells are not involved in space-associated osteoporosis. It is noteworthy that, like endothelial cells, MSC upregulate stress proteins, some of which are implicated in osteogenesis.

It is clear that endothelial cells and MSC sense microgravity as a stressor, and consequently activate a stress response that not only maintains the cells viable but also allows them to reach a novel homeostatic state so that they can perform, at least in part, their activity.

1. INTRODUCTION

1.1 SPACE

In evolution exploration has represented an important survival strategy, as a result of climate changes and population pressures. The human species is also intellectually urged to explore the unknown because of curiosity. Now, the ultimate frontier to explore is space.

Space exploration has always intrigued humankind. Jules Verne's book "From the Earth to the Moon", 1865, can be considered the starting point of our interest in space travel. This science fiction story deals, 100 years in advance, with the possibility of launching a "bullet capsule" with three people aboard with the aim of landing on the Moon (Figure 1).

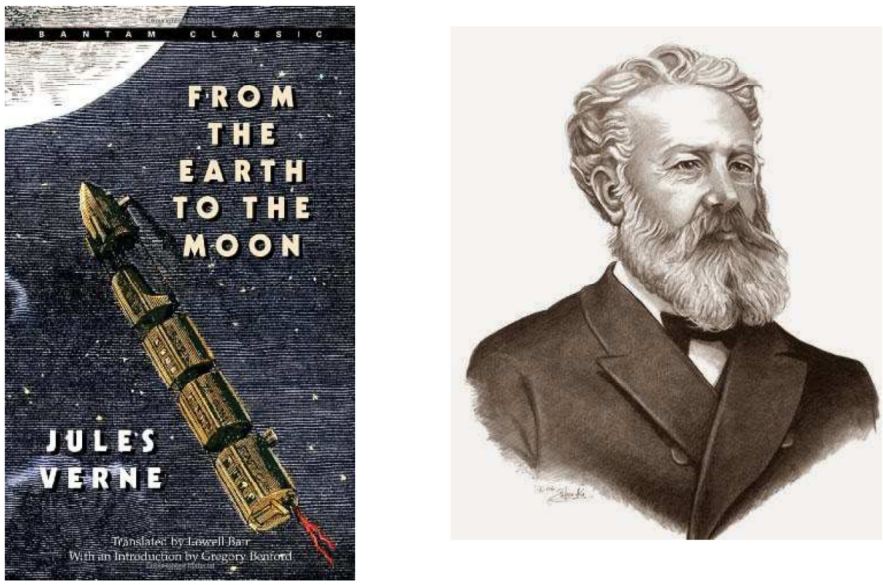


Fig.1 *Illustration of Jules Verne and his book "From the Earth to the Moon" (1865).*

The fictional vision of spaceflight became a real possibility with the advance of science. In particular, after the Second World War, new tensions between the United States and the Soviet Union lead to the space race. The first spaceflights were ballistic which, by definition, did not reach orbit and had only short periods of microgravity (10^{-3} to 10^{-6} g) lasting from a few seconds or minutes to a few hours before they return to Earth (Hughes-Fulford 2011). Major advances in spaceship technologies were achieved in the past 50 years, driven mainly by national security considerations. In 1998, the first component used to build the International Space Station (ISS) was launched into orbit. The ISS is a space platform located at 370 km from the Earth, where the intensity of gravitational pull is 90% of its value at sea level. It has a speed of 28000 km/h and completes 15.5 orbits per day, i.e. one orbit in 90 minutes (Aubert 2005). ISS offered relevant insights into the health dangers for humans in space. Indeed, space is an unforgiving,

unfriendly and dangerous environment. No human errors or technical failures are tolerated. In addition, humans remaining in space for extended periods are exposed to the near absence of gravity and to the presence of high-energy, ionizing cosmic rays. Since both zero gravity and cosmic rays have severe health implications for astronauts, their effects on cells, tissues and animal models have been intensively investigated onboard the ISS, together with astronauts' psychological, cardiovascular, vestibular, hormonal and metabolic response to space. These studies are crucial to ensure the health of a spaceship crew travelling to other planets. Even though the usefulness of space research has recently been questioned because of its high costs, it should be recalled that studies in space have sparked new scientific and technological knowledge generating social benefits and improving the quality of life on Earth (Rinaldi 2016).

1.1.1 MICROGRAVITY

Over 4 billion years many organisms have had to adapt and evolve because of physical and environmental changes on Earth, apart from gravity (g) and its acceleration (9.8 m/s^2) which remained constant. Since the establishment of the ISS, adaptation of organisms to the absence of gravity has been intensively studied.

In microgravity objects seem to be weightless but retain their mass. There is no "up" and "down" in space, astronauts define their own system of coordinates: "down" is where my feet are. In microgravity, the centrifugal force, speed of the spacecraft "forward" and the centripetal force, speed of the spacecraft "inwards", are balanced. Therefore, the astronauts on the ISS are suspended because the pull of gravity is neutralized and the forces are balanced (Aubert 2005).

1.1.2 MICROGRAVITY: HOW THE BODY ADAPTS

Microgravity has side effects on the health of astronauts and countermeasures are necessary to reduce health risk during and after spaceflight. This is not surprising if we consider that life on our planet has evolved under Earth's gravity. Consequently, all human organs are affected by gravity's absence. Tissues can adapt to changes in gravitational environment at a different rate (Figure 2).

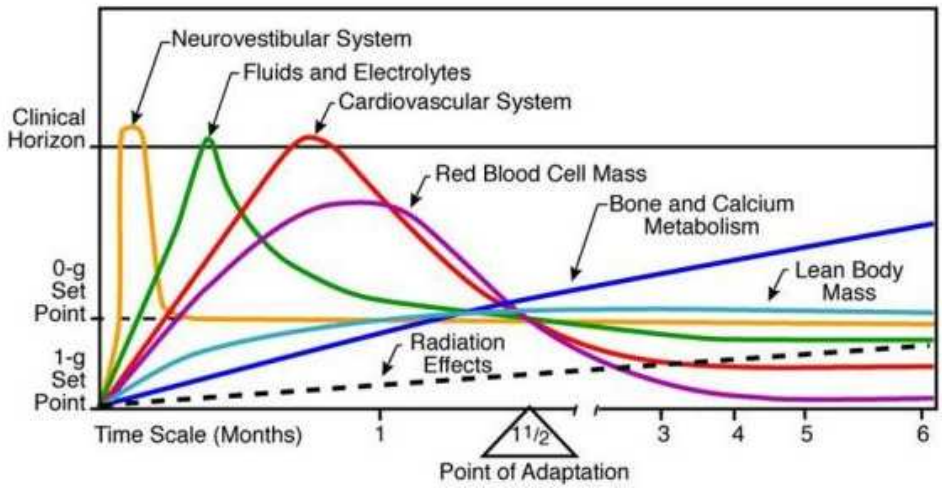


Fig. 2 *Physiological changes associated with microgravity exposure. From the Physiology Slide Set of the American Society of Gravitational and Space Biology (http://asgsb.org/slidesets/slidesets.html).*

These physiological adaptations may reduce individual fitness or have pathological consequences upon return to Earth (summarized in Figure 3 and Table 1). Cardiovascular deconditioning, immune deficiency, muscle atrophy and bone demineralization are some of the long term effects of spaceflight (White and Averner 2001).

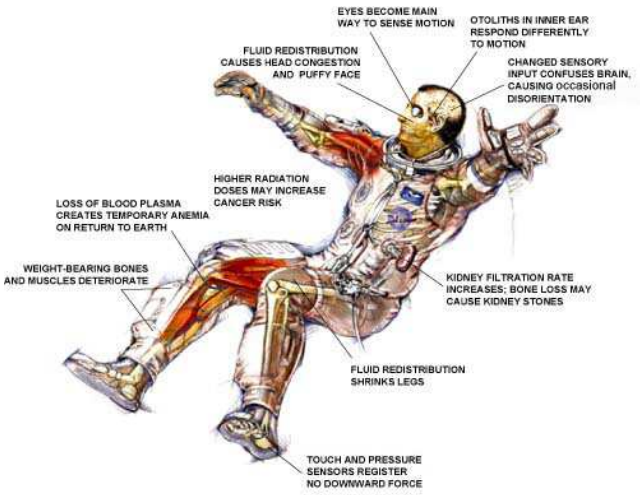


Fig. 3 *Effects of space mission on humans (Marcal 2010).*

Physiologic effects	Launch	Duration of flight				Landing	Postflight period		
		24 h	48 h 2 wk		> 1 mo		24–48 h	1–2 mo	> 1 yr
Fluid redistribution	• Redistribution of fluid to the torso and head • 10% decreased fluid volume in the legs	• 17% reduction in plasma volume	• Gradual decrease in erythropoietin secretion, leading to a 10% decrease in total blood volume			• Orthostatic hypotension from pooling of fluids in the legs	• Return of normal fluid distribution		
Neurovestibular effects	• Space motion sickness					• Space motion sickness			
Muscle changes		• Gradual decrease in muscle mass by 20%		• Gradual decrease in muscle mass by 30%		• Muscle soreness and tightness		• Full recovery of muscle mass and strength	
		• Gradual decrease in muscle strength (up to 50% loss observed)							
Bone demineralization		• 60%–70% increase in calcium loss (urinary, fecal). Reduced parathyroid hormone and vitamin D production.							• Complete or almost complete restoration of bone density
		• Gradual loss of bone density (1%–2% per month)							
Psychosocial effects	• Fatigue, sleep debt, isolation, emotional effects, stress to the astronaut's family, multicultural crew environment								
Immune dysregulation		• Possible reactivation of latent herpes viruses and impairment of cell-mediated immunity				• Numerous cellular and other changes leading to impaired immunity		• Gradual improvement in immunity (days to weeks)	

***Table 1**. Timeline of physiologic adaptation from launch to return to Earth (Williams 2009).*

While on Earth the cardiovascular system works against gravity, microgravity determines a dramatic redistribution of fluids from the legs toward the head, which results in a syndrome called "puffy face-bird legs" (Grimm 2016) (Figure 4).

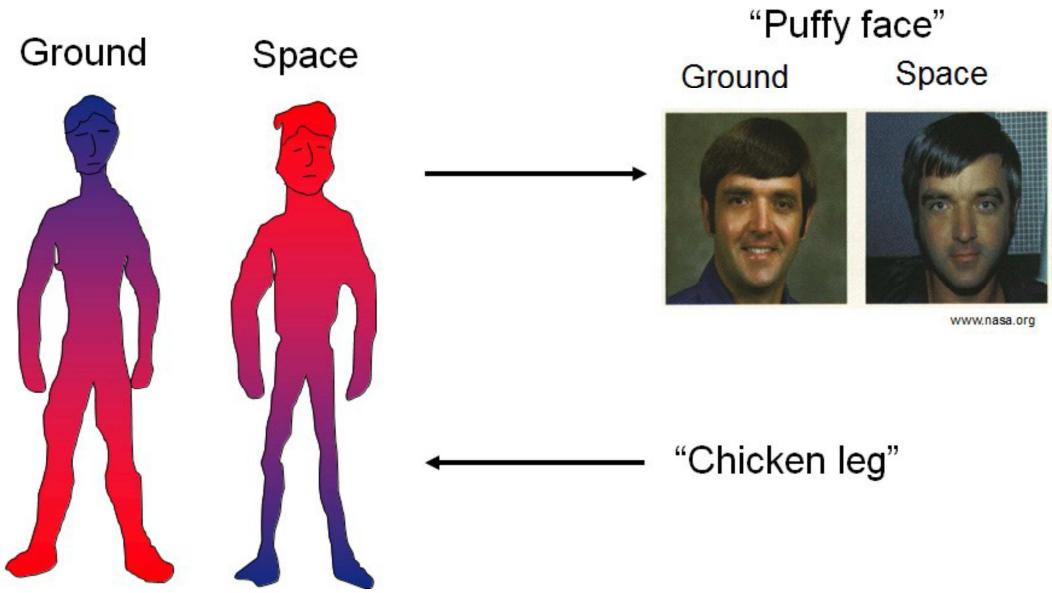


***Fig.4** Redistribution of fluids in microgravity results in "puffy face-bird legs". Photo of astronauts was taken by www.nasa.org.*

At the beginning of astronauts' space journey the first symptoms are neurovestibular and characterized by facial pallor, cold sweats, upset stomach, headache and nausea. This could happen also after return to Earth and is caused, in part, by the shift of fluids. A reduction of

plasma volume and erythropoietin secretion occurs thereafter (Williams 2009). The shift of the fluid from the lower to the upper body results in upper body blood volume expansion, causing a decrease in heart size, stroke volume, cardiac output, and aerobic capacity (White and Averner 2001). After return to Earth, orthostatic intolerance is a common problem, which means that mission members can not evacuate without assistance after spacecraft landing. This condition is characterized by an increase in heart rate and palpitation associated with the inability to assume the standing position. Orthostatic intolerance results from an increased vascular compliance, impaired arterial resistance and venous return, reduction of cardiac size and left ventricular mass, reduction of plasma volume and red blood size (Carter and West 2013) (Figure 5). It is important to find solutions for this disturbance because it could result in a variety of risks for the safety of astronauts such as an impediment to carry out an emergency landing (Aubert 2005).

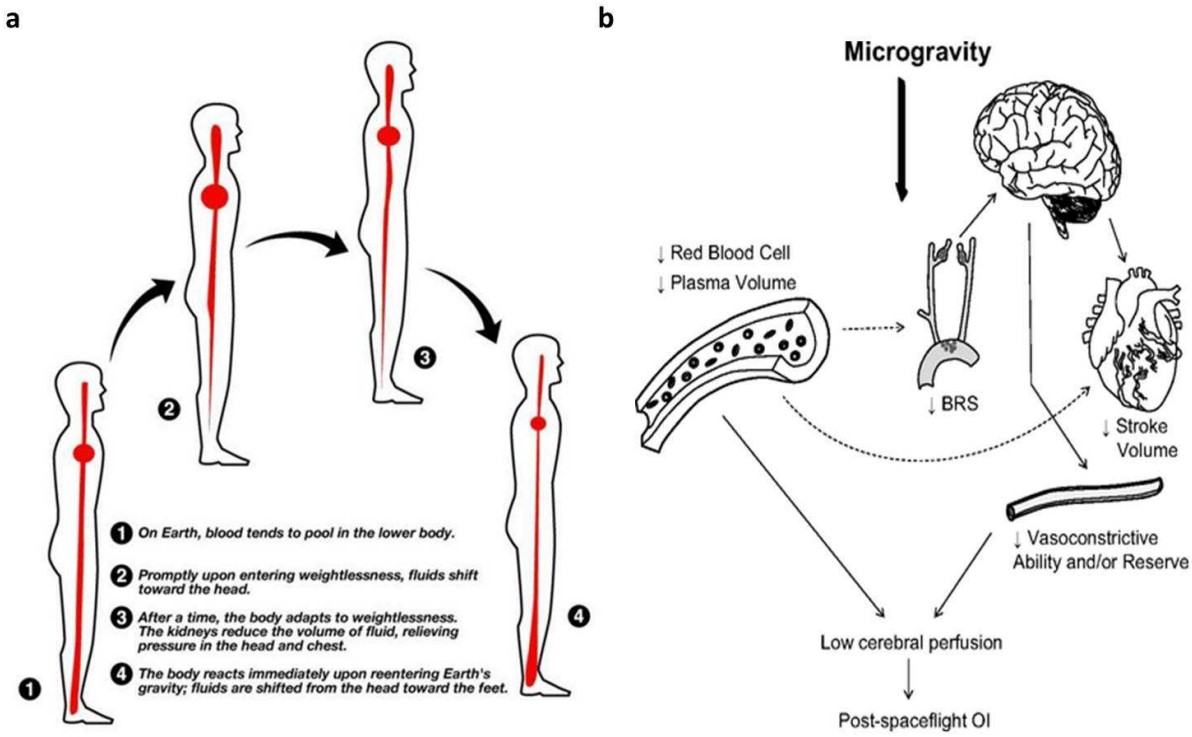


Fig. 5 *a) Fluid Redistribution pre- during- after spaceflight. From the Physiology Slide Set of the American Society of Gravitational and Space Biology (http://asgsb.org/slidesets/slidesets.html). b) Cardiovascular deconditioning leading orthostatic intolerance (OI) baroreflex sensitivity (BRS) (Carter and West 2013).*

On Earth also the muscles resist gravitational force. Muscle atrophy occurs in space mainly because of gravitational unloading (Williams 2009), which also reduces blood supply to the legs. Moreover, astronauts do not walk, the neuromuscular innervation to the legs is minimally used and muscular contraction does not occur (Vernikos and Scheneider 2009).

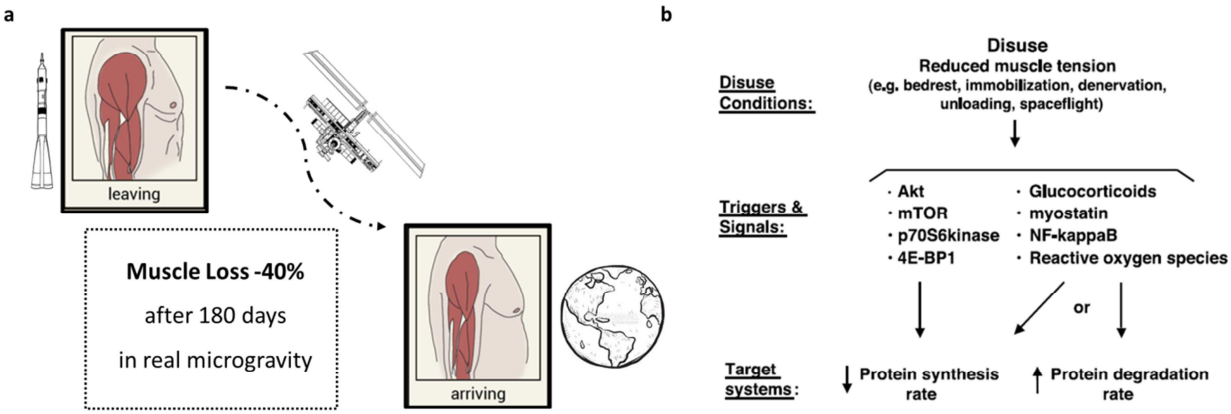


Fig. 6 *a) A diagram detailing the extent of muscular atrophy after a typical 6-months mission. b) A summary of the mechanisms leading to protein synthesis or degradation (Carter and West 2013).*

Atrophy is characterized by the reduction of muscle fiber size due to the increase of protein degradation associated with reduced protein synthesis (Figure 6). Elevated levels of circulating cytokines, such as interleukin (IL)-6, IL-1 and Tumor Necrosis Factor (TNF)-α, are associated with the reduction of muscle strength. In addition, the increase of cytokines could be responsible for the reduction of insulin like growth factor (IGF)-1, which is implicated in several phases of muscle development mediating myoblast proliferation, survival and terminal differentiation (Narici 2011). It is noteworthy that a discrepancy between lost mass and decreased force exists and seems to be due to alterations of motor unit recruitment (Figure 7). Astronauts lose 20-30% of muscles mass, and, after return to Earth, report muscle soreness and tightness, but they recover through physical exercise and physiotherapy (William 2009). Pharmacological interventions have been proposed to fight against muscle atrophy in space such as the somministration of IGF-1. At the moment, the primary countermeasure against muscle atrophy during spaceflight is through exercises such as a treadmill workout and weightlifting.

- Greater atrophy of Type II fibers
- Atrophy primarily due to decline in muscle synthesis (minimal muscle breakdown defected in excretion)
- ↓ peak force of limb skeletal muscles
- ↑ muscular fatigue and damage after return to Earth.

Fig. 7 *Key muscular adaptations associated with microgravity. MHC, myosin heavy chain (Carter and West 2013).*

Gravity also loads the bones. Accordingly, bone demineralization occurs in space immediately after arrival on the ISS. The bone loss range is 1% to 2% per month in weight-bearing bones, such as lumbar vertebrae, pelvis, femoral neck, trochanter, tibia and calcaneus (Cappellesso 2015, Williams 2009), leading to space-associated osteoporosis (Figure 8). Increased amounts of calcium and other bone minerals are excreted with the urine (LeBlanc 2007). There is an increase of 30% of calcium in plasma and a decrease of about 50% in parathyroid hormone (PTH) after 8 days of spaceflight. These values do not return to their physiological measurements before 16 days in microgravity (Hughes-Fulford 2011).

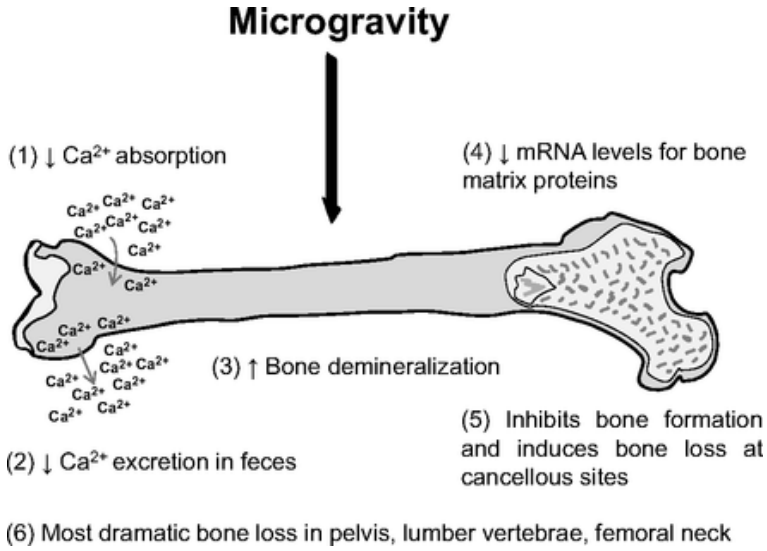


Fig. 8 *Key bone adaptations associated with microgravity (Carter and West 2013).*

Astronauts slowly recover after return to Earth, and after long-duration space missions this recovery can be longer than the period spent in space (Table 1). Most astronauts will fully recover their bone density within 3 years. However, some will never regain preflight levels. Moreover, the recovered bone shows different structure and mineralization (Williams 2009).

Immunity is dysregulated during spaceflight. Lack of gravity inhibits T-cell activation, alters the synthesis of cytokines, decreases the activity of natural killer cells, impairs the function of granulocytes, among others (Williams 2009). Part of these alterations can be determined by the high level of stress experienced by astronauts, as supported by the evidence of elevated urine cortisol. Several in-flight studies have shown that microgravity blunts signal transduction pathways essential for early T-cell activation. A recent study on 23 astronauts on the ISS for 6 months demonstrated a reduction in $CD4^+$ and $CD8^+$ T-cell function, which persisted after landing. Significant reductions in mitogen-stimulated production of cytokines, i.e. interferon (IFN)-γ, IL-10, IL-5, TNFα, and IL-6, persisted during spaceflight (Crucian 2015). No changes of immunoglobulin levels are detected in the serum of astronauts (Rykonova 2008). All the aforementioned alterations contribute to astronauts' higher susceptibility to infections during spaceflights (Klaus and Howard 2006).

Interestingly, the disorders which stem from adaptation to space closely reflect some common diseases on Earth, such as osteoporosis, muscle waste and immune deficiency (Table 2).

Table 2 A comparison of some space-related health concerns with medical issues on Earth

Research area	Space	Earth
Bone	Bone loss and increased fracture risk Increased kidney-stone formation Injury to soft connective tissue	Osteoporosis and other bone disorders
Cardiovascular	Postflight orthostatic intolerance Cardiac atrophy Heart rhythm disturbances	Orthostatic intolerance Heart disorders, such as sudden cardiac death due to heart rhythm disturbances
Performance/Sleep	Errors due to sleep loss and disruption of the biological clock	Sleep problems due to jet lag, shift work and extended work schedules Accidents due to sleepiness
Immunology/Infection	Activation of dormant viruses in the body Increased infection risk Space flight-related anaemia Interference with wound healing	Immune system disorders Viral outbreaks due to stress conditions (shingles, cold sores) Anaemia and other blood disorders
Muscle	Muscle loss and atrophy	Muscle wasting diseases Muscle weakening due to prolonged bed rest, immobilization, nerve crush injury and ageing
Neurovestibular	Space motion sickness and body orientation problems Re-entry vertigo Postflight dizziness, balance, posture and gaze stability	Vertigo and other balance disorders
Radiation effects	Cancer Damage to central nervous system Cataracts and other diseases	Risks from exposure to naturally occurring and work-related radiation

Table 2 *Comparison between space risks and medical issues on Earth (Ronald 2001).*

1.1.3 MICROGRAVITY: HOW MAMMALS AND THEIR CELLS ADAPT

One of the objective of the ISS is to advance knowledge by fostering scientific research. The astronauts on board the station serve as subjects for further studies on how microgravity affects human health, since they represent an excellent opportunity to address our persisting knowledge gaps related to human physiology during space travel.

The first biological experiments were performed on microbes and demonstrated that bacteria and fungi grew faster in microgravity than in 1G-conditions. Moreover, some bacterial species became more resistant to common antibiotics (Klaus and Howard 2006). Furthermore some pathogens such as Salmonella typhimurium became more virulent compared to those grown in 1G-conditions (Wilson 2007).

Several experiments have been performed on mice and mammalian cells, thus offering new insights into mechanosensing, transmission and transduction signals modulated by physical forces. In particular, while space experiments that involve animals give the opportunity to study the health effects of microgravity in ways that cannot be done with astronauts, experiments on cells allow to dissect the molecular pathways leading to cell dysfunction.

Given the significant similarity between murine and human genomes, mice are widely considered an excellent model to study human diseases. Recently, three mice lived on the ISS for 91 days, the longest period that rodents have ever spent in space (Mice Drawer System Experiment). Here the main findings are summarized:

- MUSCLE: muscle atrophy was present in all fiber types. Most of the sarcolemma ion channels were overexpressed, while Ca^{2+}-activated K^{+} channels were down-regulated. Gene expression studies demonstrated the upregulation of ubiquitin-ligases in spaceflown muscles, whereas autophagy genes were not modulated (Sandonà 2012). As mentioned above, similar results were reported in humans (William 2009, Hughes-Fulford 2011, Grimm 2016).
- SKIN: space induces skin atrophy with a 15% reduction in the thickness of the dermis mainly because of an increase in collagen degradation. Space also deregulates hair follicle cycle (Neutelings 2015). Indeed, while 1 out of every 9 hairs on a control mouse's body is in the growth phase, in ISS-mice all the hairs were in the growth phase. This event could be due to a perturbation of the program of the stem cells. One previous study on a human astronaut also showed thinning of the skin, as well as changes in

elasticity and reduced healing, after 6 months in orbit (Tronnier 2008), but no data about hair growth in astronauts are available. Skin dryness and itching are rather common in space and make the skin more susceptible to scratches and irritation (Lowrey 2014)

- BONE: bone loss during spaceflight in the weight-bearing bones of these mice was reported. A decrease of the trabecular number was observed after flight, but trabecular thickness did not change. The expression of specific bone marker indicate that microgravity-induced bone loss was due to an increased bone resorption and a decreased bone deposition. Interestingly, osteocytes, mature stellate cells that act as mechanosensors and are responsible for bone integrity, acquired a round enlarged shape (Tavella 2012). Moreover, the response of bone marrow cells to Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) was depressed (Chang 2012).
- IMMUNE SYSTEM: several experiments on immune cells from mice demonstrate lymphocytes' growth arrest, in particular $CD4^+$, and the inhibition of T-cell activation compared with the control on Earth. Conversely, the CD8 T-cell compartment was relatively unaffected (Chang 2015, Gridley 2009). The decrease of INF-ɣ was observed. (Chang 2012).

Turning our attention to experiments on cells, it is now clear that microgravity importantly impacts on the function of all the mammalian cells evaluated until now (Boonstra 1999, Hughes-Fulford 2003, Crawford-Young 2006). A common feature of all the cells exposed to microgravity is the remodeling of the cytoskeleton, which is considered the primary gravity sensor (Vorselen 2014). The cytoskeleton is a network of interconnected systems of filaments, the actin microfilaments, the microtubules, and the intermediate filaments. It conditions the shape of the cells and orchestrates important functions such as adhesion, proliferation, survival, polarization, migration, and gene expression (Fletcher and Mullins 2010). Consequently, cytoskeletal alterations lead to the modulation of enzymatic, genetic and epigenetic pathways, with consequent modulation of cell behavior and impairment of tissue functions (Bizzari 2015). Moreover, in microgravity, the stress fibers do not allow the correct cell-to-cell communication because the junctions are interrupted (Louis 2014).

A crucial role in cytoskeletal alterations of space flown cells is played by RhoGTPases, which seem to act as mechanosensitive switches. They represent a family of small GTP-binding proteins responsible for the integration of biochemical and mechanical signals (vescicular

transport, traffic, cytoskeleton turnover) and are involved also in the enhancement of reactive oxygen species (ROS) production (Louis 2014). Indeed, the reduction of RhoA activity explains the loss of stress fiber and the reorganization of actin (Matsumura 2005) and the reduction of cell adhesion which limits integrin signaling (Figure 9).

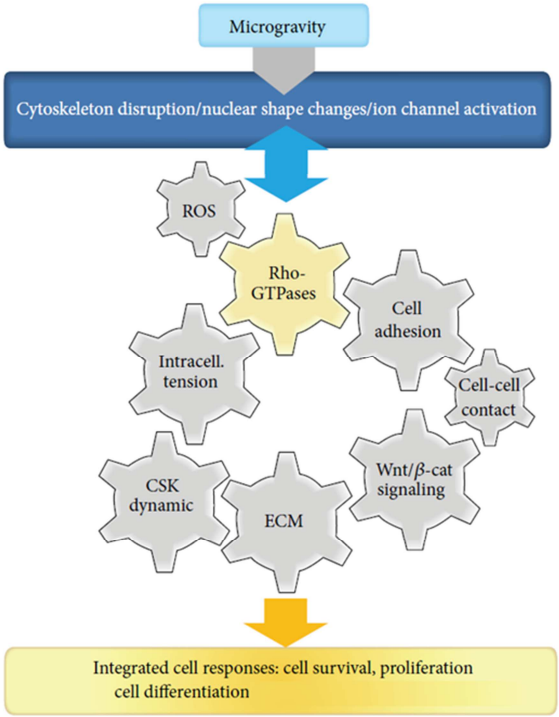


Fig. 9 *The role of RhoGTPases in the response of mammalian cells to microgravity-related conditions (adapted from Louis 2014).*

Cytoskeletal alteration in lymphocytes T, monocytes and macrophages has a role in immune dysfunction (Figure 10).

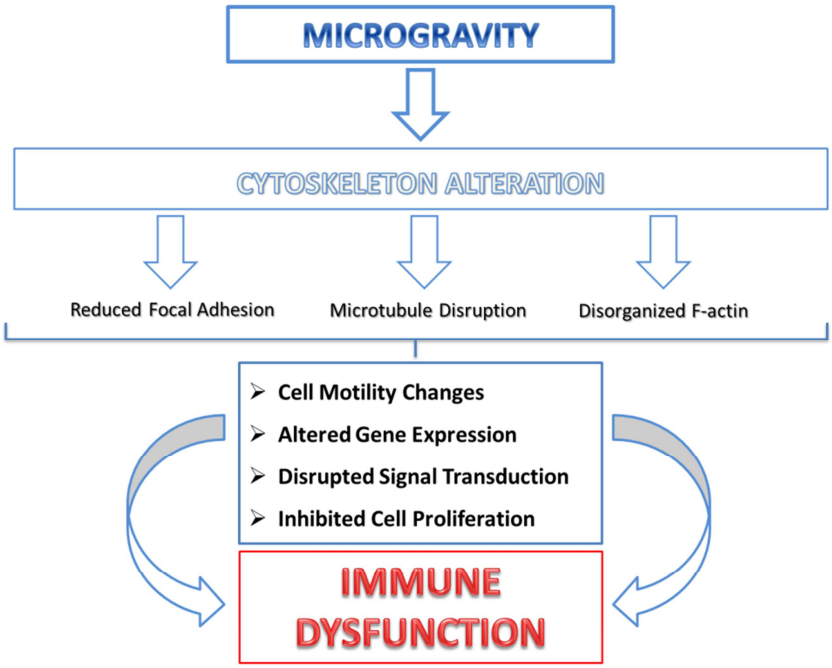


Fig. 10 *Cytoskeletal alteration and effects on the immune system.*

In T lymphocytes in microgravity, decreased growth, increased apoptosis, chromosomal aberration, inhibition of motility and alteration of cytokines production were detected (Cervantes and Hong 2015). The decrease of IL-2 and IL2Rα expressions in microgravity is in part responsible for lymphocytes growth arrest (Chang 2012). *In vitro* experiments performed on the ISS also demonstrated that microgravity promotes mitochondrial dysfunction and apoptosis in human blood peripheral lymphocytes (Battista 2012). In Jurkat cells in space, overexpression of Fas/APO-1, also called CD95, was observed. Fas/APO-1 is a member of TNF family which can activate apoptosis when it binds Fas ligand (Fas-L) (Figure 11). T cells present an anergy state that leads to maintain tolerance to self-antigen. Indeed, CD69, which is a marker of lymphocyte activation, is markedly reduced after long-space mission. The downregulation of INF-ɣ, TNF-α and IL-6 is also described (Crucian 2014). The inactivation of Rel/NF-κB pathway might also contribute to immune dysfunction (Chang 2012).

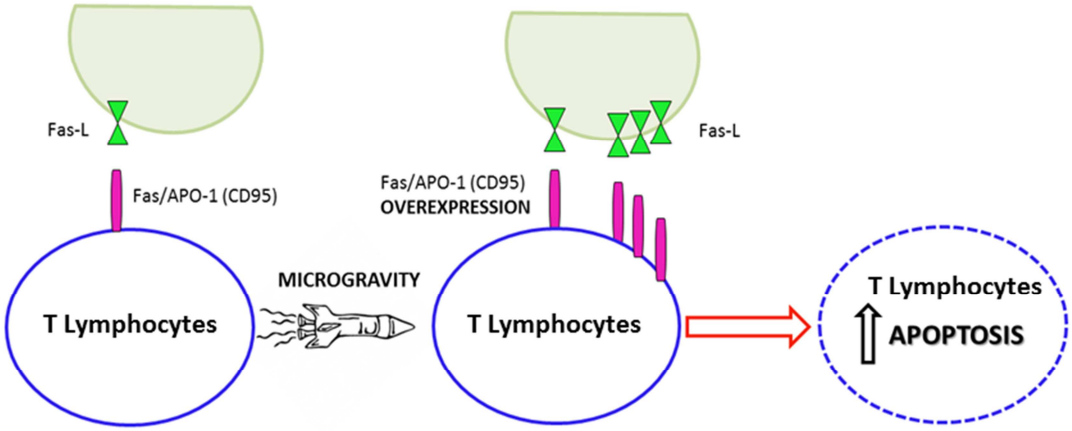


Fig. 11 *Overexpression of Fas/APO-1 in T lymphocytes exposed to real microgravity.*

In monocytes exposed to microgravity a decrease of Jun-N-Terminal kinase (JNK) is observed (Meloni 2006, Meloni 2011), which is responsible for cell death in chronic inflammation and in stress condition. No change of p38 MAP kinase activity was detected (Verhaar 2014). Moreover, in monocytes the increase of different cytokines, such as IL-10, IL-6, IL-1β and IL-8, was reported. The modulation of the expression of Intercellular Adhesion Molecule (ICAM)-1, which is essential for cell-to-cell adhesion, has also been observed in monocytes/macrophages (Paulsen 2015). The increase of ROS production, which has been observed in the majority of mammalian space-flown cells, may, in some cases, compromise cell function and, eventually, survival (Bizzari 2015).

1.1.4 HOW TO SIMULATE MICROGRAVITY ON EARTH: BIOREACTORS

Experiments in real microgravity are difficult to perform. Flight experiments are expensive and experimental apparatus is limited. It is essential that all reagents are safe for the crew. Samples are limited in number. The environment is unique, because of the lack of gravity and cosmic radiations. Moreover, the different g forces, which occur during the launch of the rocket, up to 12 g, should be taken into account. The other possibility of performing experiments in real microgravity is to use parabolic flights and rockets, but the duration of microgravity is limited, the costs are high and the missions are not recurrent (Maier 2015). For all these reasons, several studies have been performed using various bioreactors, which can simulate microgravity (10^{-3} to 10^{-6} g). The use of these bioreactors allows to perform studies in preparation for spaceflight and to deepen the results obtained from experiments on the ISS.

There are different bioreactors, which mimic only some aspects of real microgravity (Herranz 2013).

- *Clinostat*: Clinostats are considered a reasonably effective bioreactor for simulating microgravity. The clinostat is based on one (called 1-D seldom) or two axes (called 2-D clinostat, more common), which move fast and with a constant speed in one direction. 1-D or 2-D refers to the whole area that is involved in rotation (Herranz 2013). To overcome the effects of 2-D, in which omnilateral stimulation does not reproduce fully the lack of gravity, a new clinostat bioreactor the Random Positioning Machine (RPM) has been developed (Figure 12) (van Loon 2007). It is a 3-D clinostat characterized by randomly changing rotation speed and direction (Herranz 2013). The samples have to be localized in the center of the inner axis. In particular, cells have to be seeded into flasks, which are completely filled with media to reduce the risk of shear forces and turbulence during rotation. The use of RPM cannot exclude the vibrations problem. Therefore, the control in 1G-conditions is situated on the base axis of RPM to be exposed to the same vibrations of the sample in simulated microgravity.

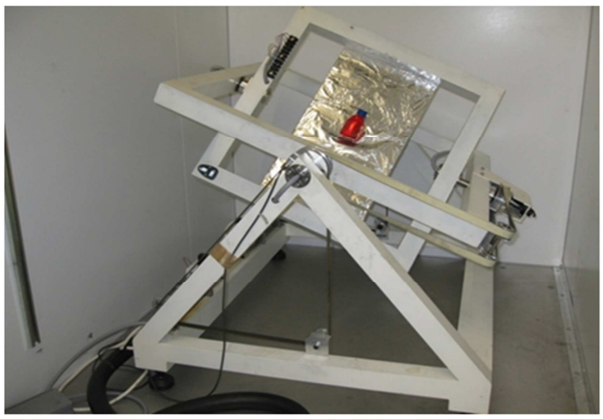


Fig. 12 *3-D Clinostat (RPM) with the flask completely filled with media, located in the center of the inner axis.*

- *Rotating Wall Vessel (RWV)*: RWV was developed by NASA (Figure 13a). The vessel maintains cells in suspension and simulates microgravity by rotation along the horizontal axis. The coaxial tubular silicon membrane for oxygenation allows the growth of cells in physiological conditions. Adherent cells have to be grown on microcarrier beads before the use of the bioreactor (Figure 13c). The vessel has to be filled with media. Rotation at the same speed (28 rpm) produces a vector averaged gravity

comparable with that of lack of gravity. The majority of the results, which were obtained using the RWV, have been confirmed with the experiments in real microgravity (Maier 2015).

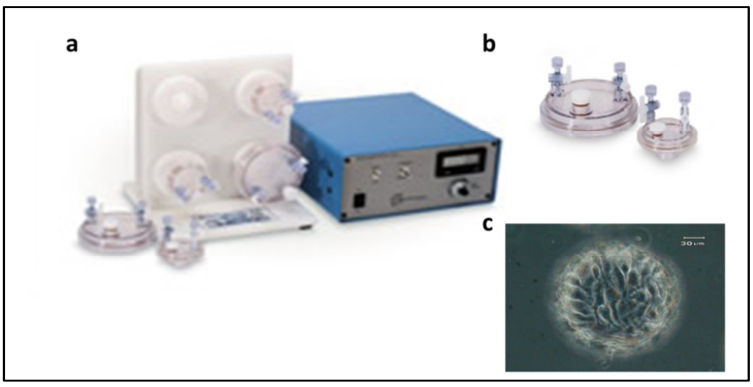


Fig. 13 *a) Rotating Wall Vessel Bioreactor b) Vessel Rotating Cell Culture System (RCCS) from Synthecon c) cells grown on microcarriers beads.*

- *Magnetic Levitation*: Magnetic Levitation is a new method of reproducing microgravity. It is based on magnetic levitation, which occurs when the magnetic and the gravitational forces are counterbalanced (Herranz 2012). In this environment the diamagnetic samples are in a simulated microgravity condition. It is necessary to underline that the magnetic field can affect the behavior of samples during experiments. Some studies have demonstrated similar changes in A431 cells cultured in Magnetic Levitation, random rotation and real microgravity conditions (Moes 2011).

1.2 THE BONE

1.2.1 BONE STRUCTURE

The skeletal system has many important functions in the human body. It is responsible for supporting the body and protecting many of the vital organs, and it allows body movements. The skeletal system participates in endocrine signaling regulating energy metabolism and is the center of hematopoiesis. Cortical bone is the outer side of the bone and is compact (Karsenty 2003). The trabecular bone is the inner part of the bone and is characterized by a unit called Osteon. The Osteon is composed of a central canal, the Haversian canal, which contains blood and nerves and is enclosed by concentric rings of matrix called "lamellae". Between the rings, into the lacune, osteocytes are located and their passage from lacune to hard matrix is allowed by small channel called canaliculi (Figure 14).

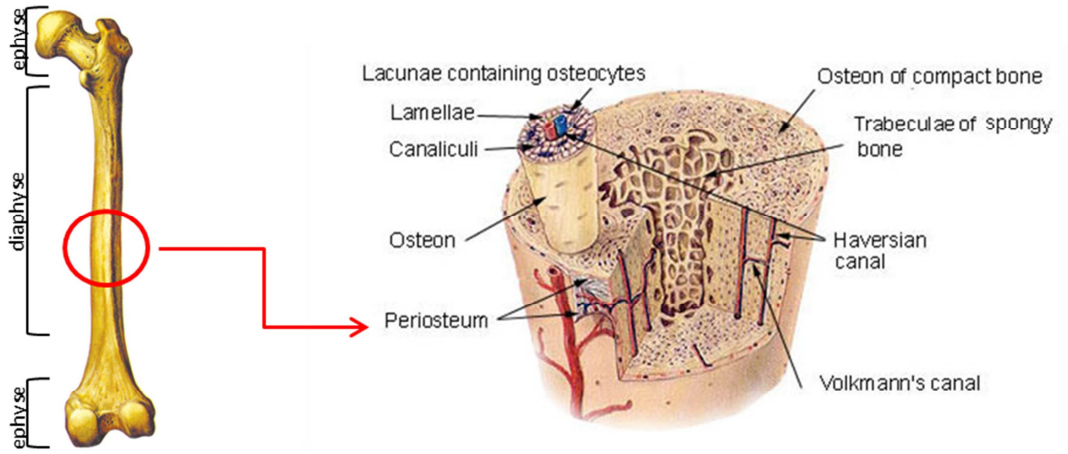


Fig. 14 *Bone anatomy and structure.*

Bone marrow is located in the trabecular bone and also in the large cavities of long bone.

The components of bone extracellular matrix (ECM) are both inorganic and organic. The inorganic component is hydroxyapatite, which is made of calcium and phosphate. The inorganic part is composed of collagenous proteins, in particular collagen type 1 (COL1A1), and non-collagenous proteins such as osteocalcin (OSC), osteopontin (OSP) and glycosaminoglycans (Young 2003). In the bone different cell types are present; i.e. osteoprogenitors, osteoblasts, osteoclasts and osteocytes (Figure 15).

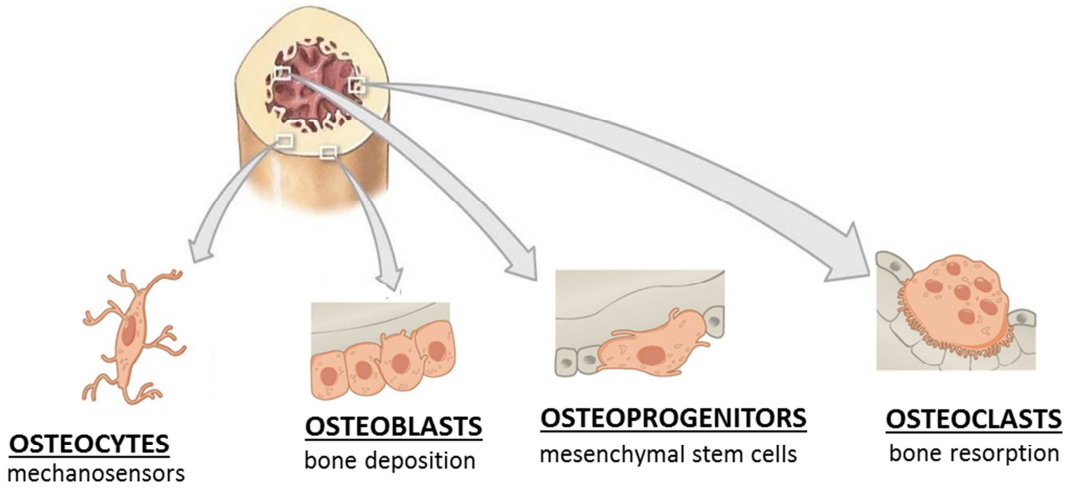


Fig. 15 *Bone cells and function.*

- **Osteoclasts** are the cells involved in bone resorption. They result from the fusion of macrophages. Indeed they are large multinucleate cells. (Suda 1992) (Figure 16). Receptor activator of nuclear factor κ B ligand (RANK-L) and macrophage colony stimulating factors (M-CSF or CSF-1) are the two cytokines required for osteoclast formation. Moreover, M-CSF is necessary also for the proliferation, survival, and differentiation of osteoclast precursors, as well as for osteoclast survival and cytoskeletal rearrangement required for bone resorption (Clarke 2008). The resorption activity is driven by integrins. The most abundant integrin in the bone is the heterodimer $\alpha_v\beta_3$ integrin, which binds osteopontin and bone sialoproteins. After the binding with bone matrix, osteoclasts form podosomes to isolate the site of resorption. Podosome are made of an actin core surrounded by $\alpha_v\beta_3$ integrin and associated with cytoskeletal proteins. Then, osteoclasts, which become polarized, produce acidified vesicles which move to the membrane and ultimately secrete H^+ ions *via* H^+-ATPase and chloride channels. Exocytosis creates an acid environment with leads to the resorption of bone matrix (Clarke 2008).
- **Osteoblasts** originate from Human Mesenchymal Stem Cells (MSC) (Figure 16). Osteoblasts are responsible for the deposition of extracellular matrix and bone formation (Pittenger 1999). Active mature osteoblasts have large nuclei, enlarged Golgi structures, extensive endoplasmic reticulum, and secrete type collagen I and other proteins. Conversely, flat-osteoblasts seem to be quiescent cells required for the

formation of the endosteum on trabecular and endosteal surfaces and underlie the periosteum on the mineralized surface (Clarke 2008).

- **Osteocytes**, the most abundant and long-lived of all bone cells, are terminally differentiated osteoblasts. During differentiation, the cell shrinks, changes from a polygonal shape to a dendritic morphology, forms many cellular projections, and remains embedded in the lacunae within mineralized bone (Figure 16). Osteocytes sense deformation of the bone due to mechanical stress, translate mechanical forces into biochemical signals and trigger the biological responses of osteoblasts and osteoclasts. Among others, mechanically-stressed osteocytes release of prostaglandin E2 (PGE_2) and nitric oxide (NO), thus impacting on Wnt signalling (Clarke 2008). Consequently, osteocytes are emerging as pivotal players in bone homeostasis.

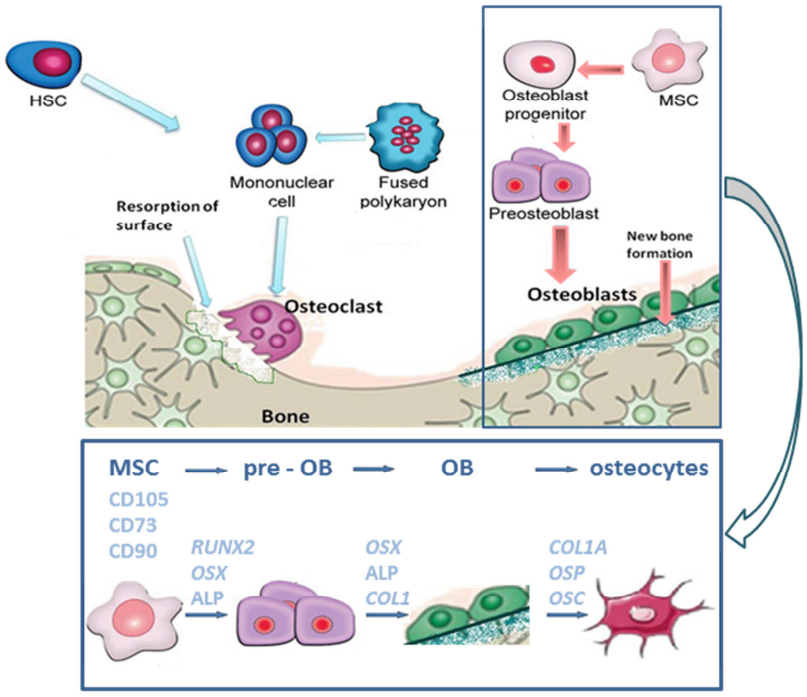


Fig. 16 *Bone cells' differentiation. Osteoclasts derive from hematopoietic stem cells (HSC). Osteoblasts derive from mesenchymal stem cells (MSC). The main genes involved in osteoblast differentiation are shown, from MSC, pre-osteoblasts (pre-OB), osteoblasts (OB) and osteocytes.*

1.2.2 BONE REMODELING

The bone is constantly remodeled. In physiological conditions, bone resorption and bone formation are balanced and osteocytes contribute to the maintenance of this homeostasis by signaling to osteoblasts and osteoclasts (Goldring 2015).

Osteoclastogenesis is strictly controlled by the CSF-1 and RANK-L, signals required for the migration of macrophage from bone marrow or peripheral circulation with the consequent fusion in multinucleate cells. RANK-L is secreted by osteoblasts and binds its receptor RANK on the membrane of pre-osteoclasts. Osteoblasts secrete also osteoprotegerin (OPG) which is a decoy receptor that binds RANK preventing its interaction with its receptor RANK-L. OPG synthesis is stimulated by anabolic agents like transforming growth factor beta (TFG)-β and bone morphogenetic protein (BMP) (Boyle 2003). If RANK-L is overproduced or is not inhibited by OPG, osteoclasts progenitors differentiate in mature osteoclasts, which bind bone matrix via integrins and resorb the bone. Hydrogen ions accumulate, creating an acidic environment that solubilizes the hydroxyapatite while the organic component of the ECM is digested by enzymes (Suda 1992, Teitelbaum 2000, Robling 2006) (Figure 17).

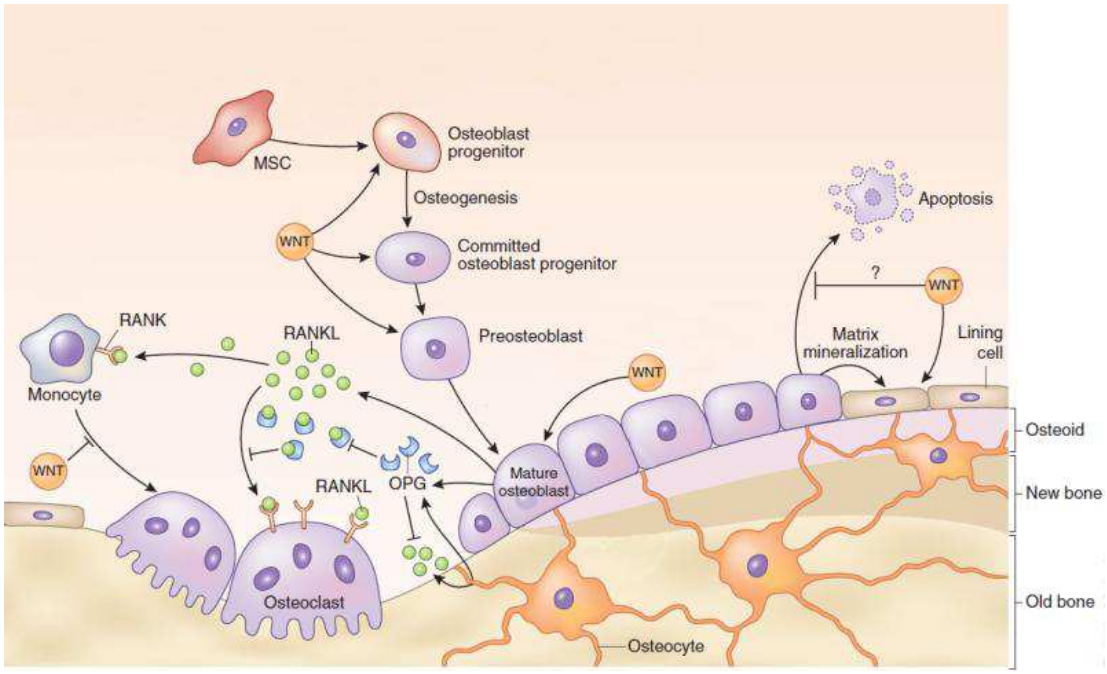


Fig. 17 *Bone remodeling (Bose 2013).*

Osteoblastogenesis requires the Wnt/βcatenin pathway. Activation of Wnt/β-catenin signaling shifts MSC fate toward osteoblastogenesis and inhibits osteoclastogenesis. (Baron and Kneissel 2013). Osteoclasts regulate osteoblastogenesis by degrading the ECM and releasing TGF-β, IGF-1, BMP and other factors that inhibit RANK-L production and therefore activate osteoblasts (Goldring 2015). Osteocytes produce sclerositin and Dikkopf-related protein (DKK)-1 which inhibit the Wnt/βcatenin pathway (Figure 18) (Goldring 2015).

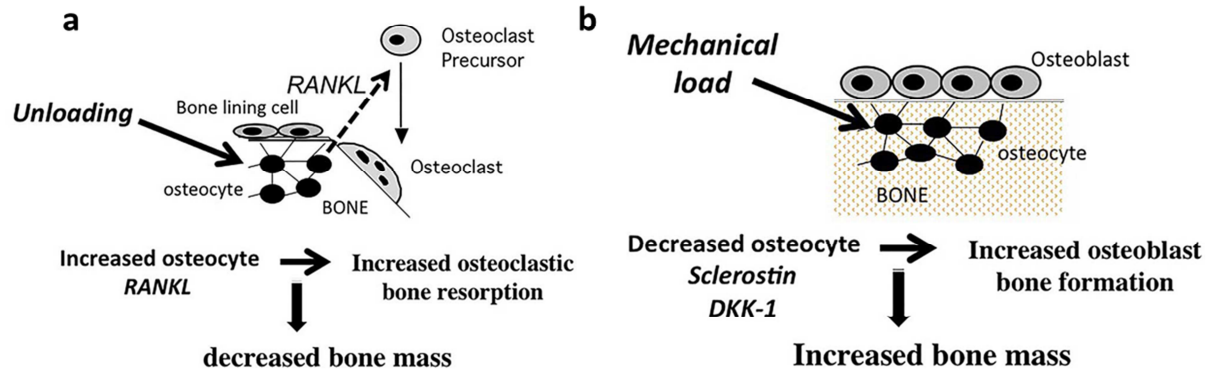


Fig. 18 *a) The increase of RANK-L by osteocytes (and osteoblasts) promotes osteoclast formation b) The decrease of sclerostin and DKK-1 induces osteoblast formation (Goldring 2015).*

The balance between osteoblasts and osteoclasts function is also controlled by hormones, i.e. parathyroid hormone (PTH), calcitonin, vitamin D3 and estrogen. PTH induces the osteoblasts to secrete RANK-L stimulating osteoclast formation. It is the most important factor for calcium homeostasis converting vitamin D3 in its active form, calcitriol (Ben-awadh 2014). Conversely, calcitonin is the antagonist of PTH and reduces blood calcium concentration. Estrogen has different roles in osteoblasts and osteoclasts regulation. It stimulates the production of RANK-L in osteoblasts but, it inhibits osteoclasts' differentiation and increases theier apoptosis (Hadjidakis 2006, Holtrop 1974).

1.2.3 BONE FORMATION

Two processes, endochondral and intramembranous ossification, are necessary for bone formation. Endochondral ossification requires the formation of cartilage and is essential for the formation of long bones. Cartilage is not present in intramembranous ossification, the process necessary for the formation of flat bones, and for bone fracture repair. Skeletogenesis begins with the migration of MSC into the site of the future new bone. MSC then differentiate in chondrocytes or in osteoblasts.

The mechanisms involved in intramembranous ossification are poorly understood. In intramembranous ossification MSC cluster and differentiate into osteoblasts forming an ossification center. They secrete proteins that form the osteoid which in combination with calcium, will form calcified bone (Percival and Richtsmeier 2013).

In endochondral ossification, MSC differentiate in chondrocytes, which proliferate until they become hypertrophic and stop to grow. Chondrocytes are aligned in columns parallel to the

direction of longitudinal growth. In a later phase, the invasion of osteoprogenitors, osteoclasts and endothelial cells from the perichondrium occurs. Some of hypertrophic chondrocytes seem to undergo apoptosis while others become osteoblasts (Berendsen and Olsen 2015). During endochondral ossification, Wnt decreases chondrocytes differentiation and increases the expression of markers of hypertrophic chondrocytes such as RUNX2 and collagen 10A1 (COL10A1) (Dong 2006).

Many factors contribute to endochondral ossification. An important role in proliferation, differentiation and apoptosis is played by Indian Hedgehog (Ihh) and parathyroid hormone-related peptide (PTHrP). Ihh is produced by pre-hypertrophic chondrocytes and stimulates hypertrophy and proliferation in the proliferative zone. It also stimulates the production of PTHrP. PTHrP, conversely, inhibits the transition of proliferative chondrocytes in pre-hypertrophic conditions, thus generating a negative feed-back which allows to control the size and the activity of the chondrocytes in the proliferative zone (Berendsen and Olsen 2015) (Figure 19).

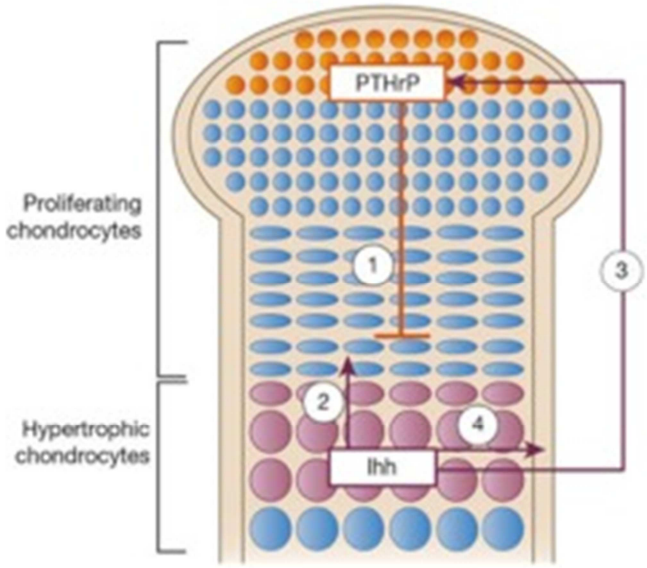


Fig. 19 *PTHrP and IHH act as part of a negative feedback loop regulating chondrocyte proliferation and differentiation. In the upper of the bone, pre-hypertrophic chondrocytes are in continue proliferation stimulated by PTHrP which inhibits the secretion of Ihh (1). Ihh, in turn, stimulates chondrocytes proliferation (2) and the expression of PHrP (3). In addition, Ihh is also responsible for chondrocytes' differentiation into osteoblasts in the bone collar (4) (Wysolmerski 2006).*

1.2.3.4 BLOOD VESSEL IN THE BONE CELLS: MOLECULAR CROSS-TALK

The bone is highly vascularized. Two subtypes of bone capillaries are described: H subtype, which is found in metaphysis, and L subtype, which is located in bone marrow cavity of the diaphysis and forms a dense capillary network (Sivaraj and Adams 2016). During the hypertrophic phase of osteochondral ossification, chondrocytes secrete pro-angiogenic factors thus stimulating angiogenesis, which is characterized by the entrance of vessels into an avascular layer to generate a new vascular network important for the ossification process (Figure 20). The most abundant angiogenic factor produced by chondrocytes is Vascular Endothelial Growth factor (VEGF). Indeed, the inactivation of VEGF impairs chondrocyte survival and growth during bone formation. Hypertrophic chondrocytes and osteoblasts are under hypoxic conditions and overexpress Hypoxia inducible factor (HIF)α, which increases VEGF synthesis and secretion by chondrocytes. Factors driving endochondral ossification including BMP and fibroblast growth factor (FGF) can upregulate VEGF. Also matrix metalloproteases (MMP) play a role in angiogenesis. They are secreted by osteoclasts and endothelial cells in the bone matrix and are responsible for the proteolytic breakdown of ECM, which facilitates endothelial cells migration (Percival and Richtsmeier 2013, Sivaraj and Adams 2016).

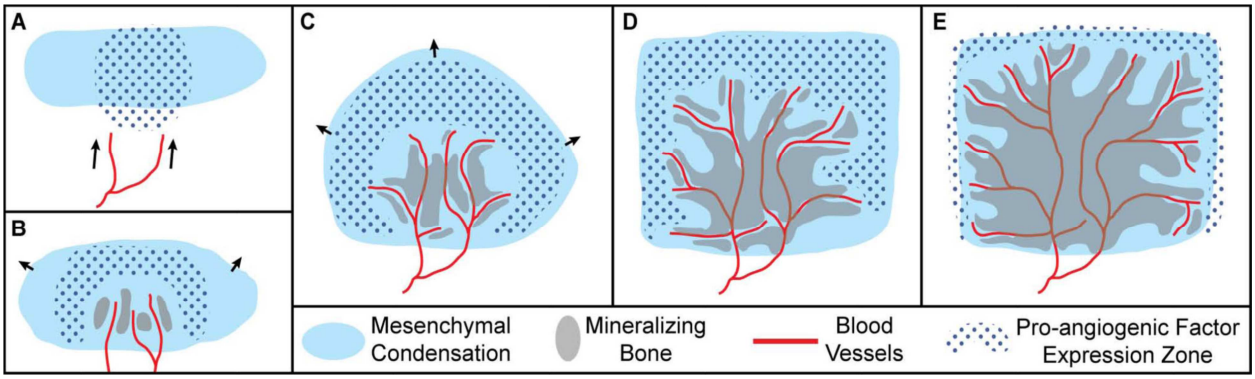


Fig. 20 *Angiogenesis and Mesenchymal stem cells. A) mesenchymal stem cells produce pro-angiogenic factors B) which recruit endothelial cells from neighboring capillaries in the avascular cluster of mesenchymal stem cells. Ossification starts around the blood vessels. C,D,E; ossification, angiogenesis and mesenchymal stem cells continue until they receive the stop signal (Percival and Richtsmeier 2013).*

Angiogenesis is important in bone development. In particular, bone cells and the endothelium cross-talk and influence each other. The upregulation of VEGF by PTH and calcitropic hormone in osteoblasts underscores the important role of the growth factor in bone physiology (Villars 2000). VEGF secreted by osteoblasts stimulates the production of Endothelin-1 by endothelial

cells which can, in turn, regulate osteogenic differentiation inhibiting the expression of osterix (OSX), Alkaline phosphatase (ALP) and OSC (Grellier 2009). The secretion of inflammatory mediators such as IL-1, IL-6, and prostacyclin by the activated endothelium has a role in the communication between osteoblasts and endothelium since they contribute to the regulation of the proliferation, differentiation, function, and survival of osteoblasts and bone resorbing osteoclasts (Villars 2000). *In vitro*, Human Umbilical Vein Endothelial Cells (HUVEC) cultured with MSC enhanced the expression and activity of ALP. In addition, co-culture with HUVEC, osteoblasts are not sensitive to the pro-differentiation activity of dexamethasone, required to induce osteoblast differentiation *in vitro*. Therefore, it is feasible that endothelial cells participate to the modulation of osteogenic differentiation (Kanclezer and Oreffo 2008). Furthermore, M-CSF, RANK-L and OPG are secreted also by endothelial cells. In particular, OPG co-localizes with P-selectin and Von Willebrand factor (vWF) in HUVEC and seems to be secreted in response to inflammatory cytokines such as TNF-α and IL-1β (Zannettino 2005).

The intricate communication between endothelial and bone cells is also supported by the evidence that hypoxia and VEGF upregulate BMP-2 in microvascular endothelial cells (Kanclezer and Oreffo 2008).

1.2.4 MESENCHYMAL STEM CELLS (MSC)

In 1960, Friedenstein's group isolated adherent stromal cells from bone marrow (Väänänen 2005). These cells were named mesenchymal stem cells (MSC). MSC are a group of clonogenic cells that can be found also in peripheral blood, umbilical cord, placenta, and adipose tissue (Zuk 2002, Gimble and Guilak 2003, Kern 2006) and have the ability to differentiate in all mesoderm-type cells: osteoblasts, chondrocytes and adipocytes (Abdallah and Kassem 2008). They are multipotent cells with the capability of self-renewal and a more restricted ability to differentiate (Figure 21).

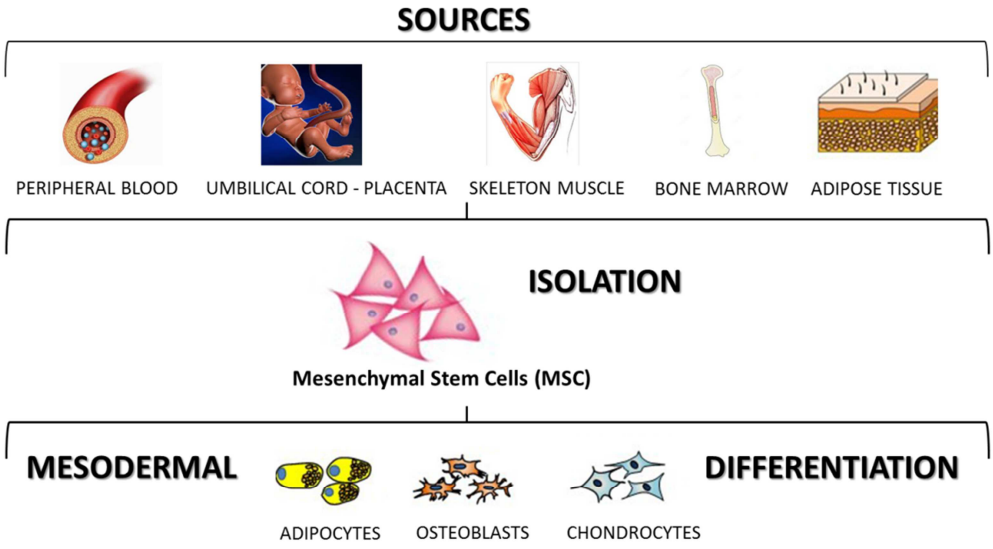


Fig. 21 *Mesenchymal Stem Cell (MSC).*

On the contrary, embryonic stem cells (ECS), which are extracted from the inner cell mass of the blastocyst, are pluripotent cells which can differentiate in the three germ layers (endoderm, ectoderm and mesoderm) (Figure 22) and have unlimited proliferative capability.

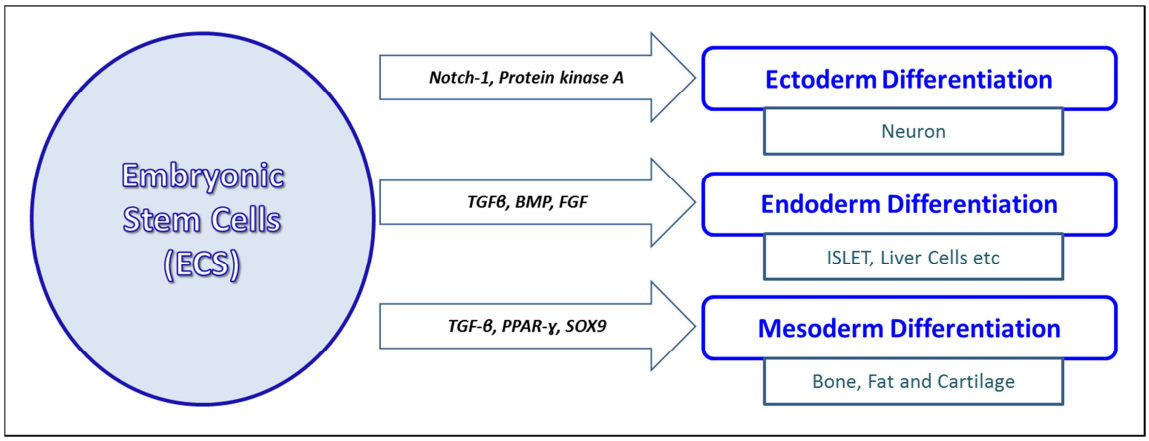


Fig. 22 *Effects of the different pathway of embryonic stem cells (ECS) differentiation. Transforming Growth Factor (TGF); Bone Morphogenetic Protein (BMP); Fibroblast Growth Factor (FGF).*

The use of ECS raises ethical problems that were circumvented by the finding that mature cells can be reprogrammed to become pluripotent - the so-called induced pluripotent stem cells (iPS). iPS are very similar to ECS in morphology, in their ability to differentiate and proliferate. In 2012 the Nobel Physiology and Medicine Prize was awarded for this discovery. To produce these cells, it is necessary to use retroviral or adenoviral transfection of a multiprotein expression vector combined with the piggyback transposon/transposase system to induce the capability of self-renewal. This genetic manipulation represents an obstacle for clinical

application (Woltjen 2009). Attention has then been pointed to MSC. Their extraction from bone marrow and their characterization are easy to maintain, and the maintenance *in vitro* does not require particular precautions.

The International Society for Cellular Therapy (ISCT) indicates three main criteria to define MSC (Dominici 2006):

- MSC have to adhere to plastic.
- MSC have to differentiate *in vitro* towards the adipogenic, osteogenic and chondrogenic cell lineage in response to specific stimuli (Figure 23).
- MSC have to be positive for CD73, CD90 and CD105, and negative for CD34, CD45, CD14 or CD11b, CD79α or CD19 and HLA-DR.

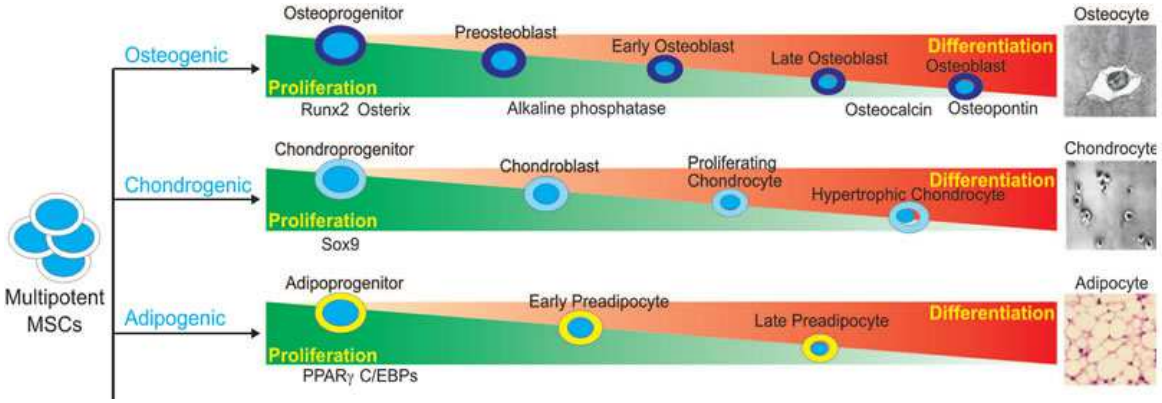


Fig. 23 *In response to specific differentiation-inducing agents, MSC differentiate into osteoblasts, chondrocytes, and adipocytes (Beederman 2013).*

Until now, adipose MSC (AT-MSC) and bone marrow MSC (BM-MSC) have been widely used. In the adipose tissue, MSC are located mainly in the perivascular adventitia (Ryu 2013). In the bone, MSC are found in niches with hematopoietic stem cells (HCS) and, in particular, at the endosteal surface of trabecular bone and in proximity of the endothelial cells of blood vessel (Nombela-Arrieta 2011). The isolation from bone marrow and the separation from HSC are rather easy. From 1mL of bone marrow it is possible to extract up to a billion of MSC that differ from hematopoietic cells because of their ability to adhere in culture dish. MSC are heterogeneous. After extraction, several clones can be detected that differ each other for size, morphology or growth rate. For this reason, after extraction of MSC from bone marrow, it is important to isolate every single clone and to test the three main criteria established by ISCT

(Bianco 2001, Bianco and Robey 2015). Their ability of self – renewal has opened new avenues in clinical application, such as in bone tissue engineering (Kasper 2009, Bianco and Robey 2015) but more studies are needed to ameliorate their clinical application.

❖ CHONDROGENIC DIFFERENTIATION

The main transcription factor responsible for the chondrogenic differentiation is factor sex determining region Y-box 9 (SOX9) (Akiyama 2002). SOX9, together with other SOX transcription factors, i.e. SOX5 and SOX6, induces the expression of proteins essential for the chondrocytic phenotype such as collagen type II alpha 1 (COL2A1) (Ikeda 2004). Several TGF-β isoforms (in particular TGF-β1) and BMPs (mainly BMP2, BMP4 and BMP14) are the main protagonists for the activation of SOX family transcription factors (Danišovič 2012). These proteins form a complex with two types of transmembrane receptors, which lead to the activation of the SMAD-signaling cascade, which induces transcription of chondrogenic genes (Furumatsu 2005). Chondrocyte progenitors can differentiate in cartilage or become hypertrophic cells. When chondrocytes are hypertrophic, there is a decrease of SOX9 and an increase of runt-related transcription factor 2 (RUNX2). Moreover, the cells synthesize collagen X type I (COL10A1) (Dong 2006). The inhibition of Wnt signalling increases early chondrogenesis by up-regulating COL2A1 and SOX9 (Im 2010) (for more details see Figure 23).

❖ ADIPOGENIC DIFFERENTIATION

The main transcription factor gene responsible for adipogenic differentiation is Peroxisome proliferator-activated receptor gamma (PPARγ) (Figure 23). No other factor has been found that can induce adipogenic differentiation in the absence of PPARγ. PPARγ is also required to maintain adipogenic differentiation state (Rosen and MacDougald 2006). CCAAT-enhancer-binding protein α (C/EBPα) is expressed in adipocytes and, with other C/EBP family members, participates in adipogenesis increasing the expression of PPARγ (Wu 1999). Insulin-like growth factor 1 (IGF-1) and several BMPs are stimulators of adipogenesis (Rosen and MacDougald 2006). The main anti-adipogenic factors are glucose transporter type 2/3, which are activated by Wnt pathway, and TGF-β, which inhibits the expression of PPARγ (Rosen and MacDougald 2006).

❖ OSTEOGENIC DIFFERENTIATION

RUNX2 is the protagonist of osteogenic differentiation. It is involved in the regulatory mechanisms of bone cell growth and differentiation, as well as in skeletal homeostasis (Stein 2004). The main activators of RUNX2 are the BMPs (in particular BMP-2, BMP-6, BMP-7 and BMP-9). RUNX2 acts early in the activation of osteogenic differentiation, together with OSX. They are responsible for the production of extracellular matrix. ALP is activated between 5 and 14 days of differentiation and its activity is used to monitor calcium deposition (Birmingham 2012). Other genes required for the full differentiation in osteoblasts are COL1A1, essential for the progression of differentiation at the middle stage, OSC and OSP, which are the most abundant non collagenous components of the bone extracellular matrix (Stein 2004).

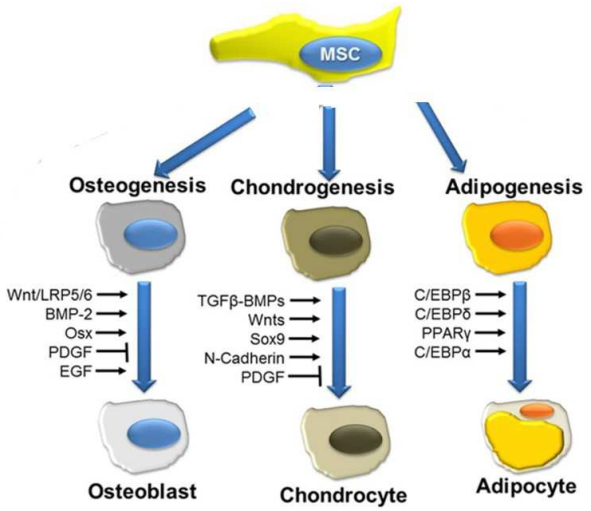


Fig. 23 *Pathways of differentiation of MSC (adapted from Karantalis 2015).*

1.2.5 BONE IN MICROGRAVITY

In the future, astronauts will perform long missions on the Moon or on Mars. To satisfy this goal we need to overcome the side effects of microgravity. The main disorder suffered by astronauts is an accelerated osteoporosis, as a result of an unbalance between osteoblast and osteoclast activity. Thus, microgravity is a challenging environment to study the mechanisms underlying osteoporosis (Cappellesso 2015). On Earth, two main forms of osteoporosis are classified, i.e. primary and secondary osteoporosis. Primary osteoporosis is caused by internal problems, for instance the decrease in the concentration of hormones such as estrogen. Secondary osteoporosis is determined by external factors (Lau and Gau 2011). Space-associated osteoporosis is a secondary form of osteoporosis and is caused by microgravity. The analyses

performed on astronauts revealed the increase of bone resorption and the decrease in bone formation. The calcium balance, which is zero on Earth, decreases to -250 mg/day during space missions. In particular, a decrease of bone mineral density and an increase of resorption markers were observed after 28-84 days. Collagen was abundant in the urine (Smith 1998) but no variation in the undercaboxylated osteocalcin concentration in serum was detected (Zwart 2011). About osteogenic markers, a decrease of OSC and ALP has been detected during space flight in cosmonauts although no hormone variation has been observed apart from one cosmonaut who experienced a decrease of PTH during spaceflight (Calliot-Augusseau 1998). Research on rodents, monkeys and mammalian cells has been performed in space.

In mice 91 days on the ISS decrease Pleiotrophin (PTN), an important marker for osteogenesis involved in bone turnover (Tavella 2012). In rats after 16 days on Cosmos 2044 Biosatellite, a lower amount of COL1A1 has been detected in the distal section of the femur diaphysis (Arnaud 2000). Several other experiments were performed on mice and monkeys in real microgravity demonstrating an increased percentage of mature enlarged osteocytes and resorption activity (Pajevic and Richtsmeier 2013). These data suggest that bone remodelling is altered in various animal models when exposed to real microgravity. Several studies were performed on mice in simulated microgravity using the “tail-suspended” method, which is characterized by forelimbs that are normally loaded while the movement of hindlimbs is free without weight bearing (Sakai 2001). Sakai et al observed a rapid reduction of trabecular bone volume (Sakai 2001). Moreover, after 3 days of simulated microgravity, osteocytes’ apoptosis in trabecular and cortical bone was detected, but it disappeared after 2 weeks of tail-suspension. In the end the number of osteocytes was increased and trabecular and cortical width was reduced (Aguirre 2006). These data are recently confirmed by Dohke et al. detecting an increase of osteoclast activation after 2 weeks in tail suspended. Animals, which returned to normal levels after 4 weeks after weight bearing (Dohke 2016).

Mammalian bone cells were studied in real and simulated microgravity. Space-flown osteoblasts have shown morphological alterations. In particular, real microgravity induces alterations of the cytoskeleton. The cortical actin is thinner and microtubules are shorter than in controls, and the nuclei fragmented or disrupted. The decrease in osteoblast cellular integrity parallels the increase of osteoclasts’ activity (Nabavi 2011). These data were confirmed by Rucci et al. (Rucci 2007), who performed the experiments with primary osteoblasts from mice in simulated microgravity. They detected that the ratio of OPG/RANK-L is altered, because of the

increased secretion of RANK-L in osteoblasts exposed to microgravity. Another pathway affected by simulated microgravity is Wnt/βcatenin pathway. Indeed, the upregulation of sclerostin and Dkk-1 is observed, which means that the Wnt/βcatenin pathway is inhibited. In the OCLAST experiments in real microgravity, Tamma et al. (Tamma 2009) cultured bone marrow macrophages in the presence of M-CSF and RANK-L, specific stimuli for osteoclasts differentiation. They observed that the exposure to real microgravity increases the levels of markers of osteoclastogenesis such as integrin β_3, calcitonin receptor, MMP-9 and catepsin K. Moreover, they measured collagen I telopeptide concentration (CTX), a marker of bone resorption activity, in mature osteoclasts in the presence of M-CSF and RANK-L and found it increased in space-flown cells vs 1G-conditions. Ultrastructure alterations were detected in young osteocytes derived from monkeys exposed for 14 days to real microgravity. The increase of collagen biosynthesis, the fibrotic osteoblast reorganization and the increase in osteocytes activity ultimately result in their death (Rodionova 2002). Aguirre et al. demonstrated an increase of apoptotic phenomena in osteoblasts and osteocytes simulated microgravity with the consequent decrease of bone formation (Aguirre 2006).

The results obtained in osteoclasts and osteoblasts in simulated microgravity confirmed those reported in real microgravity such as the reduction of osteoblasts growth and the alteration of cellular morphology and nuclear structure. In addition, the decreased release of some anabolic factors such as TGF-β is observed, which means inhibititon of osteoblastogenesis (Zhang 2015). Turning our attention to pre-osteoclasts in simulated microgravity, the overexpression of autophagic markers such as Atg5 and LC3-II demonstrates the formation of autophagosome, an event which enhances their survival and increases their activity (Sambandam 2014).

An issue that is still controversial concerns the effects of microgravity on MSC differentiation. Zayzafoon et al. (Zayzafoon 2004) observed that simulated microgravity inhibits MSC osteogenic differentiation and promotes their differentiation in adipocytes. MSC in simulated microgravity cultured in osteogenic medium overexpressed *PPARɣ*, the master regulator of adipogenesis. Similar results were obtained by Saxena et al. who observed the increase of adipogenic differentiation in parallel with the decrease of osteoblast markers such as *RUNX2*, *COL1A1*, *OSP* and *OSC* in MSC cultured in simulated microgravity in the presence of osteogenic medium (Saxena 2007). They also detected cytoskeletal reorganization with disruption of F-actin stress fibres, as confirmed by Mao (Mao 2016).

Since RhoGTPases are involved in the modelling of the cytoskeleton and in normal condition promote osteoblast differentiation (Meyers 2005), it is noteworthy that a decrease of RhoGTPase acitivity was described in MSC in simulated microgravity (Zhang 2015).

In disagreement with these authors, others have shown that simulated microgravity stimulates MSC proliferation and sustains their capability to differentiate along the osteogenic pathway (Yuge 2006). Sakai et al. showed that simulated microgravity potentiates the chondrogenic differentiation of MSC (Sakai 2009). On these bases, bioreactors simulating microgravity have been suggested as a useful tool for tissue engineering (Barzegari and Saei 2012).

1.3 THE ENDOTHELIUM

1.3.1 ENDOTHELIUM, GATE-KEEPER OF VASCULAR INTEGRITY

Endothelial cells (EC) form the internal lining of all the vessels and are crucial for vascular homeostasis (Cines 1998). In addition, they contribute to the regulation of vascular tone, coagulation and fibrinolysis, leukocytes' trafficking, and also participate in inflammatory reactions (Figure 24).

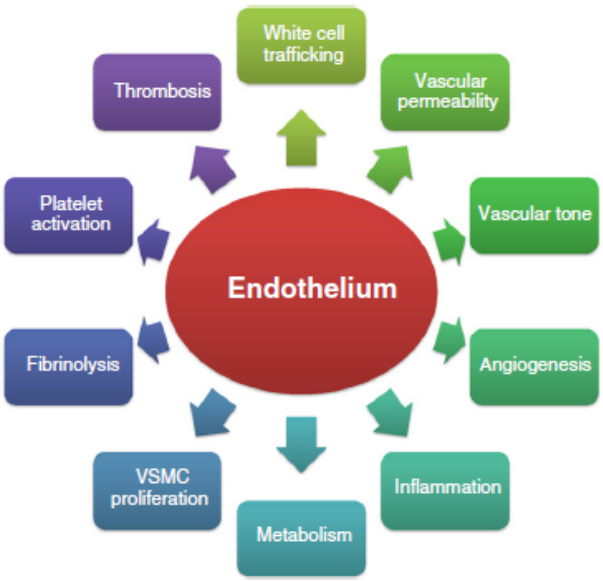


Fig. 24 *Functions of the endothelium (Sena 2013).*

In adults, EC are about 10^{13} and account for about 1 kg (Galley and Webster 2004). Roughly 600 g of them line the capillaries (Hinsberg 2012).

Endothelial and haematopoietic cells derive from haemangioblasts, bipotent blasts which differentiate in pre-endothelial cells that further differentiate in haematopoietic or endothelial cells. Mature endothelial cells express specific markers such as vWF, platelet-endothelial cell adhesion molecule 1 (PECAM-1), endothelial nitric oxide synthases (eNOS) and vascular endothelial-cadherin (VE-cadherin) (Huang 2008, Bai 2010). EC can transdifferentiate in mesenchymal cells and intimal smooth muscle cells (Galley and Webster 2004).

EC are normally quiescent *in vivo* with a turnover rate of approximately once every three years (Foreman 2003). Most of EC in the adult have a cell cycle variable from months to years, unless injury to the vessel wall or angiogenesis occur. In this case, VEGF and Fibroblast Growth Factor

(FGF) induce endothelial proliferation and migration. In particular, VEGF is highly specific for the endothelium.

The endothelium is heterogeneous. Indeed, the morphology and the function of endothelial cells differ depending on the segment and the type of vessel they line and also on the organ where they are located (Regan and Aird 2012). The arteries and the veins are characterized by a continuous layer where the cells are in contact each other through tight junction (Aird 2012). In the capillaries, the endothelium is continuous, fenestrated or discontinuous and this depends on the needs of the surrounding tissue (Hinsberg 2012) (Figure 25).

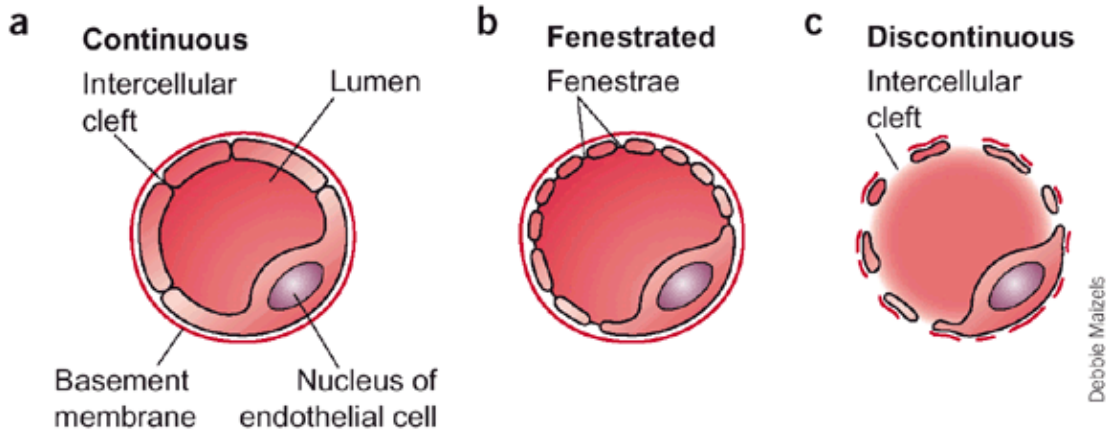


Fig. 25 *Types of capillaries: a) Continuous capillary b) fenestrated capillary c) discontinuous capillary (Cleaver 2013).*

In addition, shear stress and different blood pressure along the vascular tree direct the function and the structure of the endothelium in the arteries, veins, arterioles, post-capillary venules and capillaries (Hinsberg 2012).

1.3.2 ENDOTHELIAL FUNCTION

The concept of endothelium as a passive barrier lining the inner side of blood vessels has been overcome by the finding that EC are involved in a variety of biological responses and physiological functions.

Endothelial cells regulate exchanges between the blood and the surrounding tissues by allowing the passage of micro and macro-molecules through two different pathways, i.e. the para-cellular and trans-cellular pathways. The first one depends on the tight junctions of the EC, which are a selective barrier for the egress of molecules from the blood to the tissues. The para-cellular pathway can be considered as a gate necessary to maintain the polarity of the

cells. The trans-cellular pathway depends on the caveolae, which are vesicle carriers located in the cell membrane and responsible for transcellular transport. Caveolin-1 is a scaffolding protein, which is inserted in the cytoplasmic side of the plasma membrane to regulate caveolar internalization and is responsible for the regulation of nitric oxide (NO) diffusion and calcium signalling (Galley and Webster 2004). These two pathways are variously controlled along the vascular tree. NO is one of vasoactive molecules released by the endothelium to control vasomotor responses. Indeed, the endothelium importantly contribute to the regulation of blood pressure (Figure 26). EC also synthesize endothelin (ET)-1, a potent vasoconstrictor. It is noteworthy that EC also control smooth muscle cells proliferation, thus playing a role in the control of intimal thickness. EC also release molecules that impact on the coagulation and fibrinolytic systems as well as on platelets. In physiological conditions, endothelial cells prevent coagulation through the production of PGI_2, thrombomodulin, heparin-like molecules, NO and lipoxygenase products. Conversely, endothelial cells can be activated by inflammatory cytokines such as TNF-α and IL-1 to upregulate different genes involved in coagulation, among which tissue factor, a crucial mediator of the intrinsic pathway of coagulation. Moreover, EC are involved in the fibrinolytic pathway since they produce tPA, uPA and plasminogen activator inhibitor (PAI) and express the receptor for plasminogen activator.

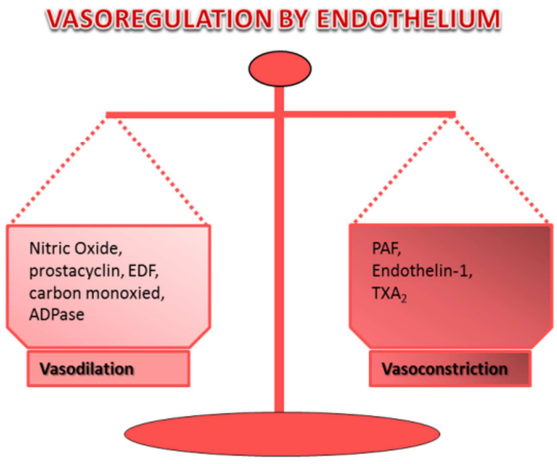


Fig. 26 *Balance between vasodilators and vasoconstrictor factors synthetized by the endothelium.*

EC participate to inflammation and immunological responses. The activation of NF-kB by cytokines, oxidative stress or other stimuli induces the expression of new genes required for the inflammatory response such as leukocytes recruitment (Hinsberg 2012). Through the secretions of specific chemokines and the expression of adhesion molecules, the endothelium

recruits and activates leukocytes. Leukocyte trafficking involves several steps: rolling, adhesion and migration (Figure 27).

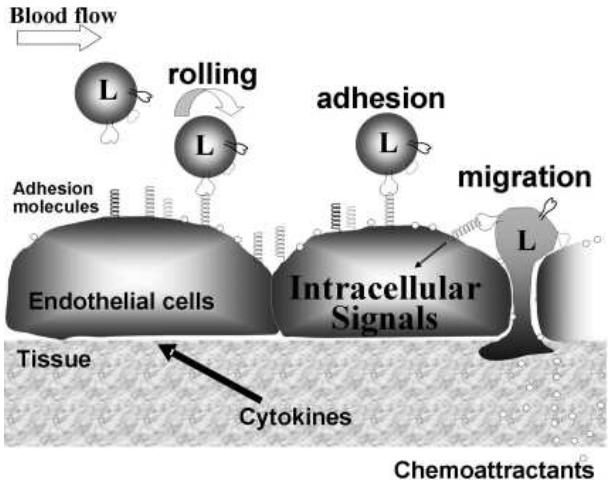


Fig. 27 *Leukocyte trafficking induced by inflammatory cytokines; L = leukocyte (Cook-Mills 2011).*

These steps are regulated by endothelium through the coordinated and finely tuned expression of adhesion molecules such as E-selectin, P-selectin, intracellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 (Aird 2012).

Microvascular EC are protagonists of angiogenesis, the most dynamic function of the endothelium. Angiogenesis occurs either physiologically (e.g. during development and growth, in the proliferating endometrium, in wound healing and repair of bone fractures) or pathologically, particularly in neoplastic and inflammatory diseases. The process of angiogenesis requires the release of angiogenic factors by the surrounding tissues or growing tumor, the migration of EC, their ability to degrade the extracellular matrix, their proliferation and differentiation, ultimately leading to functional capillaries (Galley and Webster 2004) (Figure 28).

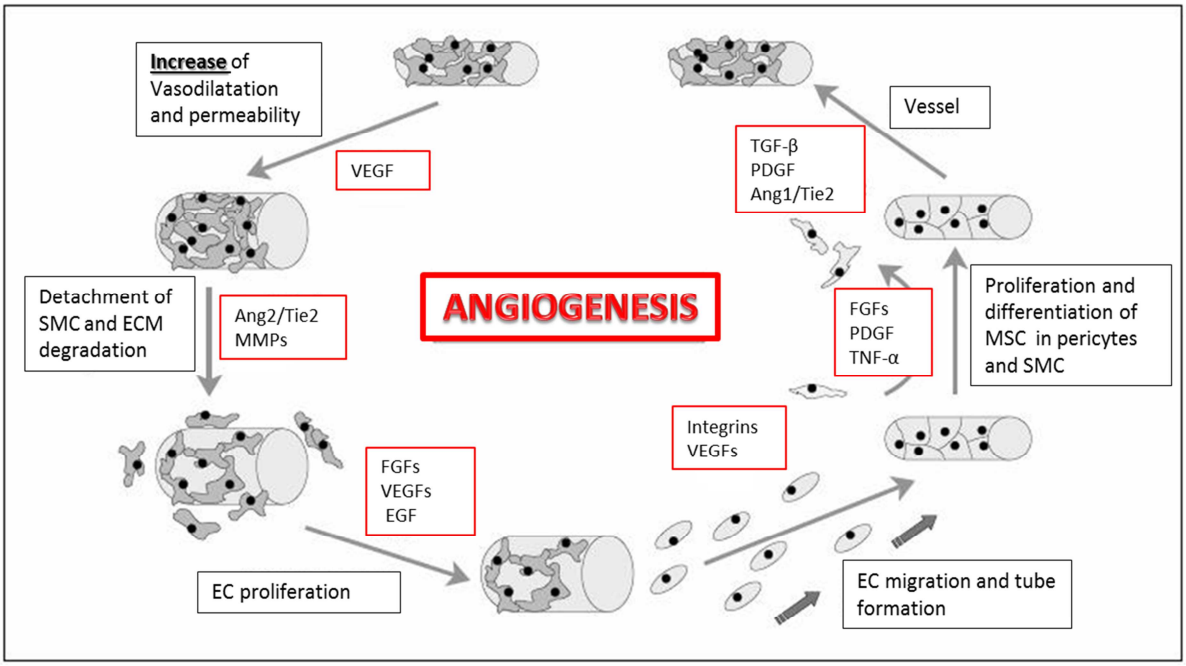


Fig. 28 *Angiogenesis process: principle regulators of angiogenesis are shown. EC, endothelial cells; MSC, mesenchymal stem cells; PC, pericytes; SMC, smooth muscle cells; ECM, extracellular matrix; Ang, Angiopoietin; Tie, Tyrosine kinase with immunoglobulin-like and EGF-like domains; PDGF, Platelet-derived growth factor.*

1.3.3 ENDOTHELIUM IN MICROGRAVITY

Cardiovascular deconditioning is common in astronauts during and after spaceflight and is characterized by cardiac dysrhythmias, cardiac atrophy, orthostatic intolerance. This disorder is due to the reduction of 15-23% of cardiac size after spaceflight (Convertino 2009) and to an altered endothelial function. In search for countermeasures against cardiovascular deconditioning, experiments in real microgravity have been performed using HUVEC, widely considered a model for macrovascular endothelial cells (Kapitonova 2011, Kapitonova 2012, Versari 2013). Space-flown HUVEC showed an irregular surface and variable size and shape. Through fluorescent staining, a lower intensity in mitochondria and in tubulin was detected in comparison with the controls on Earth. Prominent bundles of tubulin-positive structures are accumulated in the peripheral cytoplasmic surface (Kapitonova 2011, Kapitonova 2012). The last spaceflight with HUVEC was performed in 2010 by Versari at al. and investigated the modulation of gene expression by micro-array analysis. The significant upregulation of Thioredoxin Interacting Protein (TXNIP) suggests that HUVEC in real microgravity generate a pro-oxidative environment, since TXNIP inhibits the anti-oxidant function of Thioredoxin (TRX). Moreover, array profiles show the increase of inflammatory response (Versari 2013). Because

of the heterogeneity of the endothelium, also Human macrovascular endothelial cells (HUVEC) and human microvascular endothelial cells (HMEC) were flown to the ISS in late 2015 and studies are in progress. More experiments are needed but, because of the high costs, the limited number of missions and technical restraints of experiments in space, endothelial cells have been widely studied using microgravity simulators on Earth.

Experiments on HUVEC in the RWV and in the RPM yielded similar results (Carlson 2003, Versari 2007). In RPM and in RWV the rate of proliferation is higher and the morphology of the cells is altered. HUVEC in the RPM and in the RWV (Carlsson 2003, Versari 2007) showed a rapid cytoskeletal reorganization associated with reduction in the amounts of actin, which has been explained as an adaptive mechanism to prevent an excess of useless actin fibers (Figure 30).

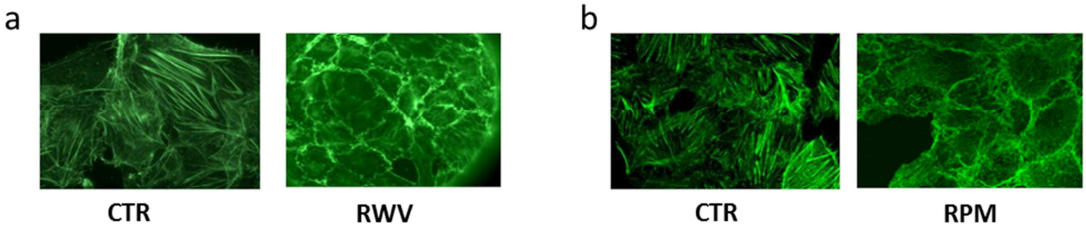


Fig. 30 *Cytoskeletal reorganization. a) alteration of the cytoskeleton in HUVEC exposed to RWV (Carlsson 2003) b) alteration of the cytoskeleton in HUVEC exposed to RPM (Versari 2007). Cells were stained with phalloidin.*

The upregulation of caveolin 1 and eNOS may be connected with the alteration of cytoskeleton, and these events might affect the behaviour of endothelial cells in migration, adhesion and in the production of extracellular matrix (Grenon 2013, Maier 2014). The increase of NO has been observed both in the RPM and in the RWV in several studies (Versari 2007, Carlsson 2003, Grenon 2013) and has been linked to the upregulation of eNOS. Wang et al. found enhanced amounts of NO in HUVEC in simulated microgravity for 24 hours as the result of an increase of inducible NOS through a mechanism dependent on the suppression of the activity of Activator protein (AP)-1 (Wang 2009).

The downregulation of the pro-angiogenic factor FGF-2 and the proinflammatory cytokine IL-1α was observed (Griffoni 2011, Carlsson 2003). The upregulation of Heat Shock Protein (HSP)70 was detected immediately after microgravity exposure. HSP70 blocks the expression of pro-inflammatory cytokines, is responsible for the survival of EC and prevents apoptosis by inhibiting the release of cytochrome c. IL-1α has multiple functions such as the inhibition of cell

growth and its downregulation can explain, in part, the increase of HUVEC proliferation in microgravity (Carlsson 2003, Versari 2007, Maier 2014) (Figure 31).

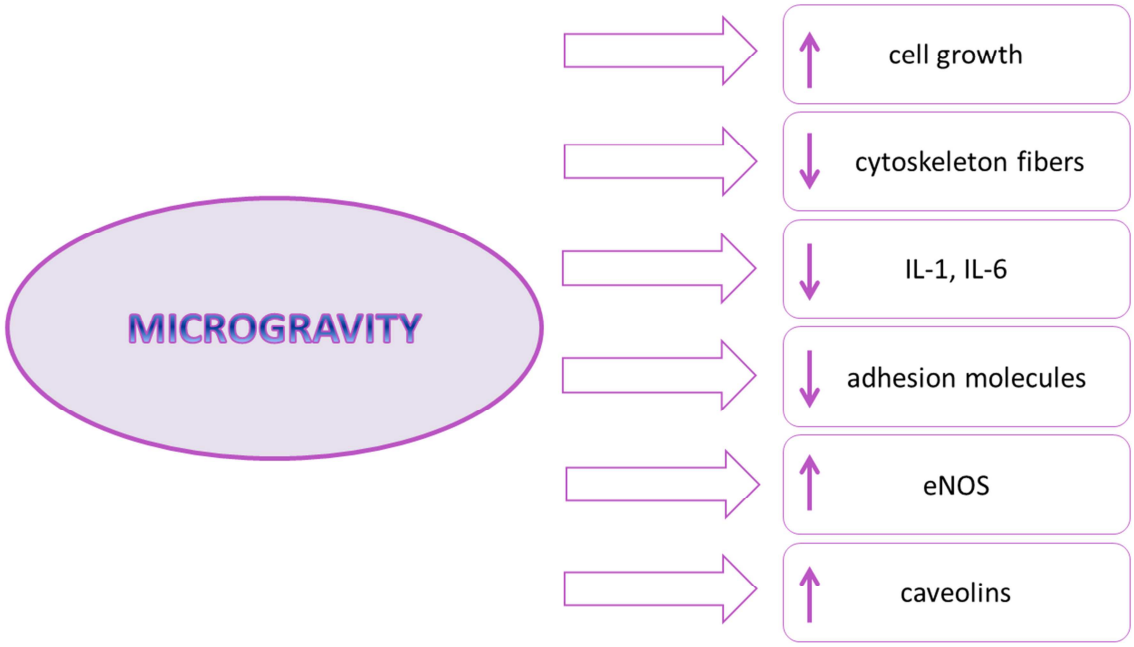


Fig. 31 *Effects of microgravity on human macrovascular endothelial cells (HUVEC).*

Human microvascular endothelial cells (HMEC) exposed to simulated microgravity shown an inhibition of proliferation, which is determined by the upregulation of p21. The upregulation of HSP70 is observed also in HMEC, as in HUVEC, and this protects the cells from apoptosis (Maier2014). The downregulation of IL-6 was described, while NO synthesis is increased (Cotrupi 2005). All these data were confirmed also in the animal model. In rats, microvascular endothelial dysfunction was detected during spaceflight (Vernikos and Scheneider 2009).

Because i) endothelial cells are crucial to maintain the integrity and the function of the vessel wall and ii) vessels are responsible for the vehiculation of oxygen and nutrients to all the tissues, it is pivotal to continue studies on endothelial cell in microgravity since that the alteration of endothelial function can contribute to cardiovascular deconditioning and other disorders observed in space, from bone loss to muscle atrophy.

2. MATERIALS AND METHODS

2.1 CELL CULTURE

Human Mesenchymal Stem Cells (MSC) were isolated from adult human bone marrow of healthy male volunteers via bilateral punctures and were donated by prof. Berti (Policlinico in Milan), according to institutional guidelines approved by the IRCCS Policlinico Milano (donor 1). The cells were tested for purity by flow cytometry (Quirici 2002), they are positive for CD29, CD44, CD105, and CD166 and negative for CD14, CD34 and CD45. MSC were grown in Dulbecco's Modified Eagle's Medium with 1000 mg/L glucose, 10% fetal bovine serum (FBS) and 2 mM glutamine 1 mM penicillin and streptomycin (culture medium, CM). To stimulate the osteogenic differentiation 2 x 10^{-8} M 1α,25-Dihydroxyvitamin D3, 10 mM β-glycerophosphate and 0.05 mM ascorbic acid were added to CM.

Human Umbilical Vein Endothelial Cells (HUVEC) were from American Type Culture Collection (ATCC). They were cultured in the medium M199 (Euroclone) containing 10% FBS, 2 mM glutamine, 1 mM penicillin and streptomycin, 150 mg/mL Endothelial Cell Growth Factor (ECGF), 1 mM sodium pyruvate and heparin (5 units/mL).

Human Microvascular Endothelial Cells (HMEC) were obtained from CDC (Atlanta) and were grown in MCDB131 (Gibco, Thermo Fisher Scientific) containing 10 ng/mL Epidermal Growth Factor (EGF), 1 mg/mL hydrocortisone, 10% FBS, 2mM glutamine, 1 mM penicillin and streptomycin.

Normal human osteoblasts (NHOst) (Lonza) and Saos-2 (ATCC) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Euroclone) containing 10% FBS, 2 mM glutamine, 1 mM penicillin and streptomycin. All the reagents were from Sigma-Aldrich.

All cell lines were cultured at 37°C and 5% of CO_2.

2.2 DANIO RERIO

Danio Rerio embryos used for each experiment were obtained by natural spawned and natural staging by crossing of isolated male and female couples using dedicated nursery tanks (Pasqualetti 2013).

2.3 CELL CULTURE IN SIMULATED MICROGRAVITY

2.3.1 RANDOM POSITIONING MACHINE (RPM)

Simulated microgravity was performed using RPM. Confluent MSC were cultured in T25 flasks. The flasks had to be completely filled with the media added with HEPES 12.5 mM. The samples in simulated microgravity were positioned as close as possible to the center of the inner rotating frame of the machine, while the controls in 1G-conditions were positioned on the basis of the machine. The experiment were performed at different time points (24 h – 4 days – 10 days).

2.3.2 ROTATING WALL VESSEL (RWV)

Before the use of RWV, HMEC and HUVEC have to be seeded on beads (Cytodex microcarrier beads from Sigma-Aldrich).

The cells grown on the beads were then moved into specific vessel which rotate around a horixontal axis at 28 rpm reproducing 10^{-3} to 10^{-6} g. The controls were positioned in the vessels which not undergoing rotation. Both RWV sample and the control were cultured at 37°C and 5% CO_2. The experiment was performed at different time points.

2.3.3 EXPERIMENTAL UNIT (EU)

The experimental units were developed by Kayser Italia and were used to perform experiments in real microgravity. EU are composed of a brick of biological compatible plastic (PEEK®) with a cell culture chamber, five cylindrical reservoirs to store media and chemicals, and a fluidic pathway for exchanging fluids. The culture chamber is designed to accommodate cells cultured in monolayer on 2.3 cm^2 Thermanox coverslips (Nunc, Roskilde, Denmark) with 1.3 mL of culture medium. Each reservoir has a piston which injects fresh fluids into the culture chamber so that the wasted fluids are collected in the empty cylinder (Figure 32).

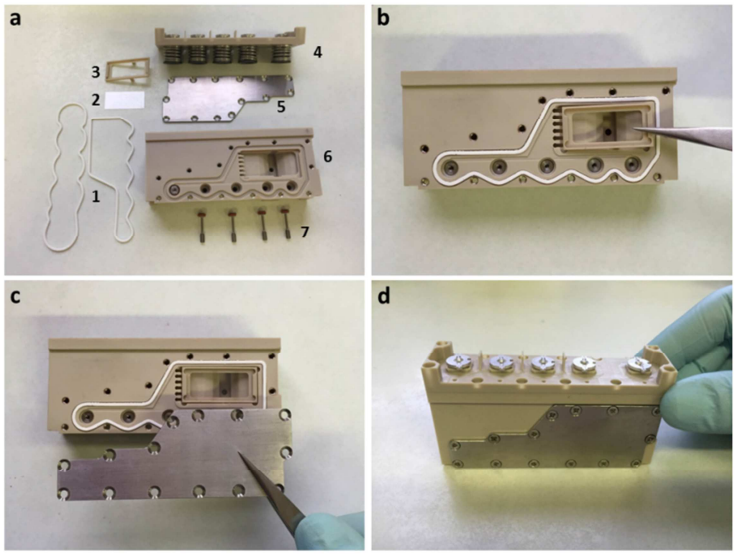


Fig. 32 *Experimental Unit (EU). EU and its components are shown before (a), during (b-c) and after (d) the assembling. (a). Gaskets (1) prevent contamination and liquid leakage; Thermanox coverslip (2) are used to culture the cells and are inserted into the Thermanox coverslip support (3) which is then assembled into the main body (6) and covered with the lateral cover (5); pistons (4) compress the fluids and valves (7) regulate the exchange of fluids.*

These EU were used to evaluate their possible effects on MSC, HUVEC and HMEC behaviour. The experiments were performed at different points from 24 h to 96 h. The medium was added of HEPES 12.5 mM. To perform the experiment the protocol in Figure 33 was set up. The change of reagents has to be manual. After two washes with phosphate buffered saline (PBS) solution, the cells were fixed with RNA later, for RNA extraction, otherwise cells are maintained in PBS solution for cell counting and ROS detection or they are fixed with 4% paraformaldehyde (PFA) for electronic microscopy evaluation.

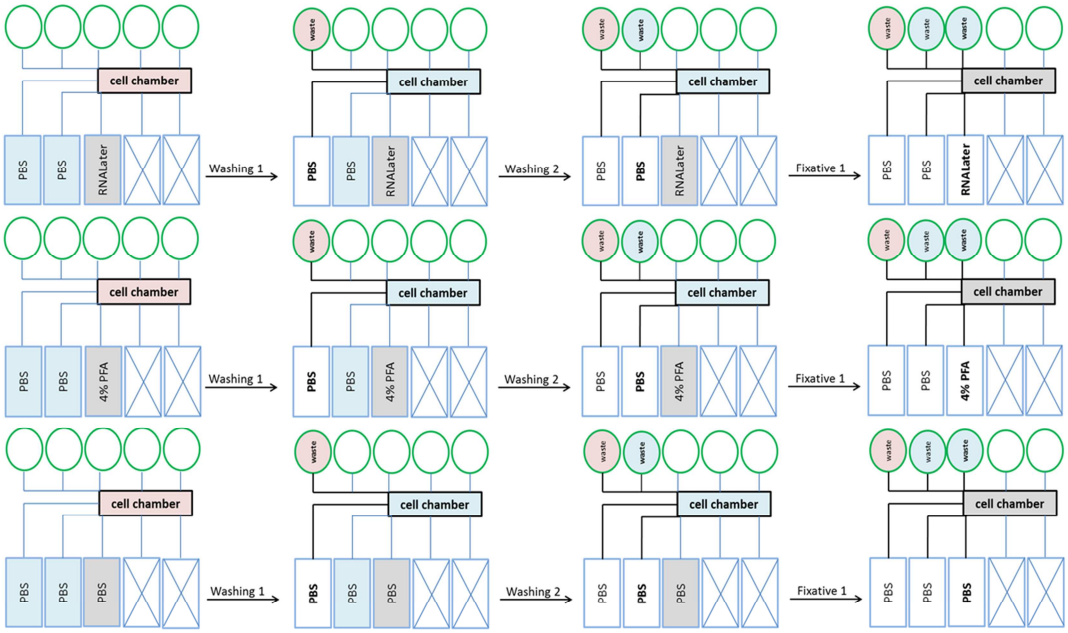


Fig. 33 *Protocol system: exchanges of washing and fixative solution using EU.*

2.4 CELL PROLIFERATION

HMEC, HUVEC, MSC and Saos-2 were trypsinized and the viable cells counted using an automated cell counter or a Burker chamber after staining with trypan blue solution (0.4%).

MTT assay and Neutral Red analysis were performed to evaluate cells viability. MTT assay was performed on NHOst and Saos-2 at 50% of confluence in 96-wells plate for 24 h before being exposed for different times to the media collected from HMEC. Cell viability is directly proportional to the formazan crystal generated by the reduction of yellow tetrazolium salt by succinate dehydrogenase located mainly in the mitochondria. Formazan crystal production is detected at 550 nm wavelengths. Neutral Red analysis, cells were cultured in 96 –wells plate from 24 to 72 h. Cell viability is detected through the ability of live cells to incorporate Neutral Red into lysosome. Absorbance was measured at 550 nm.

2.5 APOPTOSIS

HMEC cell death was evaluated using the cell death detection ELISA (Roche) which determines cytoplasmic histone-associated DNA fragments. Briefly, after 48 and 72 h in the RWV or under 1G-conditions, the cells were lyzed, centrifuged and the supernatant was analyzed according to the manufacturer's instruction. As a positive control, HMEC were exposed for 30 minutes to H_2O_2 (10 µM) and were cultured for additional 48 h in their growth medium.

2.6 CELL MORPHOLOGY

Hematoxylin-eosin staining was performed on MSC. The cells were fixed with 4% PFA for 10 minutes and washed with PBS three times. After adding hematoxylin for 3 minutes, the cells were dyed with eosin, dehydrated with gradient ethanol, soaked with xylene and mounted with neutral balsam. Stained cells were photographed with a Zeiss Imager M1 microscope equipped with the AxioCam MRc5 camera using AxioVision 4.6 software.

2.7 REACTIVE OXYGEN SPECIES PRODUCTION (ROS)

ROS production was evaluated on MSC, HUVEC and HMEC in EU and in HUVEC after RWV exposure. The cells were trypsinized, resuspended and incubated for 30 minutes with 20µM of the fluorescent probe 2'-7'-dichlorofluorescein diacetate (DCFH). Fluorescence emission is detected at 529 nm.

2.8 OSTEOBLAST ACTIVITY

Osteoblast activity was evaluated quantifying alkaline phosphatase (ALP) enzymatic activity. The quantification was performed on NHOst and Saos-2 cells at 80% of the confluence in 24-wells plate. The absorbance was measured at 405nm.

The deposition of calcium was observed on NHOst, Saos-2 and MSC cells by Alizarin Red S (pH 4.2). The absorbance is measured at 562nm.

2.9 ELISA

ELISA from GE Healthcare was used on HMEC media both from simulated microgravity and in 1G-conditions according to the manufacturer's instructions. The increment of the concentration of tissue inhibitor of matrix metalloprotease (TIMP)-2 and interleukin (IL)-6 was quantified by interpolation from a standard curve.

2.10 Real Time PCR

Total RNA was extracted by the PureLink RNA Mini kit (Ambion, Thermo Fisher Scientific) from MSC and HUVEC. cDNA is reverse-transcribed using High Capacity cDNA Reverse Transcription Kit, with RNase inhibitor (Applied Biosystems, Thermo Fisher Scientific) according to the manufacturer's instructions. Real-Time PCR is performed three times in triplicate on the 7500

FAST Real Time PCR System instrument. TaqMan Gene Expression Assays (Life Technologies) was used on MSC: Hs00231692_m1 (*RUNX2*), Hs01866874_s1 (*OSX*), Hs00164004_m1 (*COL1A1*), Hs01587814_g1 (*OSC*) and Hs00959010_m1 (*OSP*).

On HUVEC TaqMan Gene Expression Assays (Life Technologies) was utilized: Hs00197750_m1 (*TXNIP*), Hs00247263_m1 (*SIRT2*), Hs00165563_m1 (*PON2*), Hs00167309_m1 (*SOD2*), Hs00359147_s1 (*HSP1A1*). All the genes were normalized with the housekeeping gene Hs99999905_m1 (*GAPDH*).

The modulation of genes expression was calculated by the $2^{-\Delta\Delta Ct}$ method.

2.11 WESTERN BLOT

Western Blot was performed on HMEC and HUVEC at different time points. The cells were lysed in lysis buffer (Tris-HCl 50mM PH 7.4, 1% of NP40, NaCl 150 mM, 0.25% of NaDeoxycholate). Protein concentration was determined using the Bradford protein assay (Bio-Rad). Cell extracts (80 µg/lane) were separated on SDS-PAGE, and transferred to nitrocellulose sheet at 400mA for 2 h. Western blot analysis was performed using several antibodies: p21 (Tebu Bio-Santa Cruz), p53 (Tebu Bio-Santa Cruz), TXNIP (Invitrogen), SIRT2 (Invitrogen), PON2 (Invitrogen), HSP70 (Tebu Bio-Santa Cruz), GAPDH (Tebu Bio-Santa Cruz). Secondary antibodies were labelled with horseradish peroxidase (GE Healthcare). Danio Rerio embryos were centrifuged and lysated. Protein concentration was determined using the Bradford protein assay (Bio-Rad). Extracts (80 µg/lane) were separated on SDS-PAGE, and transferred to nitrocellulose sheet at 400mA for 2 h. Western blot analysis was performed using antibody HSP70 (Tebu Bio-Santa Cruz). The SuperSignal chemiluminescence kit (Pierce) was used to detect immunoreactive proteins. Densitometric analysis was performed by ImageJ software calculating ration between the protein of interest and GAPDH on three separate experiments.

2.12 PROTEIN ARRAY

Human stress protein array (R&D systems, Space Import Export) was performed on MSC and HUVEC after exposure to simulated microgravity. Cell lysates (80 µg) were incubated with the membrane on which 26 antibodies against human cell stress-related proteins were spotted in duplicate. The array was performed according to the manufacturer's instructions.

Human Inflammatory array (RayBiotech) was performed on HUVEC after exposure to simulated microgravity. Cell lysates (80 µg) and concentrated conditioned media were incubated with the

membrane on which 40 antibodies against human cell inflammatory proteins were spotted in duplicate. The array was performed according to the manufacturer's instructions. Densitometry was performed by ImageJ and the measurements performed according to the manufacturer's instructions.

2.13 STATISTICAL ANALYSIS

All data were representative of at least three separate experiments. Densitometric analysis was performed by the ImageJ software on different blots and expressed using an arbitrary value scale. Results are shown as the mean ± standard deviation. Statistical significance was determined using the Student’s t test and set up at p values less than 0.05. In the figures *P≤0.05; **P≤0.01; ***P≤0.001.

3. RESULTS

3.1 DOES MICROGRAVITY IMPACT ON THE CROSS-TALK BETWEEN MICROVASCULAR ENDOTHELIAL CELLS AND OSTEOBLASTS?

Bone is a highly vascularized tissue, and endothelial cells are important to maintain bone health not only because they allow the nourishment of the tissue but also because they release molecules that affect bone cells.

Because endothelial cells are sensitive to microgravity (Carlsson 2003, Versari 2007, Mariotti 2008; Versari 2013), it is feasible that altered endothelial function in microgravity impacts on osteoblasts. To investigate this topic, HMEC were used.

Initially the behaviour of HMEC in the RWV was investigated. HMEC were cultured in RWV for different times (24 – 48 – 72 – 96 h). Cell proliferation was retarded in simulated microgravity. Growth inhibition correlated with the upregulation of p21 (WAF1), an inhibitor of cyclin-dependent kinases, in a p53-indipendent fashion (Figure 34).

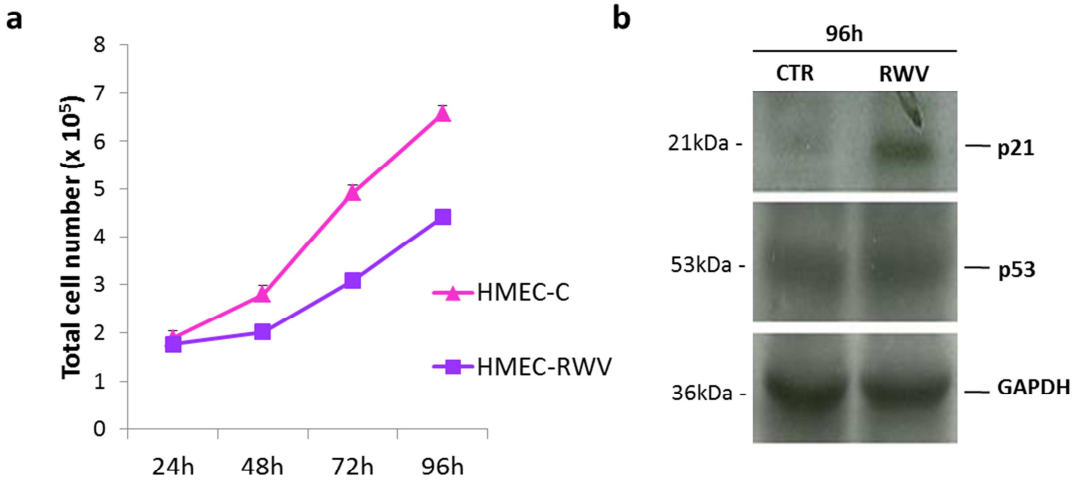


Fig. 34 *Cell growth and p21 and p53 levels in HMEC cultured on RWV and 1G-conditions. a) Cell proliferation was performed on HMEC in simulated microgravity (HMEC-RWV) and 1G-conditions (HMEC-C). Viable cells were counted using a Burker chamber. Data are expressed as the mean ± standard deviation of three separate experiments in triplicate. b) Cells lysates (50µg/lane) were analysed by western blot using antibodies against p21 and p53. The levels of p21 and p53 were visualized by chemiluminescence. Antibody against GAPDH was used as a control of loading.*

HMEC death was evaluated by an ELISA kit (Roche). Hydrogen peroxide (H_2O_2) was used as a positive control. As shown in Figure 35, no apoptosis was detected in the samples both in

simulated microgravity and in 1G-conditions, while, 30 minutes exposure to H_2O_2 are sufficient to induce apoptosis.

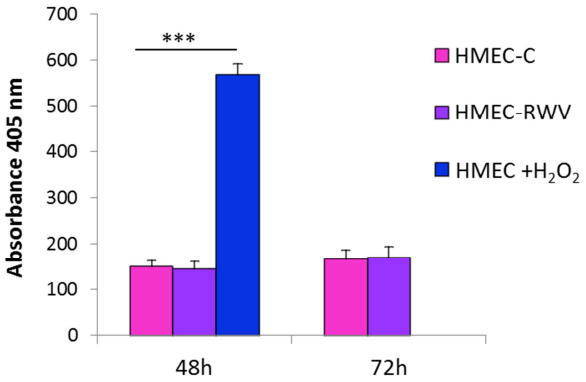


Fig. 35 *Apoptosis in HMEC cultured on RWV and 1G-conditions. Apoptosis was evaluated by an ELISA kit (Roche) after 48 h and 72 h. Positive control is represented by HMEC exposed for 30 minutes to H_2O_2. Data are expressed as the mean ± standard deviation of three separate experiments in triplicate.*

The lack of apoptosis in cells in the RWV associates with the upregulation of HSP70 which was detected after 48 h in simulated microgravity and maintained up to 96 h (Figure 36). It is known that HSP70 sustains cells survival preventing apoptosis (Zhu 1996).

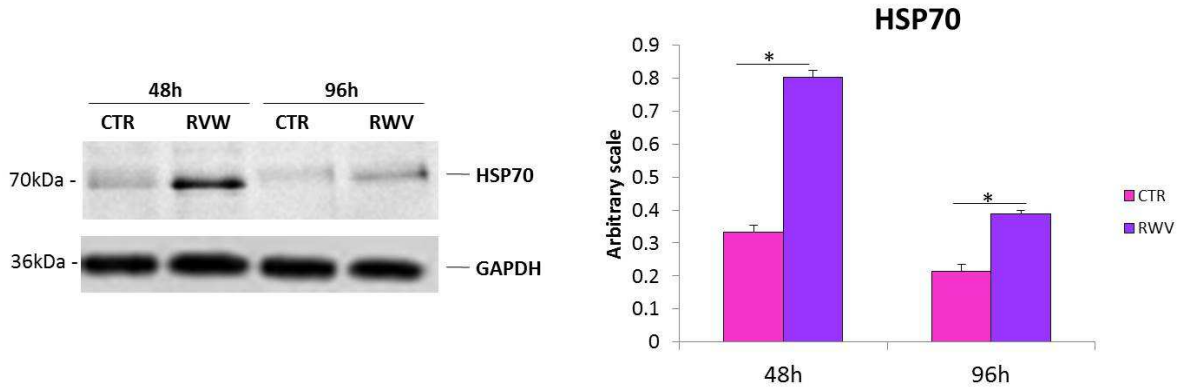


Fig. 36 *HSP70 in HMEC cultured on RWV vs 1G-conditions. Cell lysates (80µg/lane) were analysed by western blot using antibodies against HSP70 and GAPDH. Densitometric analysis was performed by the ImageJ software and HSP70/GAPDH ratio was calculated on three blots from separate experiments ± standard deviation.*

Conditioned media (CM) of HMEC were collected and analysed by protein array on 40 proteins involved in inflammation and angiogenesis. Protein array demonstrated the increase of TIMP-2 and IL-6 was detected in conditioned media from RWV (CM-RWV). This result was confirmed by ELISA (GE Healthcare) (Figure 37).

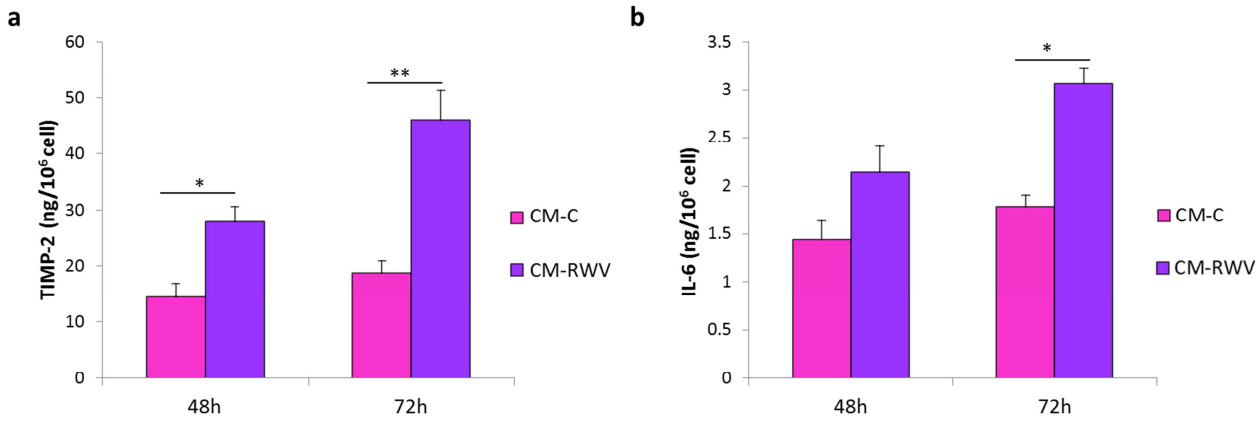


Fig. 37 *<u>Cytokines in HMEC cultured in RWV vs 1G-conditions.</u> ELISA (GE Healthcare) was used to measure cytokines in conditioned media (CM) of HMEC from 1G-conditions (CM-C) and RWV exposure (CM-RWV). CM were collected after 48 and 72 h in RWV or 1G-conditions. Data are expressed as the mean ± standard deviation of three separate experiments in triplicate.*

CM were collected from HMEC after 72 h in the RWV, centrifuged, filtered through 0.2 µm filter, diluted 1:1 with fresh culture medium to replenish nutrients and used to culture human primary osteoblast NHOst for different times. MTT and Neutral Red assay revealed a significant reduction of NHOst cell proliferation after exposure to conditioned media from HMEC in simulated microgravity (Figure 38).

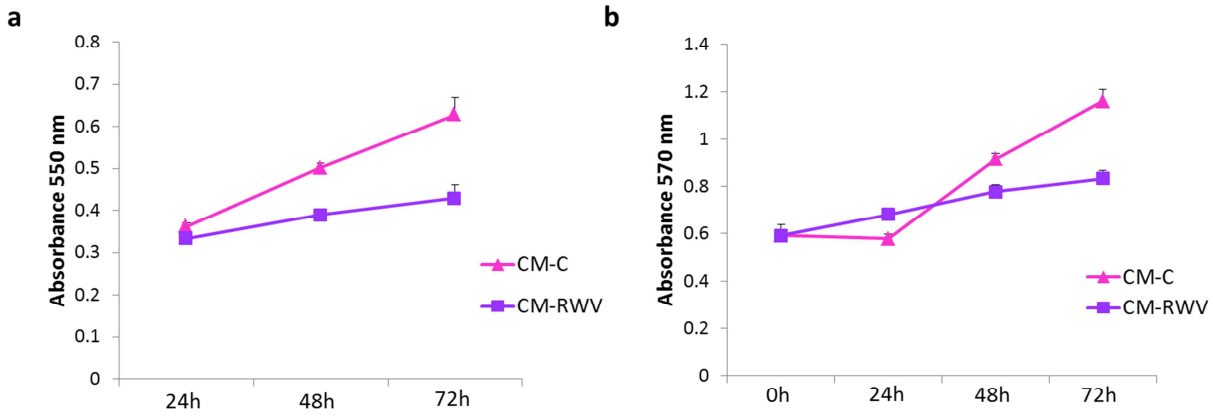


Fig. 38 *<u>NHOst viability.</u> MTT (a) and Neutral Red (b) were performed on NHOst cultured with CM-C and CM-RWV collected from HMEC. Data are expressed as the mean ± standard deviation of three separate experiments in triplicate.*

To evaluate osteoblastic activity, confluent NHOst were cultured in a 24-well plate with conditioned media deriving from HMEC in the RWV (CM-RWV) and relative controls (CM-C) additioned with 50 µM L-ascorbate-2-phosphate and 10 mM glycerophosphate. Osteoblastic

activity was evaluated by measuring alkaline phosphatase (ALP) activity using a colorimetric assay based on the hydrolysis of P-nitrophenyl phosphate and by detecting calcium deposition after staining with Alizarin Red. Figure 39 shows that media from HMEC in simulated microgravity inhibited NHOst activity.

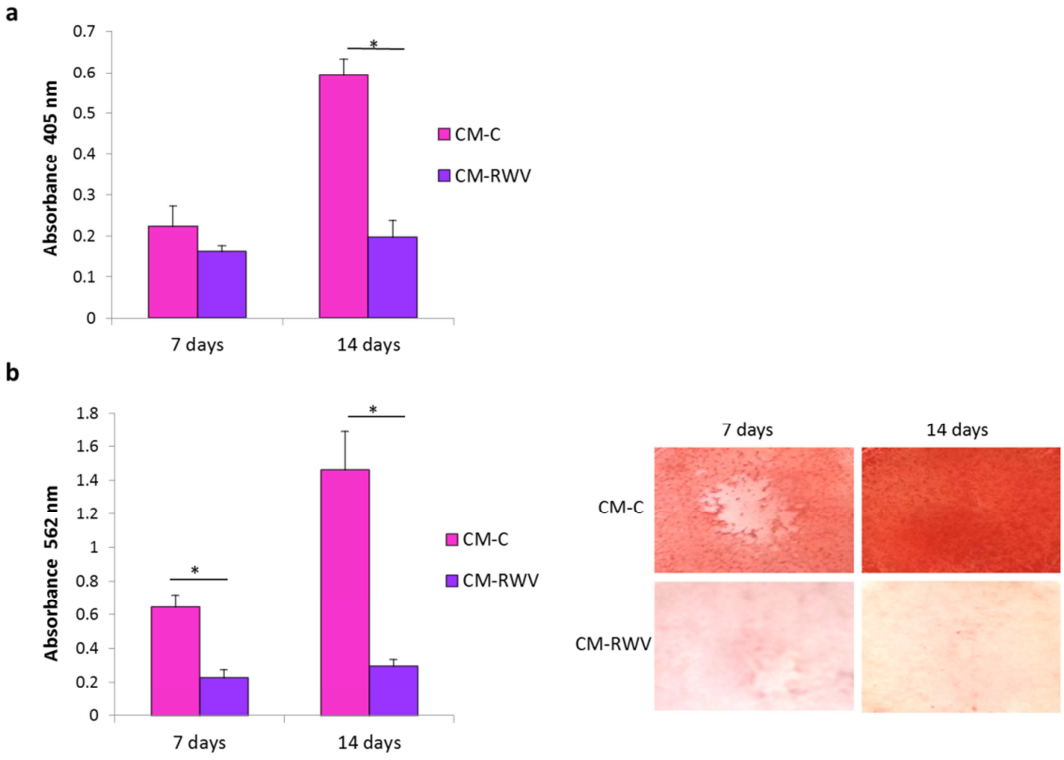


Fig. 39 *<u>NHOst acitivy.</u> a) ALP activity in NHOst cells exposed to CM and CM-RWV. b) Alizarin Red S staining was performed and quantified after acid extraction. Absorbance was measured at 562 nm. Photographs were taken before acid extraction. Data are expressed as the mean ± standard deviation of three separate experiments in triplicate.*

Since gender, age and site of isolation influence the performance of primary osteoblasts, experiments were performed also on the immortalized cell line Saos-2. These cells are used as representative of primary osteoblasts (Saldaña 2011).

Figure 40 and 41 show that Saos-2 exposed to the CM of HMEC in simulated microgravity perform very similarly to NHOst.

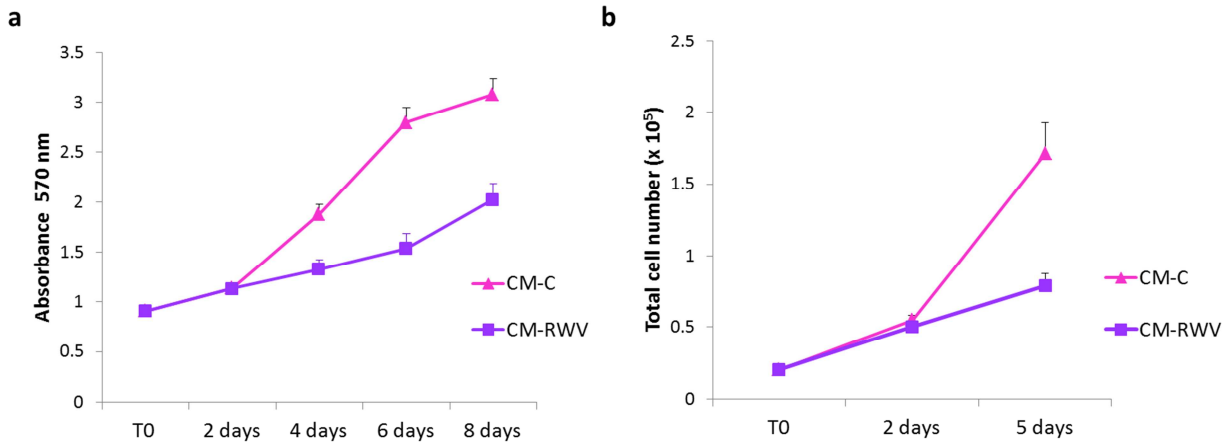


Fig. 40 *<u>Saos-2 viability and growth.</u> Cell viability was detected by MTT assay (a) and cell growth was evaluated by cell count using a Burker chamber as described (b). Data are expressed as the mean ± standard deviation of three separate experiments in triplicate.*

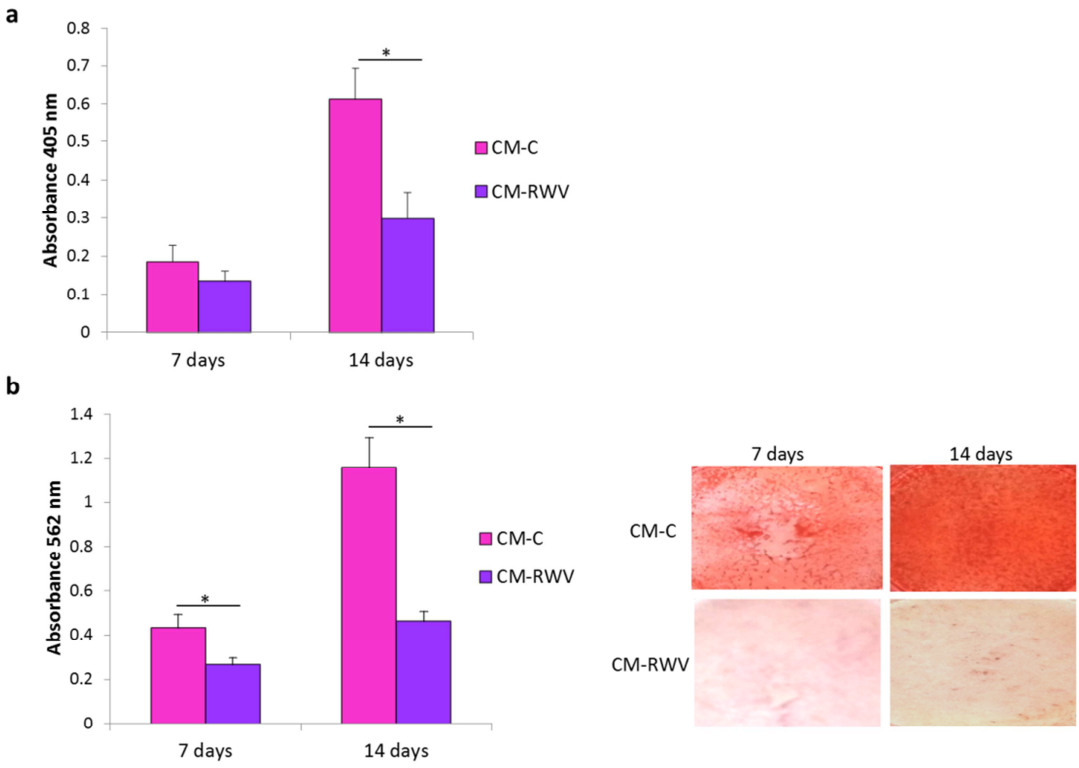


Fig. 41 *<u>Saos-2 activity.</u> a) ALP activity in Saos-2 exposed to CM-C and CM-RWV. b) Alizarin Red S staining was performed and quantified as described. Data are expressed as the mean ± standard deviation of three separate experiments in triplicate.*

In conclusion, conditioned media from HMEC in the RWV retard osteoblast proliferation and inhibit their activity. Indeed, in simulated microgravity HMEC release factors which exert

inhibitory effects on osteoblasts. Overall, microgravity might impair the communication between osteoblasts and endothelial cells.

Part of these data were published in Biomed Res Int. 2014: 857934, (2014) doi: 10.1155/2014/857934. open access. (Appendix A)

3.2 DOES MICROGRAVITY AFFECT THE OSTEOGENIC DIFFERENTIATION OF HUMAN MESENCHYMAL STEM CELLS?

Initially, the expression of *RUNX2* and *OSX*, both involved in osteogenic differentiation, was investigated under normal culture conditions by Real Time PCR; *RUNX2 is* the master switch of osteogenesis. *OSX* is required for early events of osteogenesis but it is not sufficient to reach full differentiation.

MSC were cultured for 2, 4 and 7 days with and without the addition of an osteogenic cocktail containing 10^{-8} M 1α,25-Dihydroxyvitamin D_3, 10 mM β-glycerolphosphate and 0.05 mM ascorbic acid. *RUNX2* was upregulated after 2 and 4 days while OSX expression increased at day 4 (Figure 42). At day 7 the expression of both dropped to levels comparable with the controls.

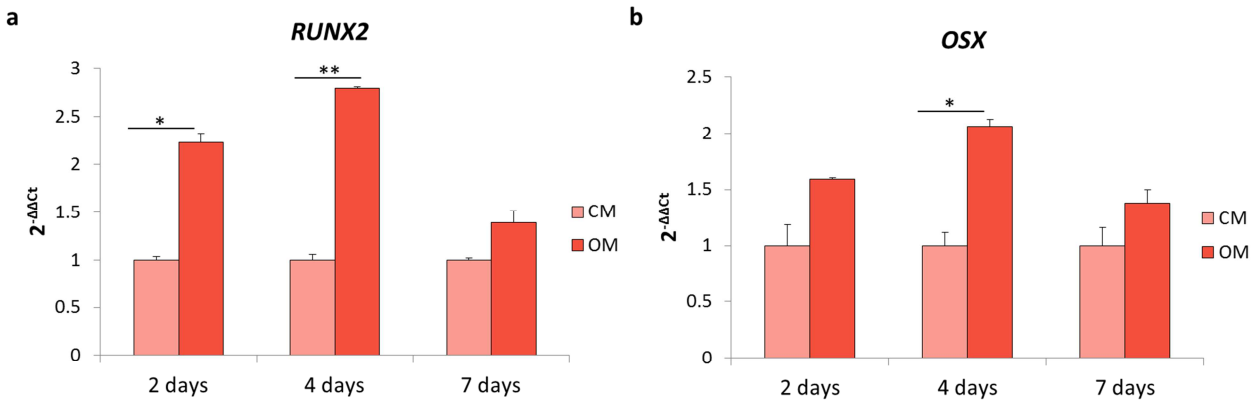


Fig. 42 *The expression RUNX2 and OSX in MSC. MSC were exposed to an osteogenic cocktail for 2, 4 and 7 days and Real Time PCR was performed on RNA samples from three different experiments in triplicate using primers designed on RUNX2 and OSX sequence.*

On these bases, MSC were cultured in simulated microgravity using RPM. Real Time PCR was performed to study the expression of the main transcription factors involved in osteogenesis (*RUNX2, OSX)* and adipogenesis (*PPARγ*) after 4 days in RPM or 1G-conditions. By Real Time PCR a significant increase of the transcripts for *RUNX2* and *OSX* was found in MSC cultured on the RPM, while no modulation of *PPARγ* was observed (Figure 43). After 10 days in simulated microgravity or 1G-conditions, the cells were stained with Alizarin Red S and no differences were observed (Figure 43). These data indicate that, even though MSC overexpress osteogenic markers in the RPM, they do not fully differentiate into osteoblasts after 10 days on the RPM. It is noteworthy that similar results were obtained using MSC from different donors.

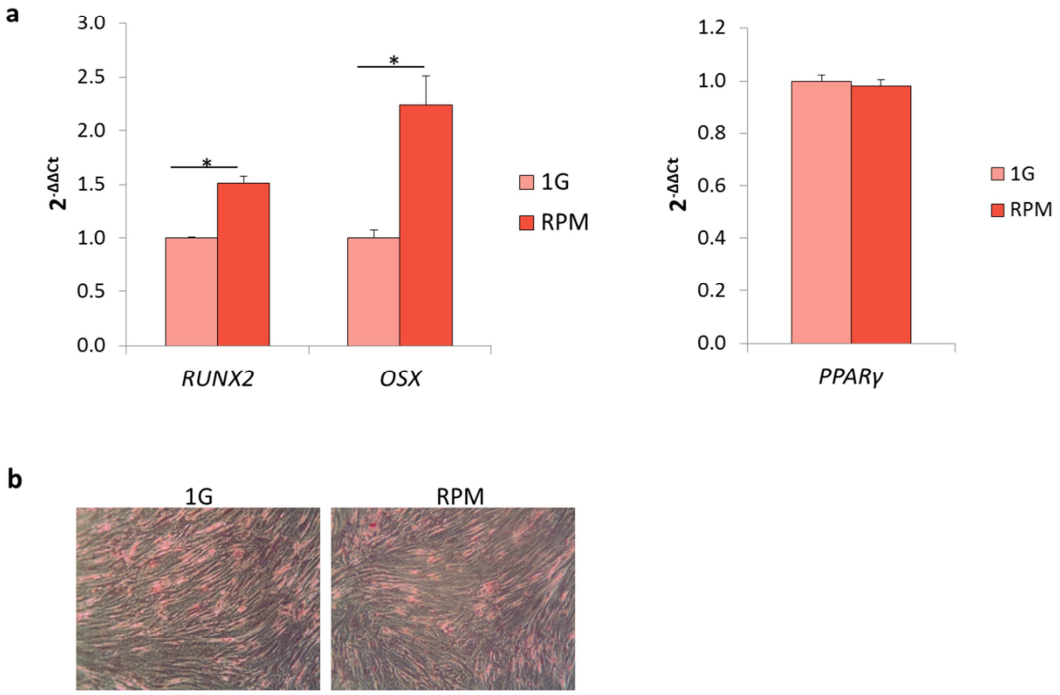


Fig. 43 *Effect of 4 days culture on the RPM or in 1G-conditions. a)The modulation of the expression of transcription factor genes related to osteogenesis and adipogenesis was investigated by Real Time PCR after 4 days in RPM. Real Time PCR was performed on RNA samples from three different experiments in triplicate using primers designed on RUNX2, OSX and PPARɣ sequence. b) Alizarin Red S staining was performed after 10 days in simulated microgravity.*

To determine how MSC adapt to simulated microgravity, the levels of stress proteins were evaluated using a protein array. After 24 h in the RPM, a significant increase of several stress proteins, i.e. HSP60, HSP70, cyclooxygenase (COX)-2, superoxide dismutase (SOD)2, was detected, while no relevant alterations of cell shape were visualized (Figure 44).

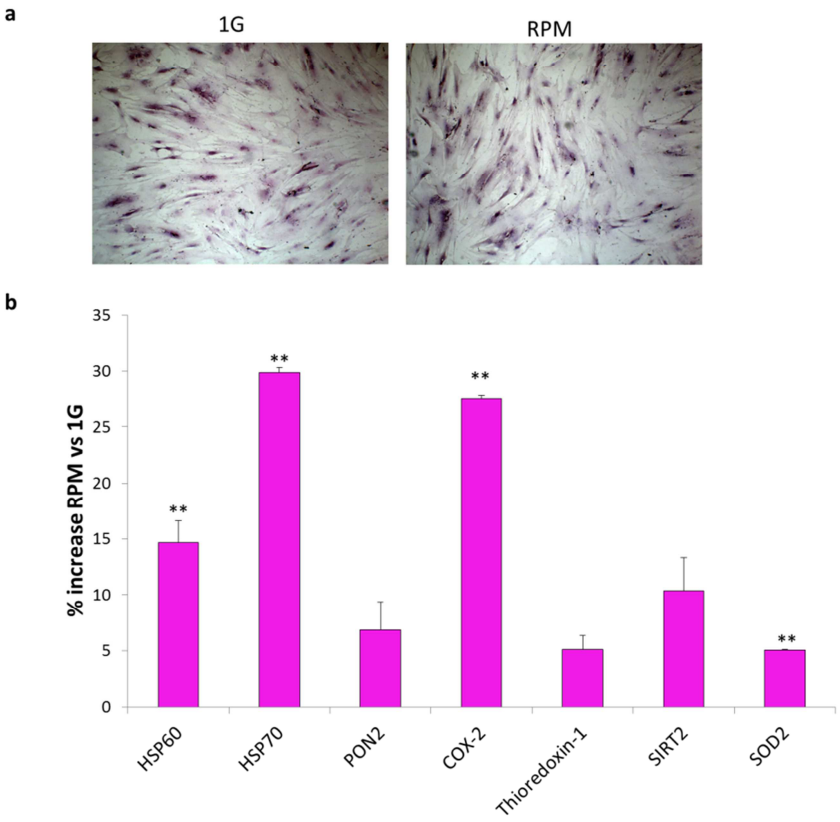


Fig. 44 *Effect of 24 h culture on the RPM or in 1G-conditions. a) MSC were photographed at 10X magnification after 24 h in RPM and 1G-conditions. b) Modulation of stress proteins was detected by stress protein array. Densitometric analysis on array spots was performed and data are expressed as % of the variation in the signal intensity of RPM vs 1G-conditions. Each bar is the mean of three separate experiments ± standard deviation.*

MSC were then cultured in the RPM or in 1G-conditions in CM or in OM for 4 and 10 days. The expression of several osteogenic markers was analyzed by Real Time PCR. Interestingly, it suffice to culture MSC in their CM in the RPM for 4 or 10 days to overexpress *RUNX2, OSX, OSP* and *OSC,* whereas *COL1A1* did not change. After 4 days of culture in the presence of the osteogenic cocktail, the expression of *RUNX2, OSX, COL1A1*, *OSP* and *OSC* was increased in the RPM and under 1G-conditions. After 10 days, both in the RPM or in standard culture conditions the expression of all the osteogenic genes apart from *COL1A1* was increased (Figure 45).

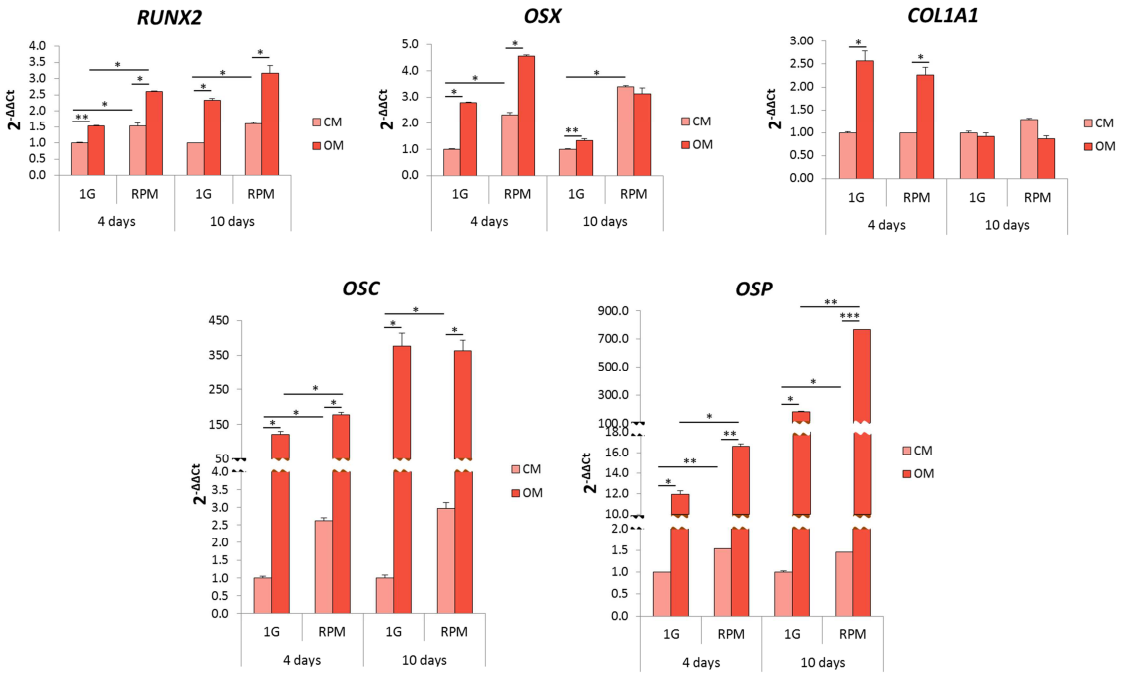


Fig. 45 *The expression of osteogenic markers after 4 and 10 days of culture in the RPM or in 1G-conditions. Real Time PCR was performed on RNA samples from three different experiments in triplicate using primers designed on RUNX2, OSX, COL1A1, OSC and OSP sequence.*

No significant differences in calcium deposition were observed between cells cultured in OM in the RPM and in static 1G-conditions (Figure 46).

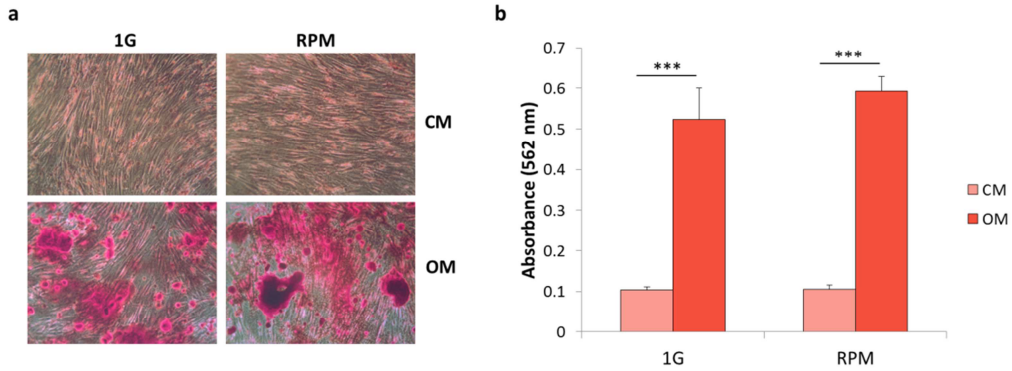


Fig. 46 *Ca deposition after 10 days of culture in the RPM or in 1G-conditions. a) Alizarin Red S staining was performed after 10 days in simulated microgravity. b) After acid extraction the absorbance was measured at 562 nm. Data are expressed as the mean ± standard deviation of three separate experiments in triplicate.*

This study demonstrates that simulated microgravity does not affect the osteogenic differentiation of human MSC.

Part of these data were published in Biochem Biophys Res Commun. 473: 181-6, (2016) doi:10.1016/j.bbrc.2016.03.075. The agreement to publish these data on the thesis is attached. (Appendix B).

3.3 HOW DO ENDOTHELIAL CELLS ADAPT TO MICROGRAVITY?

The aim of these experiments was to broaden present knowledge on the mechanism whereby HUVEC adapt and react to prolonged simulated microgravity.

In 2010 HUVEC flew to the ISS (SPHINX experiment) and were cultured for 10 days in real microgravity. Microarray analysis indicated that weightlessness modulates the expression of more than one thousand genes, among which *Thioredoxin Interacting Protein* (*TXNIP*), a stress-responsive gene encoding a protein that contrasts the antioxidant action of Thioredoxin (TRX) (Versari 2013).

Until now HUVEC have been exposed to simulated microgravity for various times but never for longer than 96 h (Carlsson 2003, Versari 2007, Griffoni 2011, Grenon 2013). In this study HUVEC were cultured in simulated microgravity for 4 and 10 days. Since no data are available about TXNIP expression in HUVEC in simulated microgravity, *TXNIP* expression was evaluated by Real Time PCR. In agreement with the results obtained in SPHINX, *TXNIP* transcript was markedly upregulated after 10 days of culture in the RWV (Figure 47a).

While experiments in space are subject to several restraints, among which the impossibility to culture enough cells to perform quantitative studies at the protein level, culture in simulated microgravity is less demanding and western blot on cell extracts is feasible. Western Blot were performed and TXNIP resulted upregulated after 10 days of simulated microgravity (Figure 47b).

It is well accepted that HUVEC proliferate faster and reach confluence earlier in the RWV than controls in 1G-conditions (Carlsson 2003, Maier 2014). After 10 days in the RWV the cells are confluent. To understand whether gravitational unloading was responsible for the late TXNIP upregulation or confluence was involved, HUVEC were cultured at different density (25% - 50% - 100%) in 1G-conditions for 24 h. Western blot demonstrated that the modulation of TXNIP is inversely proportional to confluence. These data indicate that simulated microgravity is responsible for the upregulation of TXNIP after 10 days in RWV (Figure 48).

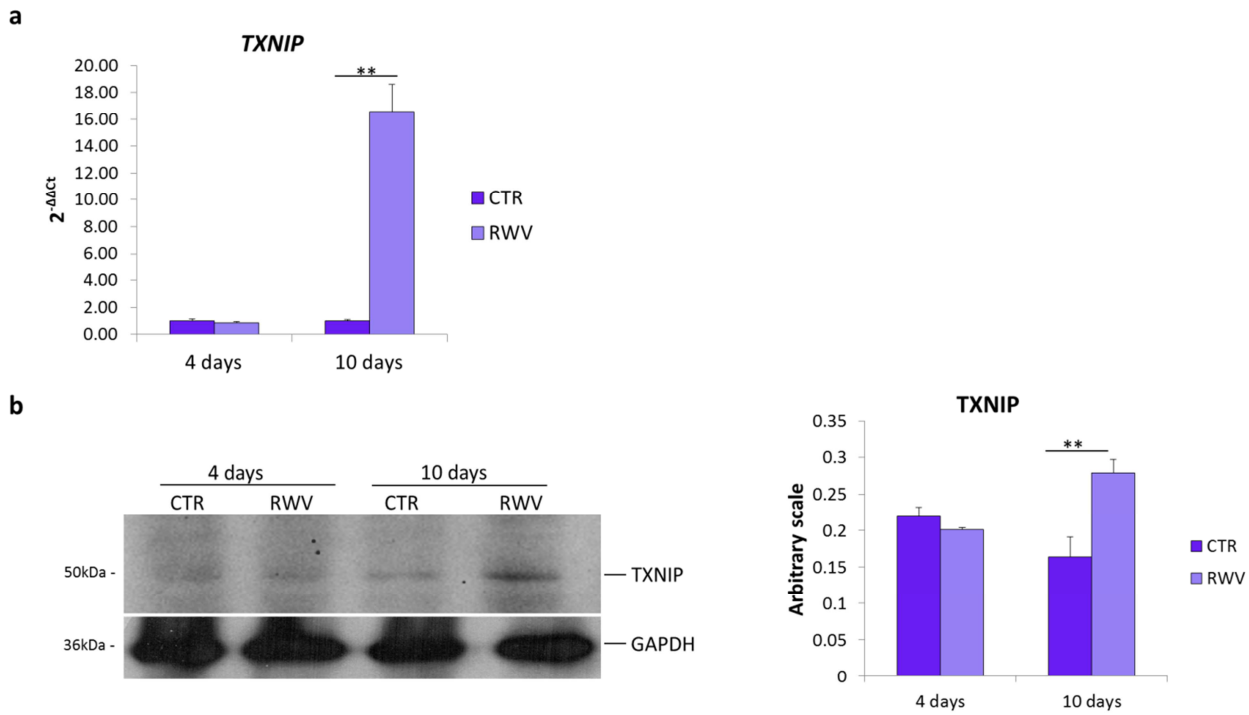


Fig. 47 *TXNIP in HUVEC cultured in the RWV vs 1G-conditions. HUVEC were exposed to simulated microgravity for 4 and 10 days. a) Real Time PCR was performed on RNA samples from three different experiments in triplicate using primers designed on TXNIP sequence. b) Cell lysates (80µg/lane) were analysed by western blot using antibodies against TXNIP and GAPDH. Densitometric analysis was performed by the ImageJ software and TXNIP/GAPDH ratio was calculated on three blots from separate experiments ± standard deviation.*

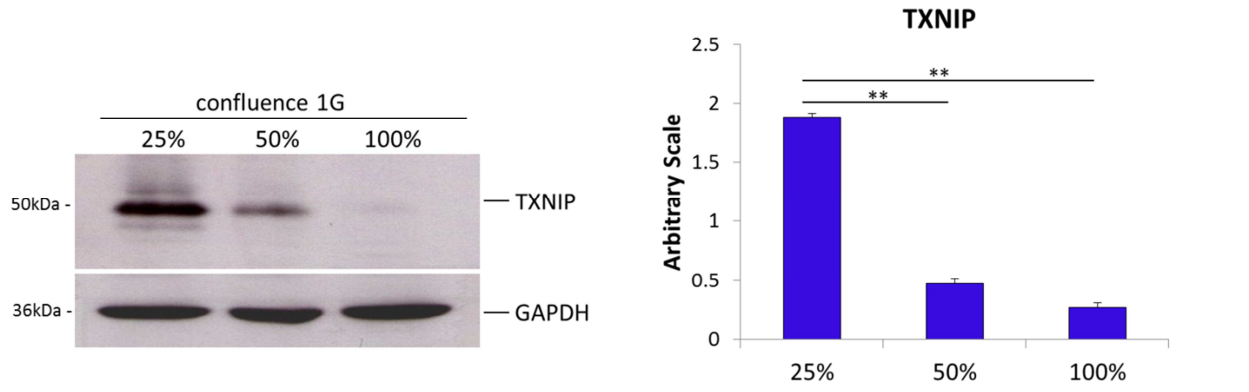
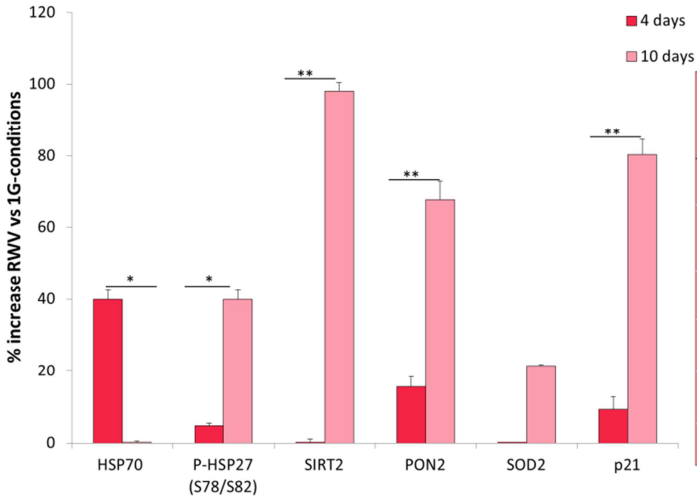


Fig. 48 *TXNIP in sparse or confluent HUVEC. Cell lysates (80µg/lane) were analysed by western blot using antibodies against TXNIP and GAPDH. Densitometric analysis was performed by the ImageJ software and TXNIP/GAPDH ratio was calculated on three blots from separate experiments ± standard deviation.*

Simulated microgravity and spaceflight activate stress proteins in plant and animal cells as well as in Drosophila and Daphnia (Taylor 2014, Trotter 2015). Stress proteins are induced in response to different strains and help the correct folding of newly synthesized proteins or fix

misfolded proteins. To investigate stress response in HUVEC in simulated microgravity, a protein array specifically tailored for stress proteins was used. 80 µg of lysates from cells cultured in the RWV and 1G-conditions for 4 and 10 days were utilized. Out of 26 proteins investigated, HSP70; P-HSP27 (S78/S82); SIRT2; PON2; SOD2; p21 were upregulated (Figure 49).



Stress Protein	Fold increase (%) ± SD 4 days	Fold increase (%) ± SD 10 days
HSP70	39.9 ± 2.6	0.2 ± 0.3
P-HSP27(S78/S82)	4.7 ± 0.7	40 ± 2.6
SIRT2	0.2 ± 0.07	98 ± 2.4
PON2	15.5 ± 2.8	67.8 ± 5.0
SOD2	0.2 ± 0.3	21.5 ± 0.2
p21	9.4 ± 3.4	80.3 ± 4.3

Fig. 49 *Stress proteins in HUVEC cultured in the RWV. Modulation of stress protein detected with protein array. Densitometric analysis was performed on array spots and data are expressed as % of the variation of the signal intensity of RWV compared to 1G-conditions. Each bar is the mean of three separate experiments ± standard deviation (SD). Measurements were calculated as indicated by the manufacturer.*

The results obtained by protein array were validated by western blot. To see if the modulation of the protein correlated with transcriptional activation, also Real Time PCR was carried out. Initially HSP70 was studied, since in space the decrease of *HSPA1A* was observed (Versari 2013), while HSP70 was upregulated early after exposure to simulated microgravity (Carlsson 2003). Real Time PCR and western blot demonstrated that HSP70 is still upregulated at day 4 thus confirming the results by Carlsson et al. (Carlsson 2003) and returns to basal levels at day 10 both at RNA and protein level (Figure 50).

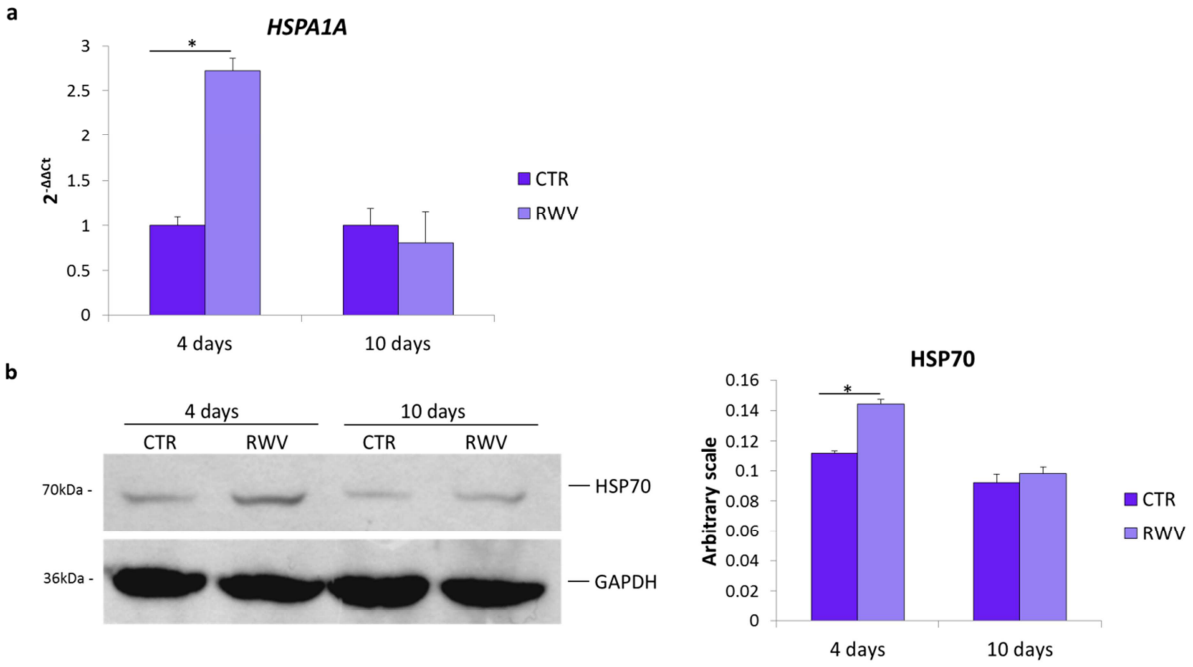


Fig. 50 *<u>HSP70 expression in HUVEC cultured in the RWV vs 1G-conditions.</u> HUVEC were exposed to simulated microgravity for 4 and 10 days. a) Real Time PCR was performed on RNA samples from three different experiments in triplicate using primers designed on HSPA1A sequence. b) Cell lysates (80µg/lane) were analysed by western blot using antibodies against HSP70 and GAPDH. Densitometric analysis was performed by the ImageJ software and HSP70/GAPDH ratio was calculated on three blots from separate experiments ± standard deviation.*

These results are in disagreement with data obtained in space. This suggests that real and simulated microgravity might differently impact on the levels of HSP70. Unfortunately, no data are available on the early response of spaceflown in HUVEC. Interestingly, HSP70 increased also in HMEC in simulated microgravity for 72 and 96 h (Figure 36) and in MSC under mechanical unloading for 24 h (Figure 44).

It is nothworthy that HSP70 was upregulated in Japanese medaka (Oryzias latipes), a model fish used for studying space adaptation, after two months on the ISS (Murata 2015). Moreover simulated weightlessness elevates liver HSP70 expression both at protein and mRNA levels in rats (Cui 2010). Similar results were obtained in Danio Rerio larvae maintained for different times in the RWV (Figure 51).

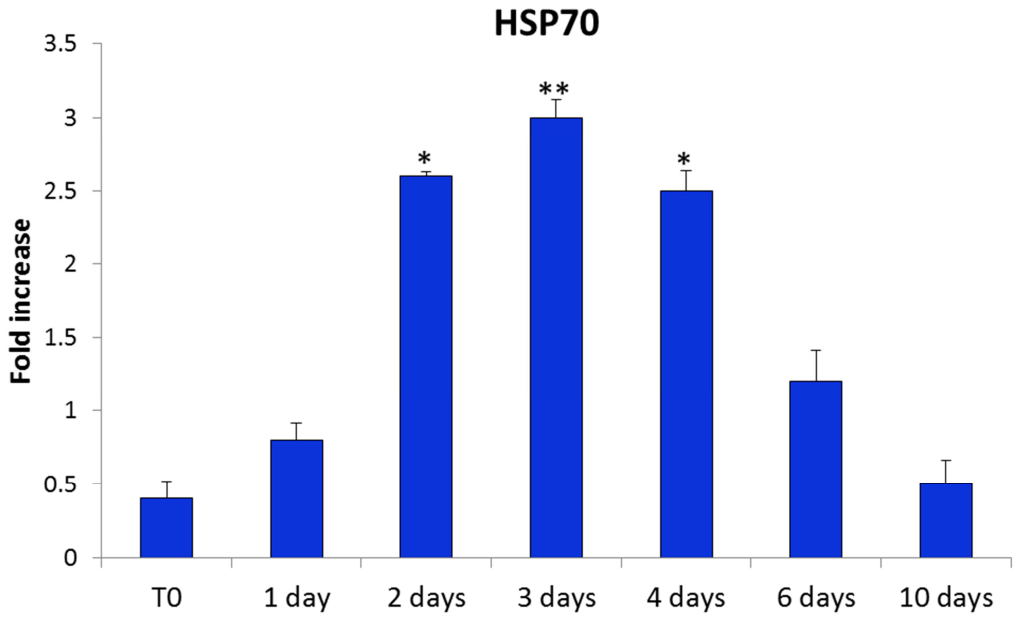


Fig. 51 *HSP70 expression in Danio Rerio embryos. Danio Rerio embryos were centrifuged and lysated. Lysates (80µg/lane) were analysed by western blot using antibodies against HSP70 and GAPDH. Densitometric analysis was performed by the ImageJ software and HSP70/GAPDH ratio was calculated on three blots from separate experiments. Data are presented as mean ± standard deviation and represent the fold increase in HSP70 expression compared to control embryos.*

From these results in cell culture and in animal models it is possible to conclude that the induction of HSP70 is a general response to microgravity. HSP70 protects cells from apoptosis event and has the ability to inhibit inflammatory cytokines (Zhu 1996). In EC, HSP70 is rapidly upregulated in simulated microgravity and this might be considered as an early adaptive response of the cells to survive. It is reasonable to propose that under mechanical unloading alterations of protein folding and/or protein aggregation might trigger the induction of HSP70.

Western blot demonstrates the increased amounts of total HSP27 and P-HSP27 in HUVEC in simulated microgravity (Figure 52). The increase is significant after 4 days of culture in the RWV and more accentuated after 10 days.

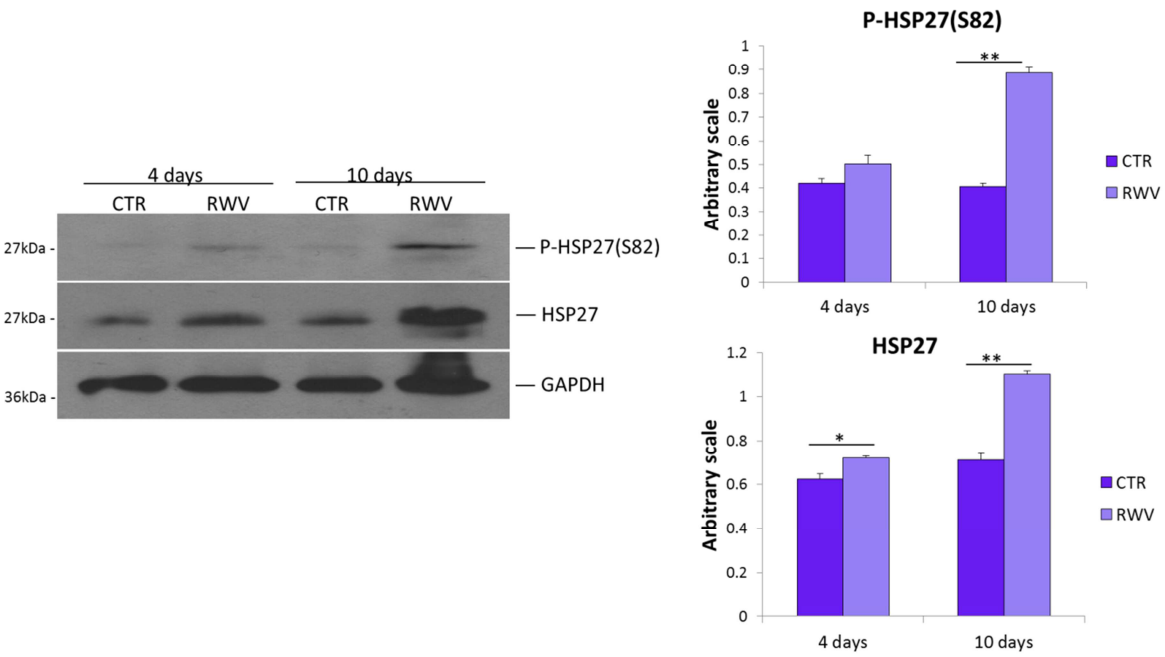


Fig. 52 *HSP27 an P-HSP27 expression in HUVEC cultured in the RWV vs 1G-conditions. Cell lysates (80µg/lane) were analysed by western blot using antibodies against HSP27 and P-HSP27(S82) and GAPDH. Densitometric analysis was performed by the ImageJ software and HSP27/GAPDH and P-HSP27/GAPDH ratio was calculated on three blots from separate experiments ± standard deviation.*

While Real Time PCR did not detect any modulation of SIRT2 and PON2 transcript (Figure 53a), by western blot PON2 resulted upregulated after 10 days in simulated microgravity (Figure 53b), thus suggesting a post-transcriptional regulation. SIRT2 is downregulated in the control after 10 days of culture but remains elevated in HUVEC cultured on the RWV.

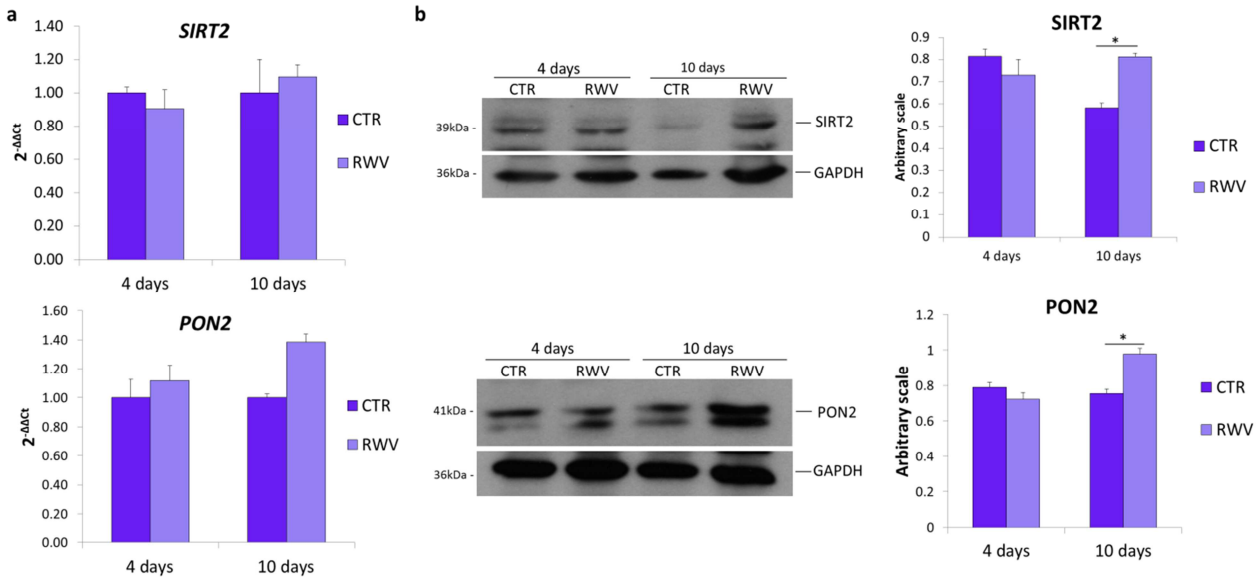


Fig. 53 *SIRT2 and PON2 expression in HUVEC cultured in the RWV vs 1G-conditions. HUVEC were exposed to simulated microgravity for 4 and 10 days. a) Real Time PCR was performed on RNA samples from three different experiments in triplicate using primers designed on SIRT2 and PON2. b) Cell lysates (80µg/lane) were analysed by western blot using antibodies against SIRT2 and PON2 and GAPDH. Densitometric analysis was performed by the ImageJ software and SIRT2/GAPDH and PON2/GAPDH ratio was calculated on three blots from separate experiments ± standard deviation.*

To evaluate if cell density modulates SIRT2 and PON2, HUVEC were cultured at different density (25% - 50% - 100%) in 1G-conditions for 24 h. Figure 54 shows that, while PON2 levels remains constant, SIRT2 progressively decreases in subconfluent and confluent HUVEC (Figure 54). On these bases, it is feasible that simulated microgravity is responsible for the upregulation of PON2 as well as for maintaining increase amounts of SIRT2 after 10 days.

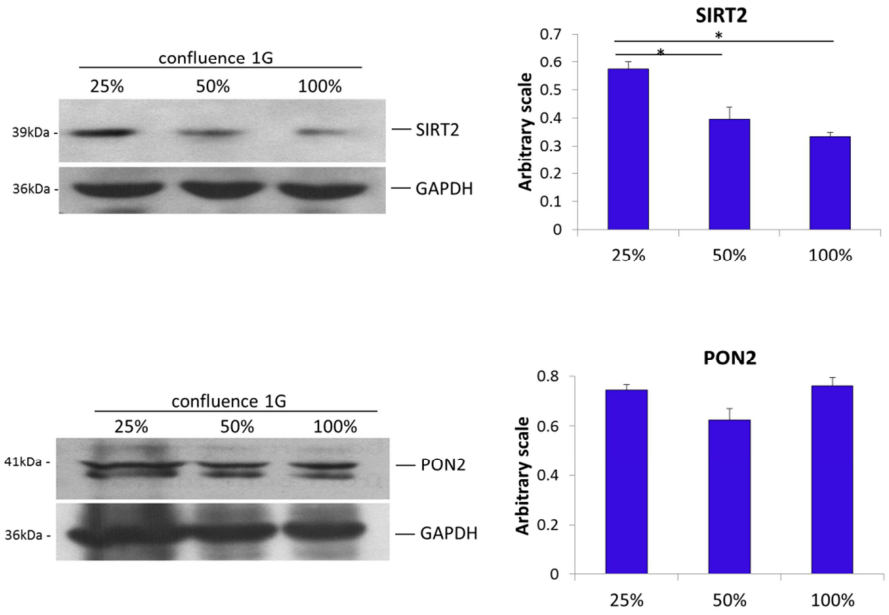


Fig. 54 *SIRT2 and PON2 in sparse or confluent HUVEC. Cell lysates (80µg/lane) were analysed by western blot using antibodies against SIRT2 and PON2 and GAPDH. Densitometric analysis was performed by the ImageJ software, SIRT2/GAPDH and PON2/GAPDH ratio was calculated on three blots from separate experiments ± standard deviation.*

Protein array revealed also the significant upregulation of $p21^{Cip1/Waf1}$, also known as cyclin-dependent kinase inhibitor 1. p21 was upregulated in HMEC in simulated microgravity after 96 h (Figure 34b) and this data is correlated with the inhibition of growth as the effects of simulated microgravity. No data are available in HUVEC. Western blot confirms the increased amounts of p21 in cells in simulated in microgravity. However, since confluence is reached earlier in simulated microgravity than in 1G-conditions, the upregulation of p21 is the result of this event. Indeed, Figure 55a shows that confluent HUVEC accumulate p21. Therefore, p21 is upregulated after 10 days in microgravity because cells reach the confluence before the controls cultured in 1G-conditions (Figure 55b).

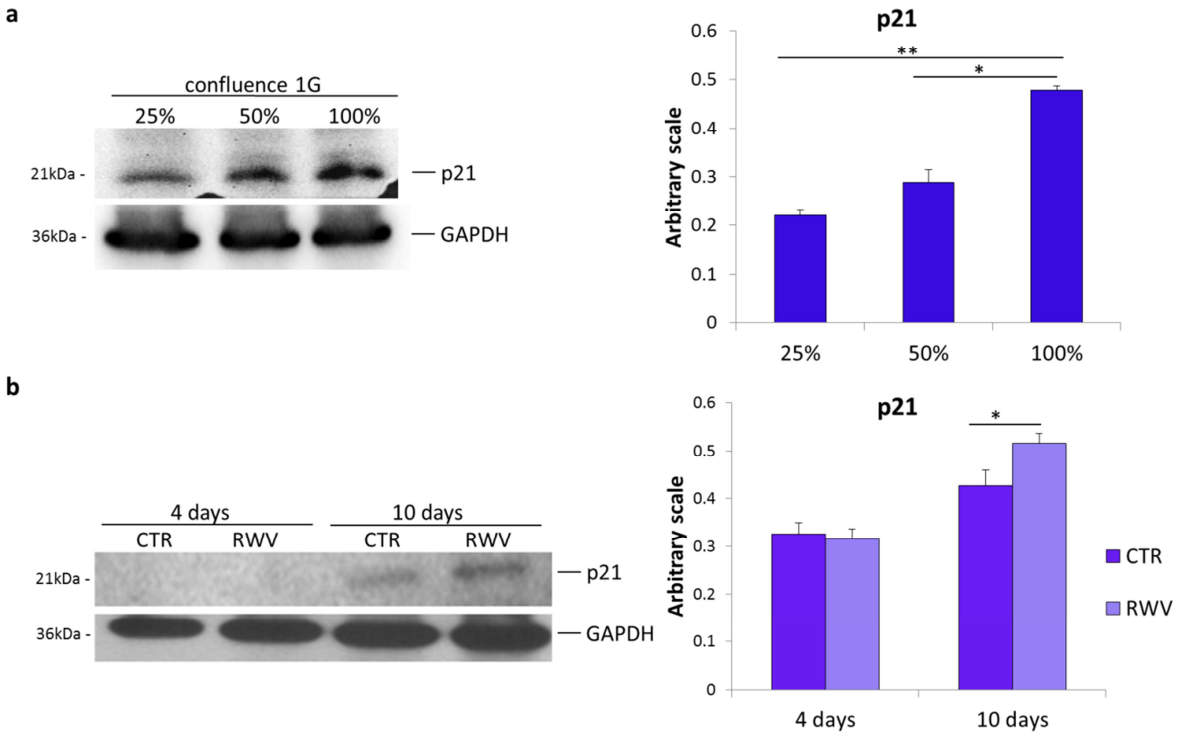


Fig. 55 *p21 in sparse or confluent HUVEC and in culture on RWV and 1G-conditions. Western blot was performed on cell lysates (80µg/lane) obtained from (a) 1G-conditions and (b) RWV experiments. The antibodies against p21 and GAPDH were used. Densitometric analysis was performed by the ImageJ software and p21/GAPDH ratio was calculated on three blots from separate experiments ± standard deviation.*

In simulated microgravity the cytokine network is altered (Carlsson 2003, Chang 2012, Paulsen 2015). In particular, IL-1α is downregulated in HUVEC cultured in the RWV up to 4 days from the beginning of the experiment (Carlsson 2003), while IL-6 is modulated in HMEC (Figure 37b). By protein array no significant modulations of several pro-inflammatory proteins was observed both in cell lysates and in the conditioned media after 10 days in simulated microgravity (Figure 56).

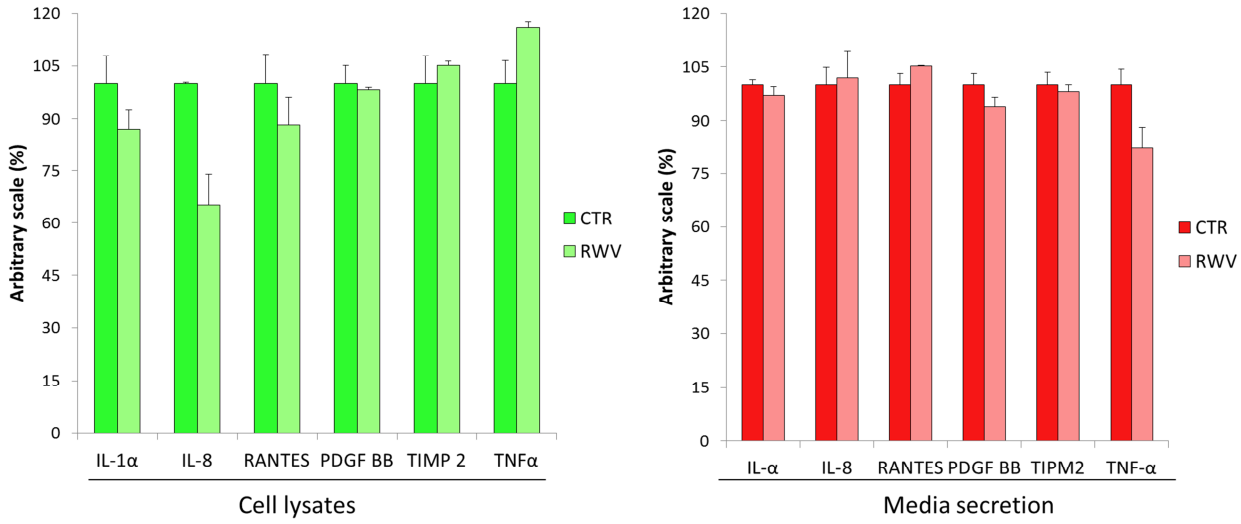


Fig. 56 *Cytokine network in HUVEC in the RWV. Modulation of pro-inflammatory proteins was studied by inflammation protein array on cell lysates (80µg/lane) and media. Densitometric analysis was performed on array spots and data were express as % of the variation of the signal intensity in samples cultured in the RWV compared to cells in 1G-conditions. Each bar is the mean of three separate experiments ± standard deviation.*

These data suggest that the adaptation of HUVEC to simulated microgravity is a dynamic process. After 4 days HSP70 is still upregulated. After 10 days, HSP70 returns to the basal levels and the upregulation of TXNIP, SIRT2, PON2, HSP27 and P-HSP27 is observed. This might explain why an increase of ROS is detected only after a few hours in simulated microgravity, while at later time points ROS generation is comparable in the RWV and in the 1G-conditions (Figure 57).

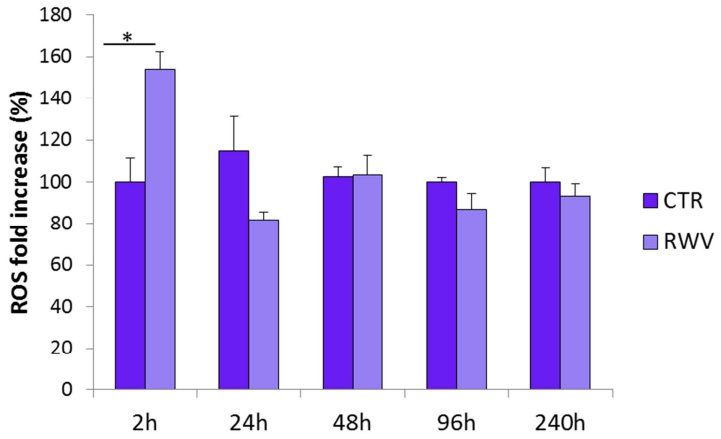


Fig. 57 *ROS production by HUVEC in RWV vs 1G-conditions. ROS measurement at different cell density. Data are shown as the fold increase in ROS levels of the samples normalized with the number of cells. Each bar is the mean of three separate experiments ± standard deviation.*

It is feasible that the pro-oxidant effects of TXNIP are counterbalanced by the overexpression of anti-oxidant proteins. In agreement with these results, no increase of pro-inflammatory cytokines was detected after 10 days in simulated microgravity. It is likely that endothelial dysfunction does not occurs, rather HUVEC gradually and dynamically adapt to mechanical unloading.

3.4 PREPARATORY WORK FOR EXPERIMENTS IN SPACE: DOES CULTURE IN THE EXPERIMENTAL UNITS FOR SPACEFLIGHT IMPACT ON CELL BEHAVIOR?

Experiments in space are complex to design. Safety requirements are very strict to minimize any risk to the crew. Cells must be cultured in closed cell culture systems (experimental units -EU-) made of biocompatible materials and with a high degree of automation. It is important to know whether culture in the EU mimics culture under normal conditions in plastic dishes. The behavior of MSC, HMEC and HUVEC cultured in EU was investigated.

Confluent MSC were cultured for 24 and 96 h in EU or in a dish as a control (CTR). The first issue to consider is whether a closed system generates oxidative stress. ROS were measured and found to be increased in cells cultured in EU (Figure 58).

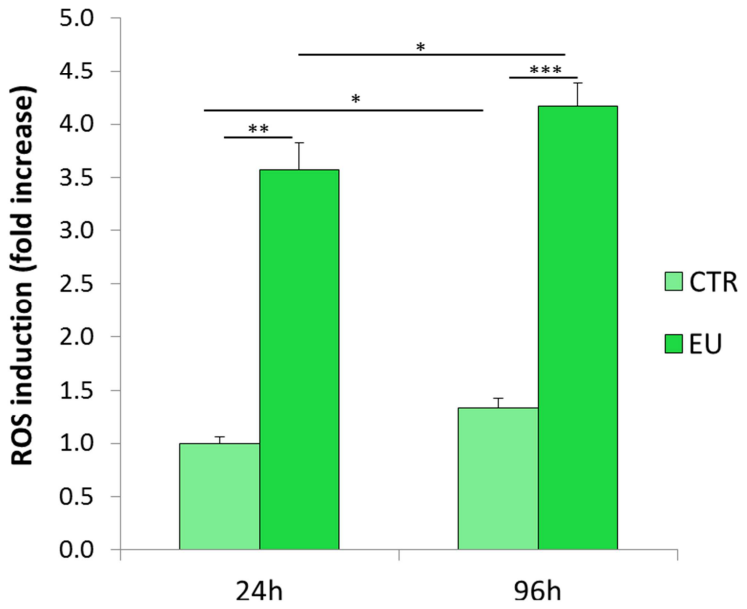


Fig. 58 *ROS production by MSC cultured in the EU vs CTR. ROS generation was measured by DCFH 24 and 96 h after assembling the EU. Data are shown as the fold increase in ROS levels of the samples compared to 24 h control cells cultured in dish. Each bar is the mean of three separate experiments ± standard deviation.*

To test whether EU induced overproduction of ROS exerted some effects on cell response, MSC were cultured in the EU in OM or CM. After 96 h, the cells were fixed, stained with

hematoxylin-eosin and observed. No differences emerged between cells in CM and OM in plastic dish, while MSC in the EU and exposed to the OM are less numerous than in the controls and tend to align and organize in bundles (Figure 59).

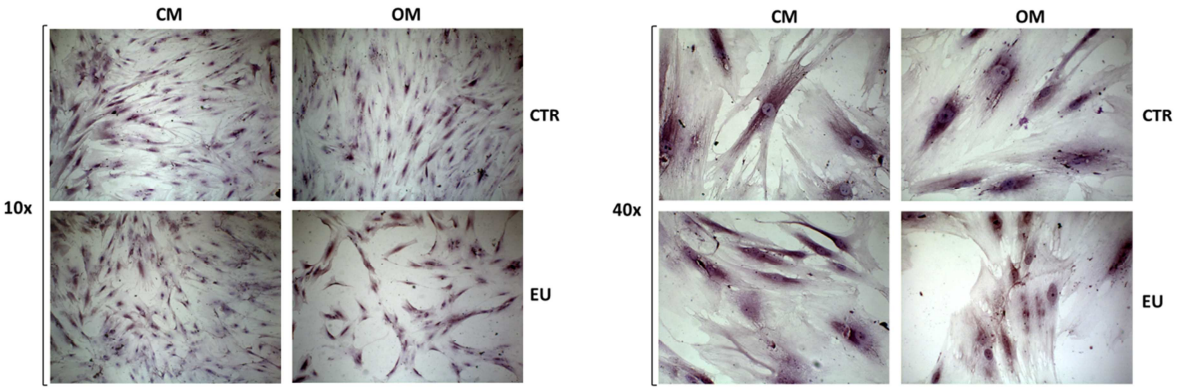


Fig. 59 *Morphology of MSC in the EU vs CTR. Microphotographs of cells were taken after 96 h culture in the EU or in control dish.*

Since after 96 h in OM osteogenic markers are overexpressed, Figure 42 Real Time PCR for *RUNX2* and *OSX* was performed. Figure 60 shows the induction of the osteogenic transcription factor genes *RUNX2* and *OSX* after exposure to OM. Interestingly, the upregulation of these transcripts is higher in MSC in the EU than in their controls.

To investigate if ROS affect the induction of *RUNX2* and *OSX*, MSC were induced to differentiate in the presence of the antioxidant *N*-Acetyl-L-cysteine (NAC). NAC decreased the expression of *RUNX2* and *OSX* in MSC cultured in the EU in OM to levels comparable with the controls cultured in a dish (Figure 60).

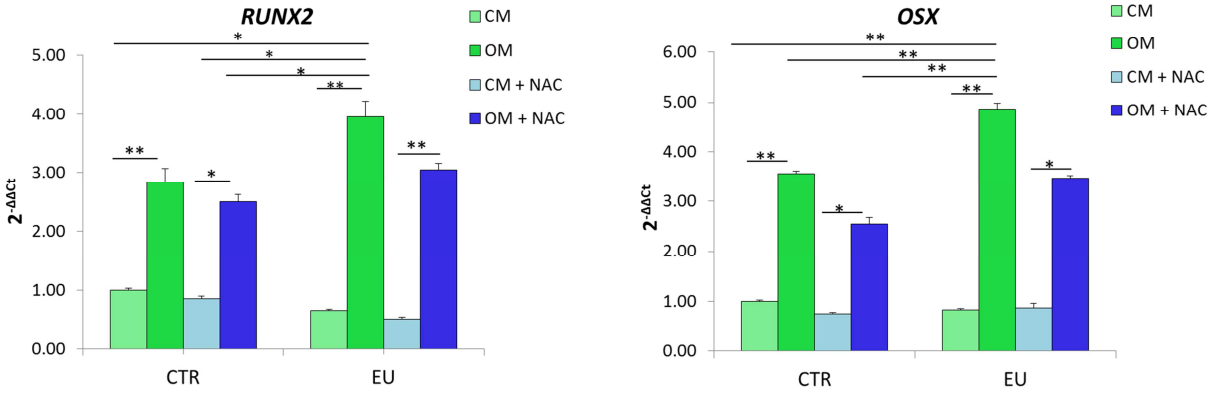


Fig.60 *<u>The expression of osteogenic markers in MSC in the EU vs CTR.</u> Real Time PCR was performed on RNA samples from three different experiments in triplicate using primers designed on RUNX2 and OSX sequence.*

These results indicate that the over-production of ROS determines the overexpression of osteogenic markers.

The behavior of HMEC and HUVEC in the EU was then investigated. 5 x 10^3 HUVEC and 10^4 HMEC were seeded on gelatin-coated Thermanox. The cells were counted after 24 and 96 h. In the first 24 h HUVEC grew faster in the EU, while after 96 h the number of cells was comparable in the EU and in the controls (Figure 61a). On the contrary, in the first 24 h HMEC in the EU were growth retarded. Similarly to HUVEC, no significant differences of HMEC number were observed after 96 h (Figure 61b).

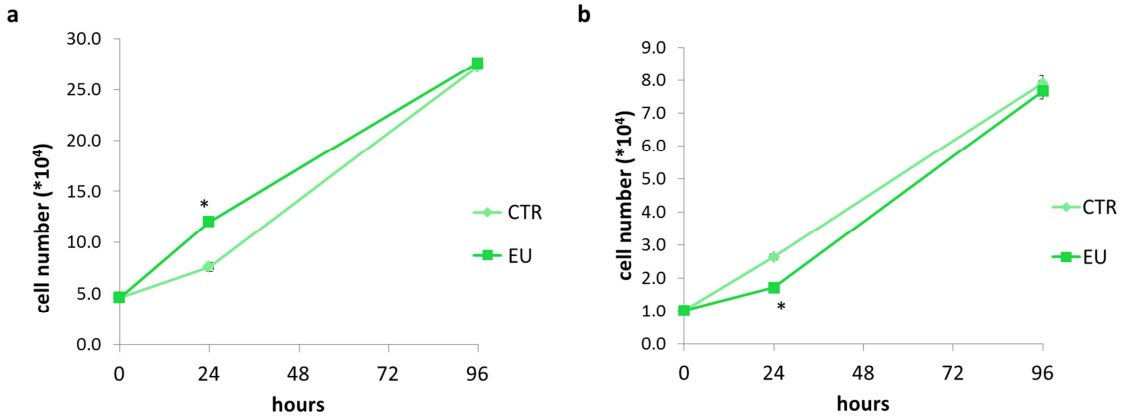


Fig 61 *<u>HUVEC and HMEC growth.</u> Effects of culture in the EU on HUVEC (a) and HMEC (b). After 24 and 96 h in the EU, viable cells were counted using an automated cell counter. Data are expressed as the mean ± standard deviation of three separate experiments in triplicate.*

Since culture in the EU generates ROS in MSC, we measured ROS also in endothelial cells by DCFH 24 and 96 h after assembling the EU. As shown in Figure 62a, after 24 h ROS generation was decreased in HUVEC in the EU, while no differences were detected between the two experimental conditions after 96 h. In HMEC, the amounts of ROS did not change after 24 and 96 h culture in the EU (Figure 62b).

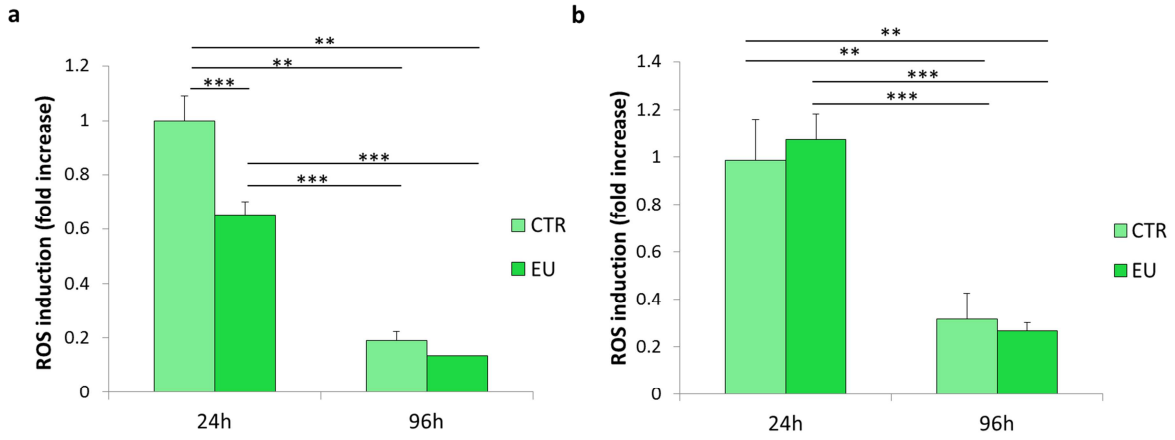


Fig. 62 *Effect of culture in the EU on ROS generation by EC. ROS production was measured 24 and 96 h after assembling the EU in HUVEC (a) and in HMEC (b). Data are shown as the fold increase in ROS levels of EU cultured cells compared to controls 24 h cultured in dish. Each bar is the mean of three separate experiments ± standard deviation.*

The reduced generation of ROS after 96 h is a common feature in HUVEC and HMEC. It is noteworthy that ROS production dropped when the cells reach confluence, suggesting that ROS production is higher in sparse vs confluent cells. To check this possibility, HUVEC and HMEC were seeded at different density (25% - 50% - 100%). After 24 h ROS production were measured. Figure 63 demonstrates that the increase of ROS is inversional proportional to the density of HMEC and HUVEC (Figure 63).

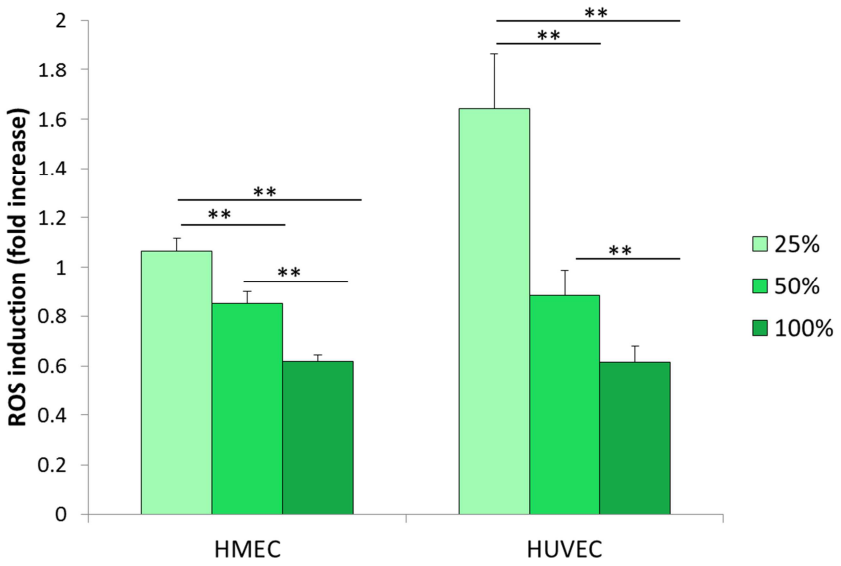


Fig. 63 *ROS measurement at different cell density. Data are shown as the fold increase of ROS levels normalized with the number of cells. Each bar is the mean of three separate experiments ± standard deviation.*

These data demonstrate that particular attention should be devoted to the choice of the Experimental Hardwares, since they affect the performances of the cells.

This is important also to facilitate the interpretation of the results obtained in space and the comparison with data obtained on earth under normal culture conditions.

Part of these data are "in press" in Experimental Biology and Medicine. (Appendix C).

4. DISCUSSION

“Look at the stars and not your feet. Try to make sense of what you see, and wonder why the universe exists. Be curious” Stephen Hawking.

4.1 RESEARCH IN SPACE, A CHALLENGE

Recently, because of the prolonged financial crisis, criticism has been raised about investments for research in space because of its high costs and because human presence in space might not be necessary due to the dramatic advances in automation and robotics (Rinaldi 2016). However, apart from “inspirational value” and “motivation for educational excellence” which have been declared for years, biomedical research in space has already offered new hints since space provides unique conditions to study mechanisms and pathways that control cell growth and function (Becker and Souza 2013). Using the microgravity environment that Earth-based laboratories cannot replicate, it is possible to explore fundamental questions about important and common health issues, from osteoporosis to muscle waste and immune impairment. Moreover, countermeasures and technologies utilized to face health concern in space have numerous application on Earth for patient care.

When the space race started in the 50s, a possible link between gravity and osteoporosis was totally unexpected. With long duration missions on the ISS, it became evident that bone loss occurs 10 times faster in space than in physiological aging. This problem prevents space exploration and jeopardizes also the mission to Mars. While these space-related changes resemble those observed during aging, they are more or less quickly restored after re-entry on Earth (Cappellasso 2015, William 2009). Experiments in space on bone cells are challenging because they allow the investigation of the pathogenic mechanisms of osteoporosis during its development but also of the mechanisms determining recovery after return to Earth. Osteoporosis is characterized by the loss of bone mass due the deterioration of bone microarchitecture and is the result of an imbalance between bone deposition and resorption. Osteoporosis affects millions people worldwide and represents a major health concern since the aging population will double over the next decade with enormous cost burden on the healthcare systems. It is expected that results from experiments in space might open new avenues in the approach to the disease, thereby alleviating costs and ameliorating the quality of life. The effects of microgravity on osteoblasts and osteoclasts have been extensively studied, but only recently consideration has been given to the role of bone mesenchymal stem cells which might orchestrate the unbalanced bone remodeling in spaceflight (Zayzafoon 2004, Saxena 2007, Mao 2016). Even less is known about the crosstalk between endothelial cells and osteoblasts, a communication that has been shown to be crucial for healthy bones (Villars 2000,

Zannettino 2005, Kanclezer 2008). These issues - behavior of MSC and communication endothelium/osteoblasts in simulated microgravity - have been the focus of part of this thesis.

In addition, to deepen the lesson learnt from studies on HUVEC onboard the ISS (Versari 2013), several experiments have been performed on these cells to delineate their performances when cultured in simulated microgravity for 4 and 10 days to reach the important conclusion that endothelial dysfunction does not occur under these experimental conditions.

4.2 NEW INSIGHTS INTO THE EFFECTS OF SIMULATED MICROGRAVITY ON BONE CELLS

Microvascular endothelial cells are protagonists in inflammation and angiogenesis (Danese 2007). In the bone, they have an important role in maintaining homeostasis, since they not only deliver nutrients and gases but also elaborate molecules that influence the behaviour of bone cells (Galley and Webster 2004).

An impairment of microvascular cells in real microgravity can explain the significant decrease of the density of small vessels in the quail chorioallanthoic membranes after spaceflight (Henry 1998) and the retardation of wound healing in rats during orbital spaceflight (Davidson 1999). To this purpose, it is also relevant that any attempt to neovascularize bioengineered tissues in microgravity has failed (Unsworth and Lelkes 1998). In agreement with the results obtained by Cotrupi et al. in murine microvascular cells (Cotrupi 2005), simulated microgravity affects also HMEC. The cells grow slower than controls and this is associated with the upregulation of p21. p21 inhibits cell cycle progression by blocking the activity of cyclin/CDK2 complexes required for the transition from the G1 to the S phase. Moreover, p21 interacts with PCNA and blocks DNA synthesis (Waga 1994). p21 is regulated by p53 dependent and independent mechanisms (Harris 2005). p53 is induced in response to DNA damage, hypoxia and redox stress (Harris 2005). In HMEC in the RWV, no increase of total amounts of p53 and P-p53 was found, suggesting that p53 is not involved in p21 induction by simulated microgravity. On the contrary, in porcine aortic endothelial cells engineered to overexpress vascular endothelial growth factor-receptor 2, hypogravity induces p53 mRNA, and this has been linked to increased apoptosis (Morbidelli 2005). In HMEC in simulated microgravity no apoptosis is observed. This might be due to the high amounts of p21, which prevents endothelial apoptosis (Mattiussi 2004), and to the increase of HSP70, which protects against cell death (Zhu 1996). Stress

proteins drive the folding of neo-synthesized proteins and correct proteins that have been misfolded after different stresses. Since HSP70 increases in various cell types (Carlsson 2003, Cui Y 2010, Zupanska 2013, Gritsyna 2015), in Drosophila and in Daphnia (Taylor 2014, Murata 2015, Trotter 2015) in real or simulated microgravity, the induction of HSP70 seems to be a general response to microgravity, probably driving adaptation to mechanical unloading.

Conditioned media (CM) collected from HMEC cultured in the RWV and in 1G-conditions were then analyzed. Studies utilizing CM can successfully lead to the identification of soluble factors which interconnect different cell types (Dowling 2011), because CM reveal the collection of proteins released through the classical and non classical secretion pathway as well as proteins shed from the cell surface. The proteins secreted in the CM are enzymes, cytokines, growth factors that together with various other soluble mediators, contribute to cell survival, growth and differentiation. To identify the proteins secreted by HMEC in simulated microgravity, a protein array specifically tailored for proteins involved in inflammation/angiogenesis was used. Increased amounts of IL-6 and TIMP-2, known to affect both endothelial cells and osteoblasts, were revealed. The increase of secreted TIMP-2 can contribute to the inhibition of HMEC growth in simulated microgravity, since TIMP-2 has been shown to suppress microvascular endothelial cell proliferation by antagonizing Fibroblast Growth Factor (FGF)2 and Vascular Endothelial Growth Factor-A (VEGF-A) (Stetler-Stevenson 2005). TIMP-2 plays an important role also in the bone because it inhibits osteoblast activity by decreasing ALP (Barthelemi 2012). Also IL-6, a pleiotropic cytokine implicated in acute phase response and inflammation (Wassmann 2004), impacts on the bone, since it stimulates osteoclastogenesis and inhibits osteoblast activity (Peruzzi 2012). Therefore, the increased release of TIMP-2 and IL-6 by HMEC cultured in simulated microgravity might be responsible for the impairment of osteoblast activity detected in osteoblast cultured with CM from HMEC in simulated microgravity. It is feasible to propose that simulated microgravity affect directly endothelial cells which indirectly impair osteogenic differentiation (Figure 64).

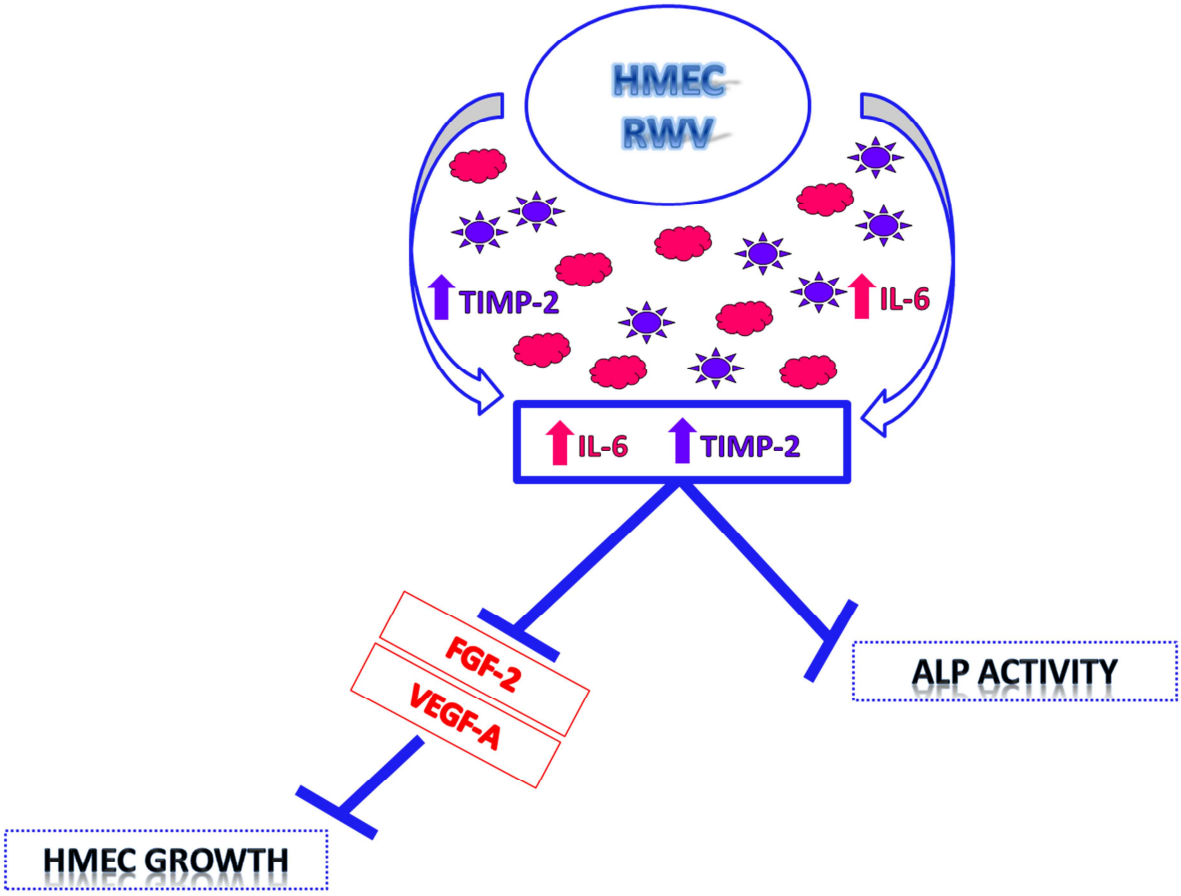


Fig. 64 *Cross-talk between HMEC in simulated microgravity and osteoblasts cells.*

On these bases, alterations of the cross-talk between the endothelium and osteoblasts might participate to generate osteopenia. This issue needs to be further studied. It will be interesting to block TIMP-2 and IL-6 with neutralizing antibodies to demonstrate their role in modulating osteoblast activity in these experimental conditions. Moreover, the effects of CM on MSC should be investigated. At the moment, MSC have never been evaluated in co-culture with endothelial cells exposed to simulated microgravity, while a few studies on MSC alone in microgravity are available (Zayzafoon 2004, Saxena 2007, Mao 2016, Zhang 2015).

I performed experiments using primary MSC isolated from healthy male donors cultured on the RPM with the aim of understanding how they adapt to simulated microgravity and how they respond to osteogenic stimuli.

Culture in simulated microgravity rapidly induces a stress response in MSC. HSP60 and HSP70 are upregulated in MSC after 24 h in the RPM. HSP70 is reported to promote the osteogenic differentiation of MSC because it induces the expression of *RUNX2* and *OSX* (Chen 2015). Accordingly, it is enough to culture MSC in the RPM to observe an increase of the expression to

RUNX and *OSX*. HSP60 is required for the maintenance of cell viability (Wang 2011) and this might explain why no apoptosis is detected and why cell morphology is maintained. Even if *RUNX*, *OSX*, *OSC* and *OSP* are overexpressed in MSC cultured in simulated microgravity, this is not sufficient to reach full osteogenic differentiation, i.e. Ca deposition in the extracellular matrix. This is due to the fact that MSC in the RPM do not overexpress *COL1A1*. It should be recalled that collagen, which represents more than 90% of organic material in the bone matrix, is necessary for calcium deposition. Only upon addition of an osteogenic medium containing vitamin D, MSC in the RPM upregulate also *COL1A1* and complete their osteogenic differentiation. These data demonstrate that there are no differences in osteogenic differentiation in the RPM and 1G-conditions, which means that simulated microgravity does not inhibit MSC differentiation in the presence of an osteogenic stimuli. These results are in disagreement with the previous study performed by Zayzafoon et al., who described that simulated microgravity prevents osteogenic differentiation of MSC and favors their adipogenic differentiation by upregulating *PPARɣ*. In my study *PPARɣ* was not increased. To explain these discrepancies, it is important to underline that mesenchymal stem cells are highly heterogeneous and that Zayzafoon performed studies in simulated microgravity using RWV, which requires to grow the cells on microbeads. The differences of bioreactors and of the methods used for culturing the cells could account for the different behavior of the cells.

The MSC used in this thesis were flown to ISS in March 2015 (Soyuz 42S spaceflight mission). It will be interesting to compare data obtained in RPM and in real microgravity as soon as the analyses will be completed.

To perform experiments in space, the cells must be cultured in specifically engineered closed experimental units which are biocompatible (to provide the optimal conditions for the cells), light (to reduce the cost of space mission), and automated (to minimize human intervention because the daily schedule of astronauts is very tight). Moreover, several rigorous safety requirements have to be met to reduce any possible hazard to the crew. Culture of MSC in the experimental units for spaceflight impacts on their behavior, since it induces the expression of *RUNX2* and *OSX* in a ROS-mediated fashion. These results indicate that the choice of the spaceflight experimental units is complex but necessary to optimize the experimental conditions so that culturing cells in space reflects as closely as possible what happens in a culture dish on Earth.

4.3 FROM SPACE TO EARTH: WHAT HAPPENS TO MACROVASCULAR ENDOTHELIAL CELLS IN MICROGRAVITY

HUVEC are widely used as a model of macrovascular endothelial cells and have been utilized both in simulated and real microgravity (Carlsson 2003, Versari 2007, Grenon 2011, Versari 2013). All these studies support the fact that microgravity profoundly influences their behaviour.

It is not easy to critically compare the results obtained in space with those generated in microgravity simulators on Earth. First of all, the constraints imposed in spaceflight limit the type of experiments that can be performed, so that, at the moment, transcriptomics is the preferred approach. In addition, time courses are difficult to be carried out. Moreover, the experimental units for spaceflight offers a limited surface to culture the cells, and this prevents the possibility of analysis at the protein level. It is important to underscore that HUVEC cultured in the experimental units used for the SPHINX experiment onboard the ISS or in culture dishes showed the same behaviour. On the basis of the results obtained in the SPHINX experiment for 10 days, more experiments were planned using the RWV to simulate microgravity for 4 and 10 days. No previous studies are available in simulated microgravity for longer than 4 days.

In real microgravity, *TXNIP* was the most overexpressed gene. TXNIP has a pro-oxidant function because it inhibits the anti-oxidant activity of Thioredoxin (TRX)-1 (Lee 2013). In HUVEC cultured in the RWV for 10 days, TXNIP was increased both at the RNA and at the protein level, while after 4 days no significant modulation was observed. One hypothesis is that the increase of TXNIP relates to cell density. Indeed, HUVEC grow faster in the RWV than in 1G-conditions, thus reaching confluence faster than controls as demonstrated by the more rapid upregulation of p21. It is noteworthy that confluent HUVEC downregulate TXNIP. Therefore, TXNIP upregulation in microgravity is independent from cell density and seems to be due to microgravity itself. Surprisingly, in spite of TXNIP upregulation, no accumulation of ROS was detected. This finding suggests the possibility of a potentiation of stress response and antioxidant defence. Therefore, the levels of some stress proteins and some anti-oxidant enzymes were investigated, starting with HSP70. Confirming previous results (Carlsson 2003), HSP70 is upregulated after 4 days in simulated microgravity and returns to the basal level

thereafter. After 10 days in simulated microgravity, the overexpression of SIRT2, PON2, SOD2 is detected. SIRT2 is a member of the sirtuin family, NAD^+-dependent deacetylases which link their enzymatic activity to the energy state of the cell. SIRT2 is primarily in the cytoplasm where it co-localizes with microtubules and is able to shuttle to nucleus in the G2/M phase of the cell cycle (North 2003). SIRT2 prevents inflammation by reducing the levels of cytokines and decreases the levels of ROS by elevating the expression SOD2, catalase, and glutathione peroxidase (Wang 2007). Accordingly, SOD2 was upregulated in simulated microgravity, as detected by protein array and also PON2 increases in HUVEC in the RWV. PON2 is part of the paraxonase family, highly conserved proteins differently located within the cell. In particular, PON2 is localized in endoplasmic reticulum, mitochondria and peri-nuclear region. PON2 has an enzymatic lactonase activity, which protects against bacterial infection, prevents apoptosis and blocks the formation of $O_2^{\bullet -}$ (Altenhöfer 2010). Another stress protein which resulted increased in simulated microgravity is HSP27. Interestingly, a protective role of HSP27 against atherosclerosis and vasculopathies was reported (Ferns 2009). HSP27 function depends upon its phosphorylation (Garrido 2002). After 10 days in simulated microgravity also P-HSP27 is increased. Wild type HSP27 lowers the levels of ROS by raising intracellular glutathione (Vidyasagar 2012). P-HSP27 prevents apoptosis interfering with the caspase cascade. The over-expression of HSP27 and P-HSP27 significantly inhibits the proliferation of endothelial cells (Trott 2009) and, conversely, confluent cells overexpress HSP27 and P-HSP27. On these bases, it is possible to propose that, upon reaching confluence at day 10, HUVEC in the RWV upregulate HSP27 and its phosphorylated form, which contribute to prevent oxidative stress and apoptosis, respectively.

Overall, the adaptation of HUVEC to simulated microgravity is a very dynamic process. Initially, it is HSP70 that is involved, while at day 10 it is the upregulation of SIRT2, PON2, SOD2 and HSP27 that counterbalances the pro-oxidant effects of TXNIP (Figure 65).

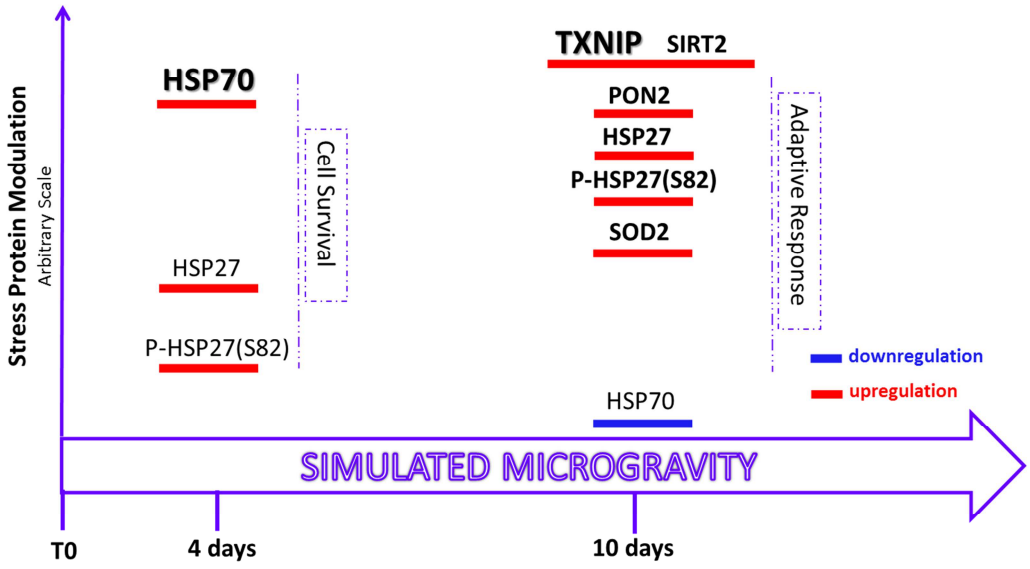


Fig. 65 Stress Protein modulation in HUVEC exposed to simulated microgravity after 4 and 10 days.

It is interesting to underline that no significant alteration of the levels of cytokines and chemokines has been observed in HUVEC in simulated microgravity, in agreement with previous studies (Griffoni 2011, Versari 2007, Grenon 2013). These data demonstrate that no endothelial dysfunction occurs in simulated microgravity thanks to the prompt adaptation to mechanical unloading.

4.4 CONCLUSIONS

Gravity shaped life on this planet and, therefore, it is not surprising that gravitational unloading promotes the activation of adaptive responses and reprograms cell activities. As of March 2015 more than 1900 experiments were performed in microgravity in the field of biomedicine. A lot has been learnt, but still many aspects need to be unraveled.

It is now clear that all the cells studied in microgravity activate a stress response. In some cell types, this is not sufficient to prevent cell death, but most cells survive and modulate their function.

Importantly, all the cells exposed to microgravity remodel their cytoskeleton. The cytoskeleton senses the reduced mechanical loading and then generates chemical signals that activate the stress response necessary to maintain the cells viable. HSP70 is reported to be rapidly upregulated in most cells cultured in microgravity, including endothelial and bone cells, and might mediate the initial cellular response to microgravity.

In macrovascular endothelial cells the adaptive response is rapidly activated (Carlsson 2003) after culture in microgravity and changes over time, thus allowing the cells to remain functional. This is crucial, because endothelial cells are the gate-keeper of a healthy vasculature, thus preventing vascular diseases. Also bone mesenchymal stem cells rapidly adapt to microgravity in order to correctly respond to osteogenic stimuli. Consequently, these cells do not seem to be involved in microgravity-associated osteoporosis. My studies indicate that, in addition to a direct detrimental effect of gravitational unloading on osteoblasts described by different authors (Rodionova 2002, Rucci 2007, Nabavi 2011, Yang 2015, Zhang 2015), an altered intercellular communication between microvascular endothelium and osteoblasts might contribute to inhibit bone deposition. It will be interesting to develop co-culture systems for experiments in microgravity.

In general, the main challenge, which has to be overcome, remains the need to increase the number of experiments in space decreasing the costs.

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APPENDIX

1. APPENDIX A

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2. APPENDIX B

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3. APPENDIX C

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Research Article

Conditioned Media from Microvascular Endothelial Cells Cultured in Simulated Microgravity Inhibit Osteoblast Activity

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Background and Aims. Gravity contributes to the maintenance of bone integrity. Accordingly, weightlessness conditions during space flight accelerate bone loss and experimental models in real and simulated microgravity show decreased osteoblastic and increased osteoclastic activities. It is well known that the endothelium and bone cells cross-talk and this intercellular communication is vital to regulate bone homeostasis. Because microgravity promotes microvascular endothelial dysfunction, we anticipated that the molecular cross-talk between endothelial cells exposed to simulated microgravity and osteoblasts might be altered. *Results*. We cultured human microvascular endothelial cells in simulated microgravity using the rotating wall vessel device developed by NASA. Endothelial cells in microgravity show growth inhibition and release higher amounts of matrix metalloproteases type 2 and interleukin-6 than controls. Conditioned media collected from microvascular endothelial cells in simulated microgravity were used to culture human osteoblasts and were shown to retard osteoblast proliferation and inhibit their activity. *Discussion*. Microvascular endothelial cells in microgravity are growth retarded and release high amounts of matrix metalloproteases type 2 and interleukin-6, which might play a role in retarding the growth of osteoblasts and impairing their osteogenic activity. *Conclusions*. We demonstrate that since simulated microgravity modulates microvascular endothelial cell function, it indirectly impairs osteoblastic function.

1. Introduction

Bone development and remodeling depend mainly upon complex interactions between osteoblasts and osteoclasts. Indeed, an intimate communication exists between osteoblasts and osteoclasts since osteoclasts control osteoblastic growth and function, while osteoblasts regulate the differentiation and the activity of osteoclasts [1]. Recently, other cells of the bone microenvironment are emerging as implicated in bone health. Among others, endothelial cells are players of the communication network in the bone [2]. In embryonic skeletal tissue, osteogenesis and angiogenesis are temporally related [3] and, in the adults, osteoblasts are always located adjacent to endothelial cells in blood vessels at sites of new bone formation [4]. The fact that older subjects with osteoporosis have decreased blood vessels in their skeletal tissue, accompanied by a parallel decrease in osteoblasts, further highlights this close relation [5]. Several lines of evidence indicate that a mutual communication system exists between the endothelium and the osteoblasts. At the cellular and molecular levels, vascular endothelial cells have been shown to regulate bone remodelling via cell signalling networks of ligand-receptor complexes and osteoblasts release growth factors that influence endothelial cells [3].

In long duration space missions, astronauts experience considerable bone loss, about 1-2% of bone mass per month in the weight-bearing regions of the leg and the spine, mainly because of an uncoupling between osteoblasts and osteoclasts [6–8]. We anticipate that endothelial-osteoblast communication might be impaired in space and contributes to bone loss. Indeed, dysfunctions in human endothelial cells cultured in simulated microgravity have been described [9–15], and alterations in the capillaries of the epiphyses and metaphyses of femoral bones of rats flown aboard the US laboratory SLS-2 were detected [16].

Cross-talk between endothelial cells and osteoblasts in simulated microgravity has not been deciphered yet. As

a first approach to investigate this issue, we exposed osteoblasts to conditioned media (CM) from microvascular endothelial cells (HMEC) cultured in the rotating wall vessel (RWV), which simulates some aspects of microgravity. Studies utilizing CM are considered a successful strategy for the identification of soluble factors interconnecting different cell types and candidate biomarkers for further validation in clinical samples [17]. Indeed, CM reveal the cell secretome, that is, the collection of proteins that are released through the classical and nonclassical secretion pathways, and also proteins shed from the cell surface. These secreted proteins include enzymes, growth factors, cytokines, and other soluble mediators and are important contributors to cell survival, growth, and differentiation [17]. We here show that CM from HMEC grown in simulated microgravity impair the proliferation and activity of cultured primary osteoblasts and osteoblast-like Saos-2 cells.

2. Materials and Methods

2.1. Cell Culture. HMEC were obtained from CDC (Atlanta, USA) and grown in MCDB131 containing epidermal growth factor (10 ng/mL) and 10% fetal bovine serum (FBS) on 2% gelatin-coated dishes. Normal human osteoblasts (NHOst) were maintained in osteoblast growth media (OGM) as indicated by the manufacturer (Lonza, Basel, Switzerland) at 37°C in a humidified atmosphere containing 5% CO_2 [18]. Saos-2 cells (American Type Culture Collection) were cultured in DMEM containing 10% FBS. Before beginning the experiments with CM from HMEC, NHOst and Saos-2 cells were gradually adapted to be cultured in 1 : 1 HMEC growth medium and OGM or DMEM, respectively. To simulate microgravity, we utilized the RWV (Synthecom Inc, Houston, TX, USA). HMEC were seeded on beads (Cytodex 3, Sigma Aldrich, St. Louis, MO, USA); as controls (CTR), HMEC grown on beads were cultured in the vessels not undergoing rotation [11]. In the RWV, the vessel rotates around a horizontal axis (28 rpm) and allows diffusion of oxygen and carbon dioxide across a semipermeable membrane. The vessel wall and the medium containing cells bound to microcarrier beads rotate at the same speed, producing a vector-averaged gravity comparable with that of near-earth free-fall orbit [19]. The beads do not form aggregates in the RWV and tend to be evenly distributed throughout the vessel. Such a rotation reduces gravity to approximately 3×10^{-2} g [10]. After 72 h in the RWV or in the vessels without rotation, the media from HMEC were collected, centrifuged, filtered through 0.2 μm filter, diluted 1 : 1 with fresh culture medium to replenish nutrients, and used to culture NHOst and Saos-2 cells. In these experiments, the medium was changed every 48 h.

2.2. DNA Fragmentation. HMEC cell death was evaluated using the cell death detection ELISA (Roche) which determines cytoplasmic histone-associated DNA fragments. Briefly, after 48 and 72 h in the RWV or under control conditions, the cells were lyzed and centrifuged and the supernatant was analyzed according to the manufacturer's instruction. As a positive control, we used HMEC exposed for 30 min to H_2O_2 (10 μM) and cultured for additional 48 h in their growth medium.

2.3. Cell Proliferation. For MTT assay, NHOst and Saos-2 at 50% confluence were cultured in 96-well plates for 24 h before being exposed for different times to the media collected from HMEC. MTT measures the reduction of yellow tetrazolium salt MTT to dark purple formazan by succinate dehydrogenase, mainly in mitochondria and it is now widely accepted as a reliable way to examine cell viability and proliferation [20]. Briefly, at the end of the experiment, the media were replaced with medium containing 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, 0.5 mg/mL) (Sigma Aldrich, St. Louis, MO, USA). Formazan crystals generated by the cellular reduction activity were dissolved in DMSO. Absorbance was measured at 550 nm.

Neutral red uptake assay was also used to estimate NHOst viability. Briefly, 24 h after seeding in 96-well dishes, the cells were exposed to CM from HMEC. After 3 days, neutral red was added to the medium to a final concentration of 50 μg/mL. 2 h later, the wells were washed with PBS and fixed. Absorbance was measured at 550 nm [21].

HMEC and Saos-2 cells were trypsinized and stained with trypan blue solution (0.4%) and the viable cells were counted using a Burker chamber.

2.4. Osteoblast Activity. NHOst and Saos-2 cells at 80% confluence were cultured in 24-well plates with conditioned media from HMEC added with 100 nM dexamethasone, 50 μM L-ascorbate-2-phosphate, and 10 mM glycerophosphate, at 37°C in a 5% CO_2 for 7 and 14 days. Osteoblast activity was evaluated quantifying alkaline phosphatase (ALP) enzymatic activity in the medium by a colorimetric assay based on the hydrolysis of P-nitrophenyl phosphate. The absorbance was measured at 405 nm [18]. To analyze calcium deposition, the cells were rinsed with PBS, fixed (70% ethanol, 1 h), and stained for 10 min with 2% Alizarin Red S (pH 4.2). Cultures were photographed with a digital camera. Alizarin Red was then released from the cell matrix by incubation for 15 min in 10% cetylpyridinium chloride in 10 mM sodium phosphate (pH 7.0). The absorbance was measured at 562 nm [18].

2.5. Measurements of TIMP-2 and IL-6 by ELISA. Conditioned media were centrifuged and filtered. The amounts of tissue inhibitor of matrix metalloprotease (TIMP)-2 and interleukin (IL)-6 were measured using a double-antibody sandwich ELISA (GE Healthcare) according to the manufacturer's instructions. The concentrations of TIMP-2 and IL-6 were determined by interpolation from a standard curve.

2.6. Western Blot. HMEC cells were lysed, separated on SDS-PAGE, and transferred to nitrocellulose sheets. Western analysis was performed using antibodies against p21, p53, and GAPDH (Tebu Bio-Santa Cruz). Secondary antibodies were labelled with horseradish peroxidase (Amersham Pharmacia Biotech). The SuperSignal chemiluminescence kit (Pierce) was used to detect immunoreactive proteins.

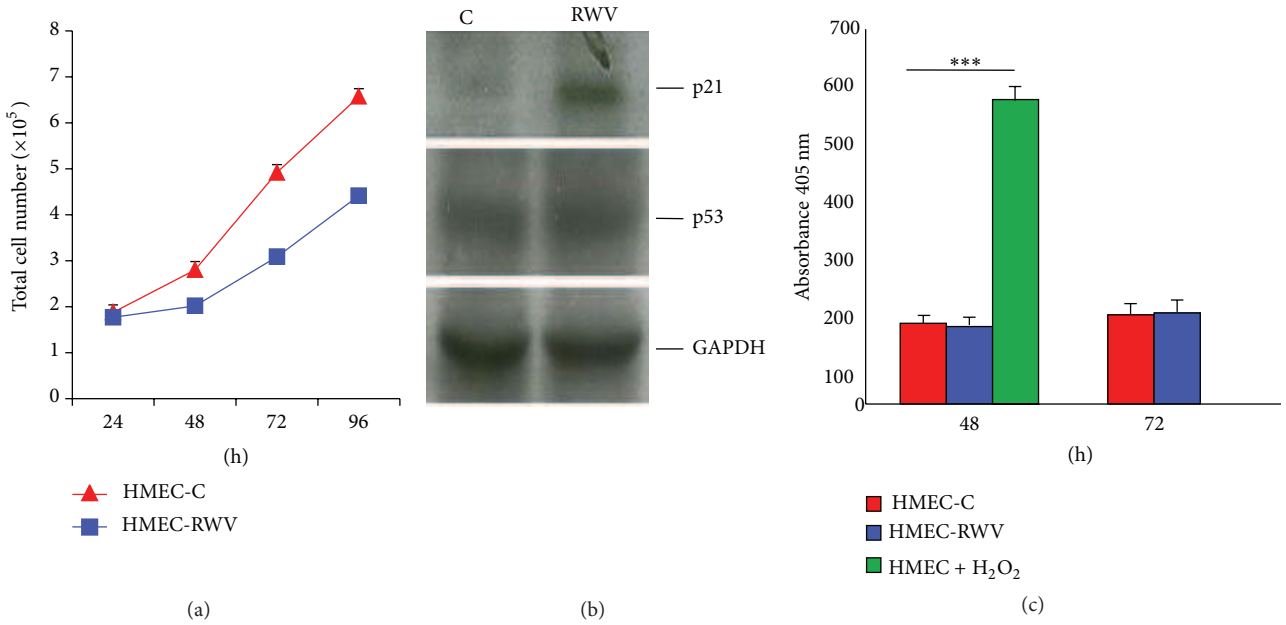

Figure 1: Simulated microgravity inhibits HMEC growth. (a) HMEC were cultured for different times in the RWV (HMEC-RWV) and trypsinized and viable cells were counted. HMEC-C: control. (b) Cell extracts (50 μg/lane) were loaded on a 15% SDS-PAGE, blotted into nitrocellulose filter, incubated with anti-p21 and anti-p53 antibodies, and visualized by chemiluminescence as described. After stripping, the blot was incubated with an anti-GAPDH antibody to show that comparable amounts of proteins were loaded per lane. (c) Apoptosis was evaluated by ELISA on HMEC lysates after 48 and 72 h in the RWV or under control conditions. Our positive control is represented by HMEC exposed to H_2O_2 for 30 min and then cultured for additional 48 h.

2.7. Statistical Analysis. All experiments were repeated at least three times in triplicate. Data are presented as means ± standard deviation. Statistical differences were determined using the unpaired two-tailed Student's t test. Consider $^{*}P < 0.05$, $^{**}P < 0.01$.

3. Results

3.1. Simulated Microgravity Alters HMEC Behaviour. Figure 1(a) shows that culture in the RWV retarded HMEC proliferation. Accordingly, growth inhibition correlated with the upregulation of p21 (WAF1), an inhibitor of cyclin-dependent kinases, as detected by western blot, and this event seems to be p53-independent since no modulation of p53 was observed in HMEC (Figure 1(b)). We also show that no cell death is detectable after 48 and 72 h culture in the RWV (Figure 1(c)). It is noteworthy that similar results were obtained when microgravity was simulated using the random positioning machine (RPM) (data not shown). On the basis of results obtained by protein array on 40 proteins involved in inflammation, we validated the increase of IL-6 and TIMP-2 in the CM from HMEC cultured for 48 and 72 h in the RWV and relative controls by ELISA. Figure 2(a) shows that TIMP-2 is significantly increased in the media collected from HMEC after 48 and 72 h in the RWV, while secreted IL-6 was increased after 72 h culture in simulated microgravity (Figure 2(b)). On these bases, we decided to use 72 h conditioned media from HMEC for the experiments on bone cells.

3.2. HMEC Secreted Factors Impact on NHOst Cell Proliferation and Osteogenic Activity. We evaluated the effects of CM from HMEC on NHOst cell proliferation. MTT assay revealed a significant reduction of NHOst cell proliferation cultured in the presence of CM from HMEC in simulated microgravity (Figure 3(a)). These results were confirmed by neutral red assay, which estimates the number of viable cells in a culture on the basis of their ability to incorporate and bind the supravital dye neutral red in the lysosomes (Figure 3(b)). We did not detect any significant difference in cell death in NHOst exposed to the conditioned media from HMEC cultured for 72 h in the RWV and relative controls (not shown).

To evaluate osteoblastic activity, NHOst cells were cultured for 7 and 14 days in a 24-well plate with CM from HMEC added with an osteogenic cocktail containing 100 nM dexamethasone, 50 μM L-ascorbate-2-phosphate, and 10 mM glycerophosphate. Two parameters were evaluated, that is, ALP activity, which has long been recognised as a reliable indicator of osteoblastic activity, and calcium deposition by Alizarin Red Staining.

ALP enzymatic activity was measured after 7 and 14 days by a colorimetric assay. Figure 4(a) shows that media from HMEC in simulated microgravity inhibited ALP activity. To analyze calcium deposition, we used the Alizarin Red S Staining. Figure 4(b) shows that CM from HMEC exposed to simulated microgravity markedly inhibited the deposition of mineral matrix.

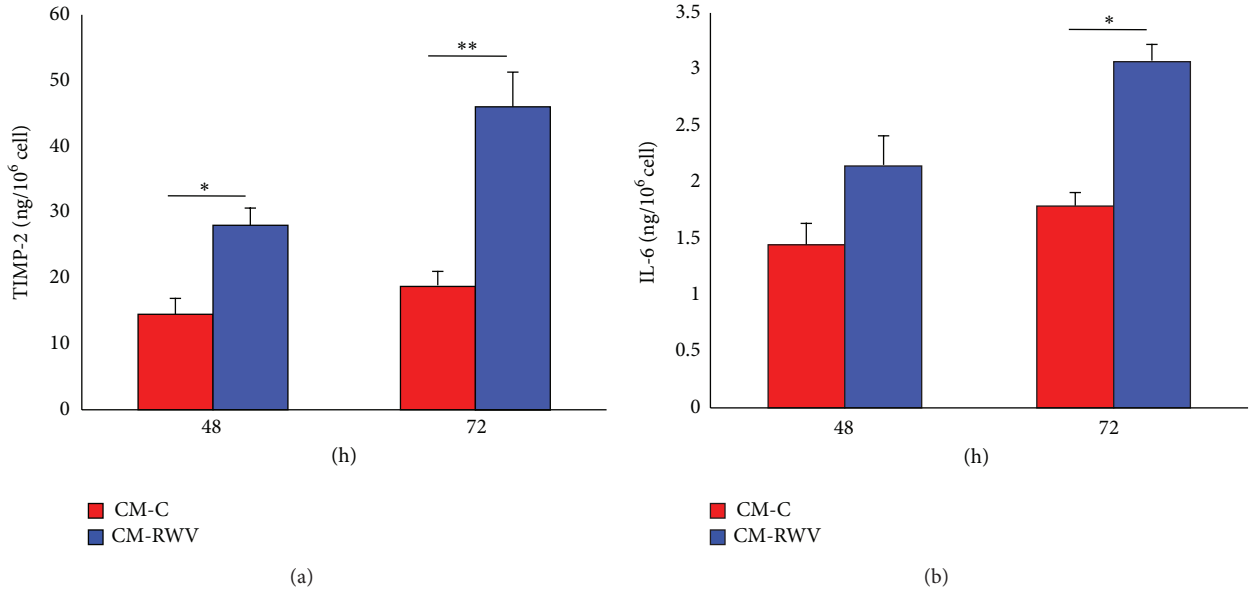

Figure 2: Simulated microgravity induces TIMP-2 and IL-6 secretion by HMEC. TIMP-2 (a) and IL-6 (b) were measured by ELISA in media collected after different times of culture in the RWV (CM-RWV) or from relative controls (CM-C).

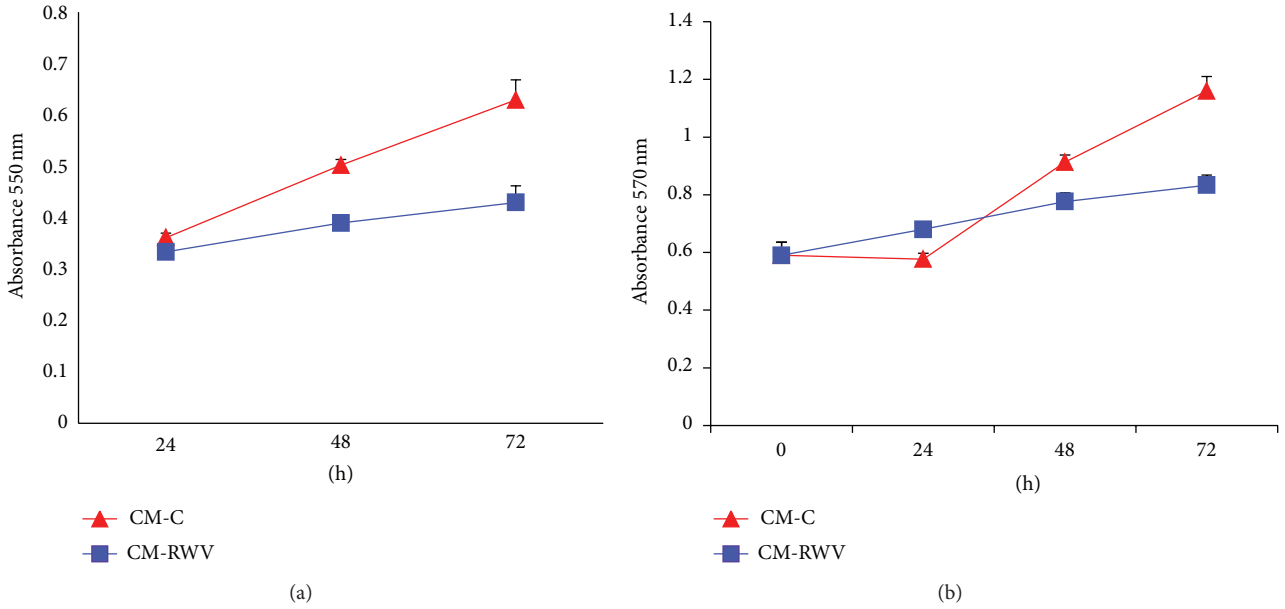

Figure 3: CM from HMEC in simulated microgravity inhibit NHOst proliferation. NHOst were cultured for different times with CM from HMEC in simulated microgravity (CM-RWV) or by HMEC controls (CM-C). Viable cells were evaluated by MTT assay (a) and neutral red (b) and the absolute absorbance values are shown. Data are expressed as the mean ± standard deviation of three different experiments performed in triplicate.

3.3. HMEC Secreted Factors Impact on Saos-2 Cell Proliferation and Osteogenic Activity. Many factors, such as age, gender, and site of isolation, influence the behavior of primary osteoblasts [22]. We therefore performed experiments also on an immortalized cell line to reproduce the results obtained in NHOst and we chose Saos-2 cells because they closely resemble primary osteoblasts [22]. Indeed, Saos-2 cells are used as representative of primary osteoblasts when standard tests are evaluated [23].

Saos-2 cells were exposed to CM from HMEC in the RWV and relative controls for different times. MTT assay shows that media from HMEC in the RWV impair cell proliferation (Figure 5(a)). These results were confirmed when the cells were counted (Figure 5(b)).

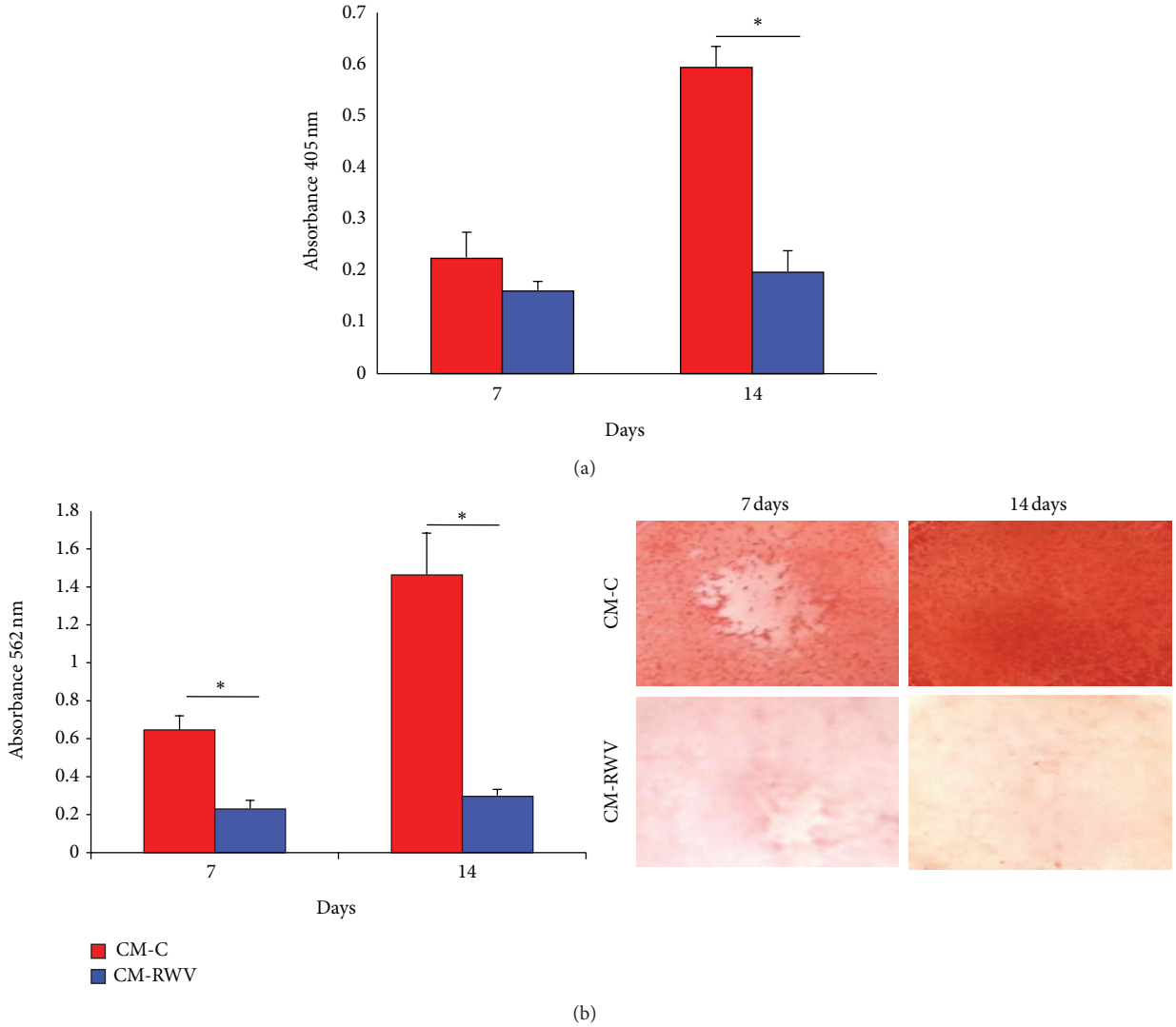

Figure 4: CM from HMEC in simulated microgravity inhibit NHOst activity. NHOst were cultured for 7 and 14 days with medium conditioned by HMEC in simulated microgravity (CM-RWV) or by HMEC controls (CM-C) both added with osteogenic stimuli. (a) ALP enzymatic activity was quantified by spectrophotometric analysis as described. Absorbance was measured at 405 nm. (b) Alizarin Red Staining was performed. Photographs were taken before acid extraction. Absorbance was measured at 562 nm.

Confluent Saos-2 cells were then cultured in CM from HMEC in simulated microgravity or HMEC controls both added with the osteogenic cocktail and were stained with Alizarin Red to evaluate the formation of calcium phosphate in culture [18]. We found that 14-day culture in the conditioned media from HMEC in the RWV inhibited ALP activity (Figure 6(a)). The inhibition of Saos2 cell activity was confirmed by demonstrating lower amounts of deposition of mineral matrix in cell cultured with the CM from HMEC in the RWV (Figure 6(b)).

4. Discussion

Bone loss in space has been reported in humans and in several experimental models [8]. All the *in vivo* results obtained in space point to major alterations of bone cells. Bone cells have been extensively studied *in vitro* both in space and on ground using different devices to simulate microgravity to conclude that microgravity alters the morphology of these cells [24], impairs the differentiation of osteoblasts [25], and increases the activity of osteoclasts [8]. All these results are not surprising since gravitational forces contribute to the maintenance of bone integrity and affect bone remodeling to adjust to mechanical demands.

Bone vasculature is important for skeletal development during the embryonic stage, postnatal growth, and bone remodeling. It supplies oxygen, nutrients, hormones, cytokines, and bone precursor cells. Moreover, the communication between bone endothelium and bone cells is vital to regulate and modulate bone homeostasis. The endothelium contributes to bone health by releasing osteogenic factors [26], and bone cells produce angiogenic factors that are crucial

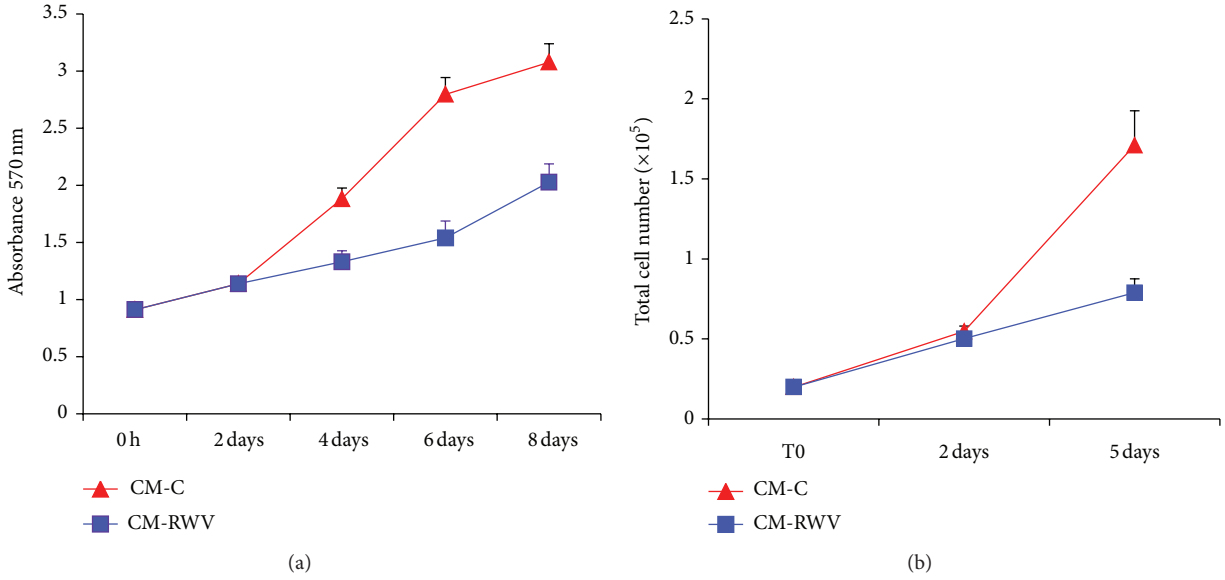

Figure 5: CM from HMEC in simulated microgravity inhibit Saos-2 proliferation. Saos-2 were cultured for different times with CM from HMEC in simulated microgravity (CM-RWV) or by HMEC controls (CM-C). Viable cells were evaluated by MTT assay (a) and the absolute absorbance values are shown. After trypsinization, viable cells were stained with trypan blue and counted (b).

for endothelial viability and survival under physiological conditions and that drive angiogenesis when needed [3].

We have shown that human endothelial cells from the umbilical vein, widely used as a model of macrovascular endothelial cells, are deeply influenced by simulated microgravity [10, 11, 27]. These results were confirmed by our recent study performed on the International Space Station (ISS) [28]. Other experiments have been performed on different types of macrovascular endothelial cells with discordant results, which can be ascribed to poor definition of the endothelial cells used [14, 15], the different culture conditions, the use of different microgravity simulators, and also the inadequate descriptions of how they were operated. Less is known about microvascular endothelial cells, which cover an area 50 times greater than that of all large vessels combined [29]. In an animal model of wound healing and in a rat fibular osteotomy model, microgravity retards neovascularization [30, 31], thus indicating the occurrence of microvascular endothelial dysfunction. Moreover, bed rest, which mimics some aspects of spaceflight, causes impairment of endothelium-dependent functions in the microcirculation [32]. We have previously demonstrated that RWV-simulated microgravity induces an antiangiogenic phenotype in HMEC [11]. In the present study, we confirm and broaden these results by showing that culture in the RWV retards HMEC cell growth without inducing apoptosis. This correlates with the upregulation of p21, an inhibitor of the cyclin/CDK2 complexes necessary for the transition from the G1 to the S phases, through a p53-independent mechanism. Our results are in disagreement with a recent report showing that culture in a clinostat induces apoptosis in pulmonary microvascular endothelial cells [12]. As mentioned above, these contrasting results might be due to differences in the cells used, in the cell culture conditions, and in the microgravity simulator utilized.

The aim of this work was to understand whether simulated microgravity impairs endothelial-osteoblast communication. To this purpose, we evaluated the effects produced on osteoblasts by CM from HMEC cultured in simulated microgravity.

We show that HMEC release factors that retard the growth of osteoblasts and severely impair their osteogenic activity. It is noteworthy that we found increased amounts of secreted TIMP-2 and IL-6, known to affect both endothelial cells and osteoblasts. Interestingly, TIMP-2 inhibits endothelial cell proliferation by a matrix metalloproteases (MMP) independent mechanism [33] and might therefore play a role in HMEC growth retardation in simulated microgravity. TIMP-2 also impairs osteoblast activity. Indeed, TIMP-2 nearly abolishes ALP expression [34] by inhibiting MT1-MMP (membrane type 1-metalloprotease) [34], a protease which is implicated in multiple steps of osteogenic differentiation and is mainly involved in ALP upregulation [35]. Interestingly, TIMP-2 inhibits cell survival of osteoblasts forced to transdifferentiate into osteocytes [36]. This result might offer a molecular explanation, at least in part, to the lysis of osteocytes in spaceflight described by Blaber et al. [37]. In media from HMEC cultured in the RWV, we also found increased amounts of IL-6, a pleiotropic cytokine implicated in acute phase response and inflammation. IL-6 not only promotes endothelial dysfunction [38] but also affects human osteoblast differentiation [39], thus contributing to osteopenia.

We therefore propose that microgravity impacts both directly and indirectly on osteoblasts. Microgravity has been shown to directly inhibit osteoblasts. In addition, by modulating microvascular endothelial cell function, microgravity indirectly exerts inhibitory effects on osteoblasts.

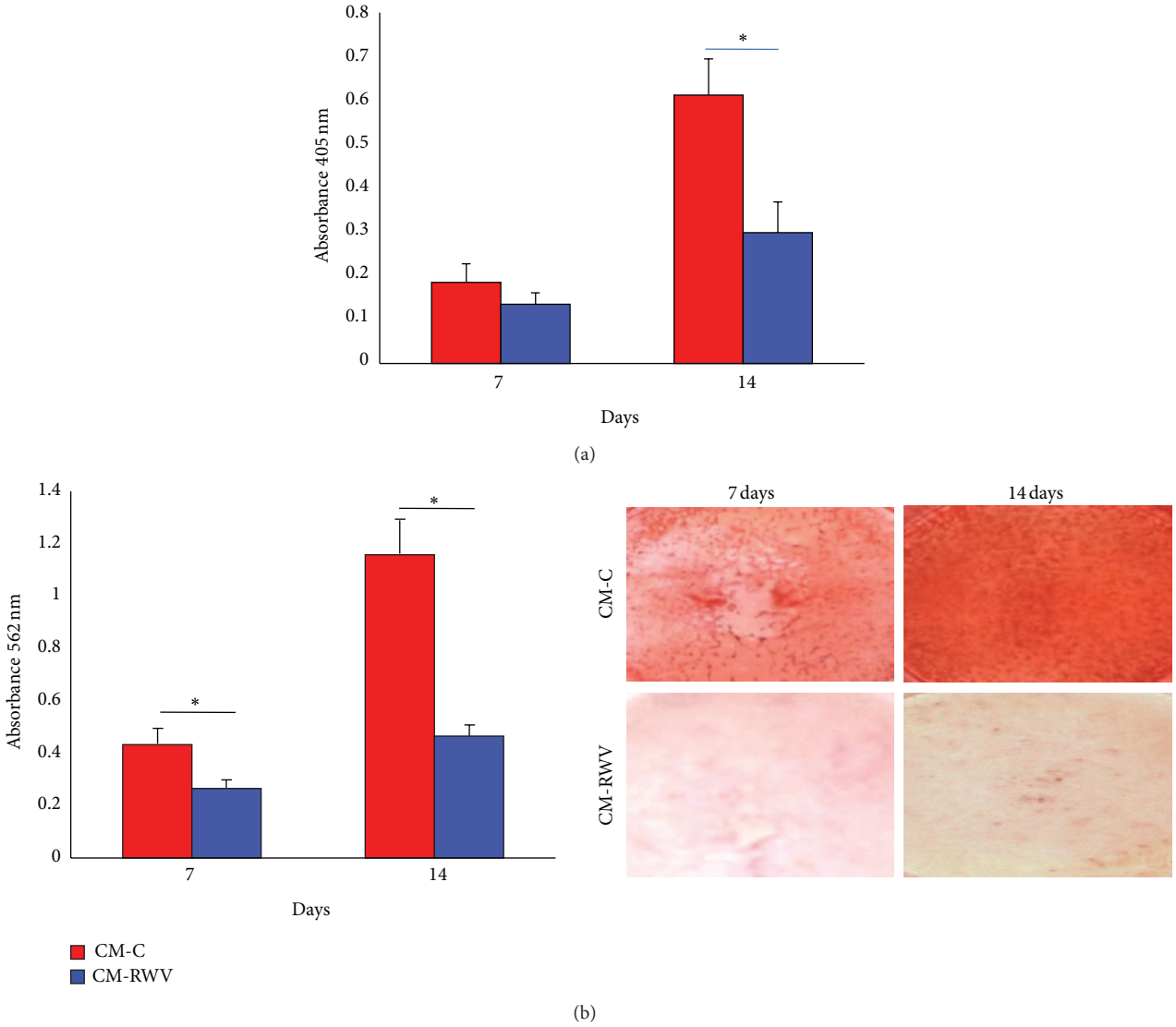

Figure 6: CM from HMEC in simulated microgravity inhibit Saos-2 activity. Saos-2 were cultured for 7 and 14 days with CM from HMEC in simulated microgravity (CM-RWV) or by HMEC controls (CM-C) both added with osteogenic stimuli. (a) ALP enzymatic activity and (b) Alizarin Red Staining were performed as above.

The current space programs onboard the ISS and the future human exploration of Mars require long duration missions. However, several biomedical issues still need to be clarified before these missions can take place without causing health problems to the astronauts. Our results suggest that endothelial dysfunction might represent a common denominator for cardiovascular deconditioning and for bone loss and offer a new light to interpret the behaviour of mammalian skeleton in microgravity. Eventually, these results might foster studies to develop countermeasures that target the endothelium to improve both bone homeostasis and vascular function.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Impact of simulated microgravity on human bone stem cells: New hints for space medicine

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ABSTRACT

Bone loss is a well known early event in astronauts and represents one of the major obstacle to space exploration. While an imbalance between osteoblast and osteoclast activity has been described, less is known about the behavior of bone mesenchymal stem cells in microgravity.

We simulated microgravity using the Random Positioning Machine and found that mesenchymal stem cells respond to gravitational unloading by upregulating HSP60, HSP70, cyclooxygenase 2 and superoxyde dismutase 2. Such an adaptive response might be involved in inducing the overexpression of some osteogenic transcripts, even though the threshold to induce the formation of bone crystal is not achieved. Indeed, only the addition of an osteogenic cocktail activates the full differentiation process both in simulated microgravity and under static 1G-conditions.

We conclude that simulated microgravity alone reprograms bone mesenchymal stem cells towards an osteogenic phenotype which results in complete differentiation only after exposure to a specific stimulus.

1. Introduction

Human bone mesenchymal stem cells (bMSC) possess capacity for self-renewal and multilineage differentiation potential into osteoblasts, chondrocytes and adipocytes [1]. Because of these properties, bMSC are crucial for tissue homeostasis and for repair after injury, and are emerging as attractive tools for tissue engineering and regenerative medicine.

Upon specific stimuli, bMSC are induced to differentiate into osteoblasts, which deposit new bone matrix. Dysfunction of bMSC in terms of self-renewal and differentiation potential has been described in senile and post-menopausal osteoporosis. bMSC derived from postmenopausal osteoporotic patients tend to differentiate in adipocytes rather than osteogenic cells [2]. In aging, alterations of the cytoskeleton and increased oxidative stress seem to be implicated in reducing bMSC response to signals from the microenvironment [3].

Bone loss (1–2% a month) is also well documented in astronauts [4] and it represents a key concern and a limiting factor for space exploration. Space-associated osteopenia is a very early event which affects especially weight-bearing bones and requires a very long time for recovery after return to earth [5]. It is linked to the impaired activity of osteoblasts and the increased function of osteoclasts as the result of gravitational unloading due to microgravity [6]. Since space flights are infrequent and expensive, several devices have been invented to simulate microgravity on earth, even though they mimic only some aspects of real microgravity. Thanks to these bioreactors, significant alterations in the behavior of osteoclasts and osteoblasts have been described [7]. Contrasting results are reported about bMSC exposed to simulated microgravity. Indeed, some authors report the inhibition of bMSC differentiation [8,9], while others demonstrate their increased differentiation [10,11] to the point of suggesting the use of bioreactors simulating microgravity for tissue engineering [12]. These discrepancies might be ascribed to the use of different microgravity bioreactors, protocols to culture the cells and induce their differentiation, and analytical procedures.

Recently, we have contributed to an experiment onboard the International Space Station (ISS) to test the behavior of cultured bMSC in space. While waiting for the cells to return to earth, we investigated the effects of simulated microgravity generated by the Random Positioning Machine (RPM) on the same bMSC that were space-flown. RPM is a 3D clinostat widely used before and after space flight experiments [13], which has yielded challenging insights into the behavior of cells and simple organisms. In particular,

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RPM proved to be a valuable tool for simulating microgravity in adherent cells [14].

We examined the expression of some genes involved in osteogenic differentiation in bMSC cultured in the RPM or in static 1G-conditions, with and without the addition of an osteogenic medium. In particular, we focused our studies on i) *RUNX2*, which is known to be the master switch of osteogenesis, since complete absence of ossification was observed in *RUNX2* knockout mice [15]; ii) Osterix (*OSX*), which is fundamental in promoting the early stages of osteogenesis but it is not sufficient to achieve a full differentiation into osteoblast; iii) collagen 1A1 (*COL1A1*), essential for progression of differentiation at early stages [15]; iv) osteocalcin (*OSC*) and osteopontin (*OSP*), the most abundant non collagenous components of bone extracellular matrix, both crucial for the osteogenic phenotype [15].

We show that culture of bMSC in the RPM i) activates the stress response and ii) accelerates the expression of several osteogenic genes, apart from *COL1A1*. Only the addition of an osteogenic cocktail containing vitamin D allows the differentiation of bMSC with deposition of bone crystals.

2. Materials and methods

2.1. Culture of bMSC

bMSC were isolated from adult human bone marrow withdrawn from bilateral punctures of the posterior iliac crests of a normal volunteer [16] and tested for purity by flow cytometry at the Policlinico in Milan according to institutional guidelines approved by the IRCCS Policlinico (donor 1). These cells were cultured at 37 °C and 5% CO_2 in Dulbecco's Modified Eagle's Medium with 1000 mg/L glucose and containing 10% fetal bovine serum and 2 mM glutamine (culture medium, CM). All the reagents for cell culture were from Sigma–Aldrich, St. Louis, Missouri, USA. The behavior of these primary cells was compared with that of bMSC purchased from Lonza, Basel, Switzerland (donor 2) which were cultured according to manufacturer's instructions. The cells were used between passage 2 and 5.

2.2. Culture in the RPM

Microgravity conditions were simulated using the RPM (Dutch Space, Leiden, Netherlands) [13]. The RPM provides continuous random change in orientation relative to the gravity vector of an accommodated experiment. Culture flasks containing confluent monolayers 72 h after seeding were completely filled with medium supplemented with 12.5 mM HEPES (Sigma–Aldrich) devoid of air bubbles and fixed in the RPM, as close as possible to the centre of the platform, which was then rotated using the real random mode (random speed and random direction) of the machine. The RPM operated at 37 °C. 1G ground control cultures, treated in parallel in identical equipment, were placed on the basis of the RPM.

2.3. In vitro osteogenic differentiation of bMSC

The cells were seeded in T25 flasks. Once the cells were confluent, an osteogenic induction cocktail was added to the medium (osteogenic medium, OM). The osteogenic cocktail contains 2×10^{-8} M 1α,25-Dihydroxyvitamin D_3, 10 mM β-glycerolphosphate and 0.05 mM ascorbic acid (Sigma–Aldrich).

To analyze calcium (Ca) deposition by bMSC, the cells were rinsed with PBS, fixed (70% ethanol, 1 h) and stained for 10 min with 2% Alizarin Red S (pH 4.2, Sigma–Aldrich) [17]. Alizarin Red S staining was released from the cell matrix by incubation in 10% cetylpyridinium chloride (Sigma–Aldrich) in 10 mM sodium phosphate (pH 7.0), for 15 min and the absorbance measured at 562 nm.

2.4. Real-Time PCR

Total RNA was extracted by the PureLink RNA Mini kit (Ambion, Thermo Fisher Scientific, Waltham, MA, USA). Single-stranded cDNA was synthesized from 0.2 μg RNA in a 40 μl final volume using High Capacity cDNA Reverse Transcription Kit, with RNase inhibitor (Applied Biosystems, Thermo Fisher Scientific) according to the manufacturer's instructions. Real-time PCR was performed three times in triplicate on the 7500 FAST Real Time PCR System instrument using TaqMan Gene Expression Assays (Life Technologies, Monza, Italy): Hs00231692_m1 (*RUNX2*), Hs01866874_s1 (*OSX*), Hs00164004_m1 (*COL1A1*), Hs01587814_g1 (*OSC*) and Hs00959010_m1 (*OSP*). The housekeeping gene *GAPDH* (Hs99999905_m1) was used as an internal reference gene. Relative changes in gene expression were analyzed by the $2^{-\Delta\Delta Ct}$ method [18].

2.5. Protein array

After 24 h of culture in the RPM or in static 1G-conditions, bMSC were lysed in lysis buffer (50 mM TrisHCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% NP-40). Protein concentrations were determined using the Bradford protein assay (Sigma–Aldrich). Cell extracts (80 μg) were utilized to incubate the membranes on which 26 antibodies against human cell stress-related proteins were spotted in duplicate (R&D systems, Space Import Export, Milan, Italy). The array was performed according to the manufacturer's instructions. Densitometry was performed by the ImageJ software. Two separate experiments were performed and data are expressed as % of the variation in the signal intensity of RPM *vs* static 1G-conditions.

2.6. Statistical analysis

Statistical significance was determined using Student's t test and set as following: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

3. Results

3.1. Culture in the RPM induces the expression of RUNX2 and OSX in bMSC from two donors

Because of individual biologic variability, initially we investigated the effects of simulated microgravity on the expression of osteogenic markers using bMSC from two healthy male donors. The cells were cultured in CM for 4 days in the RPM or in static 1G-conditions as a control. By RT-PCR a significant increase of the transcripts for *RUNX2* and *OSX* was detected in bMSC from both donors when cultured in the RPM (Fig. 1A). We found no modulation of *PPARγ*, a master regulator of adipogenesis (Fig. 1A) [19].

To understand whether the overexpression of *RUNX2* and *OSX* suffices osteogenesis, we cultured bMSC in the RPM for 10 days, which is the time usually necessary to detect calcium deposition. By Alizarin Red S staining, which reveals the formation of calcium nodules, we did not observe any deposition of calcium in the matrix of bMSC from the two donors (Fig. 1B).

Since similar results were obtained from the two donors, we continued our studies on bMSC of donor 1, which were used for experiments onboard the ISS.

3.2. Culture in the RPM activates stress response

After 24 h of culture in the RPM, no major modifications of cell

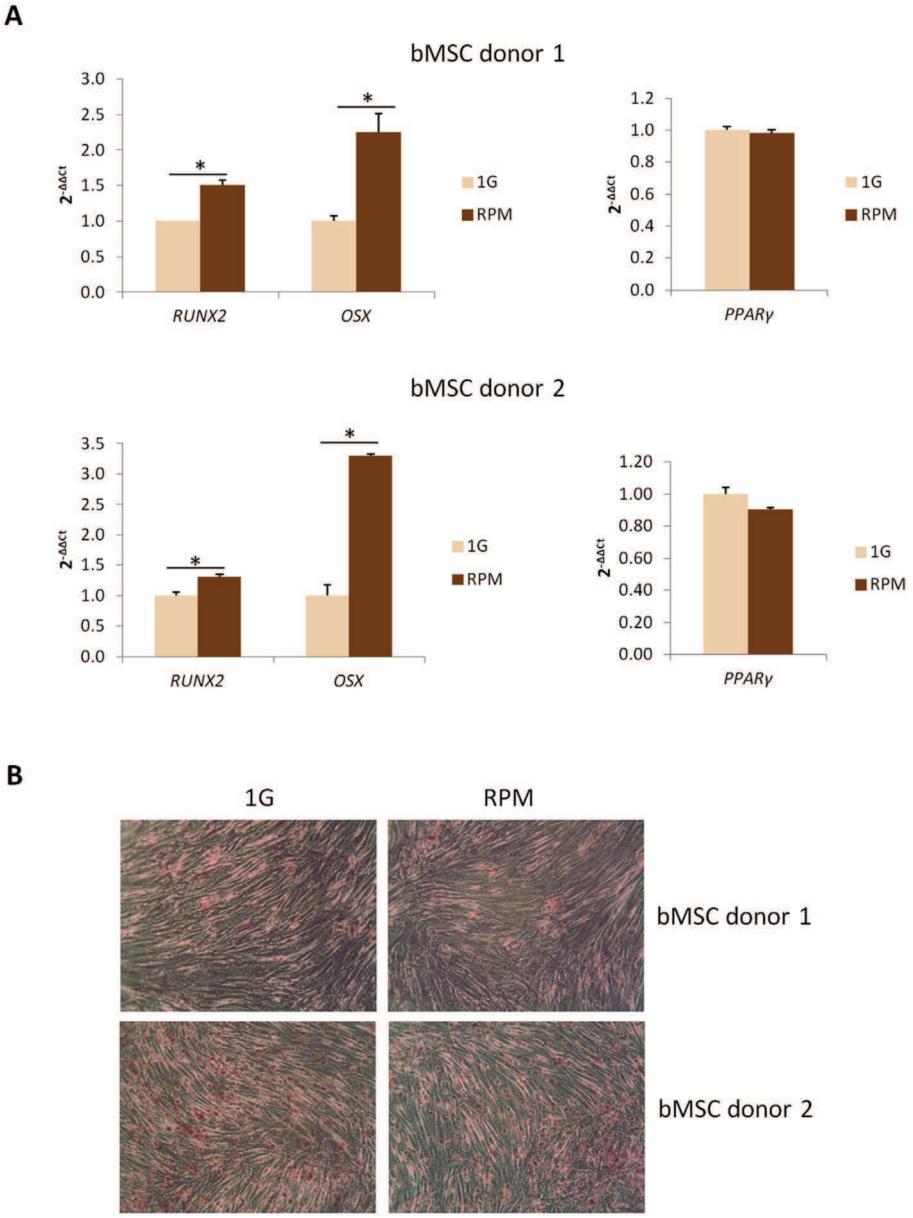

Fig. 1. bMSC from two different donors were cultured in the RPM or in static 1G-conditions. (A) After 4 days the RNA was extracted and a Real-Time PCR was performed three times in triplicate using primers designed on *RUNX2*, *OSX* and *PPARγ* sequence. (B) After 10 days Alizarin Red S staining was performed and photographs were taken at 10x magnification.

morphology were observed (Fig. 2A). We then investigated the levels of stress proteins by a targeted protein array. Out of 26 proteins involved in stress response, we detected the significant upregulation of HSP60, HSP70, cyclooxygenase (COX)2 and superoxyde dismutase (SOD)2 in bMSC cultured in the RPM (Fig. 2B).

3.3. The osteogenic medium induces the expression of osteogenic markers and Ca deposition both in simulated microgravity and static 1G-conditions

We then cultured bMSC in presence or in the absence of OM containing vitamin D for 4 and 10 days in the RPM. In addition to *RUNX2* and *OSX*, we also evaluated *COL1A1*, *OSP* and *OSC*.

Notably, it was sufficient to culture the cells in their CM in the RPM for 4 or 10 days to upregulate *RUNX2*, *OSX*, *OSP* and *OSC* while *COL1A1* did not change (Fig. 3A).

After 4 days, the osteogenic cocktail induced the expression of *RUNX2*, *OSX*, *OSP*, *OSC* and also *COL1A1* in bMSC in the RPM and under static 1G-conditions (Fig. 3A). The induction of all these genes, apart from *COL1A1*, is higher in bMSC in the RPM than in static 1G-conditions.

After 10 days, the addition of OM induced the expression of all the osteogenic genes apart from *COL1A1*. In particular, the induction of *OSP* is much more pronounced (7 fold increase) in cells in the RPM in respect to static 1G-conditions (Fig. 3A). *OSX* transcript remains elevated in bMSC in the RPM independently from the presence of OM.

Alizarin Red S staining after 10 days of culture shows the presence of calcium deposits with no significant differences between cells cultured in OM in the RPM and in static 1G-conditions (Fig. 3B).

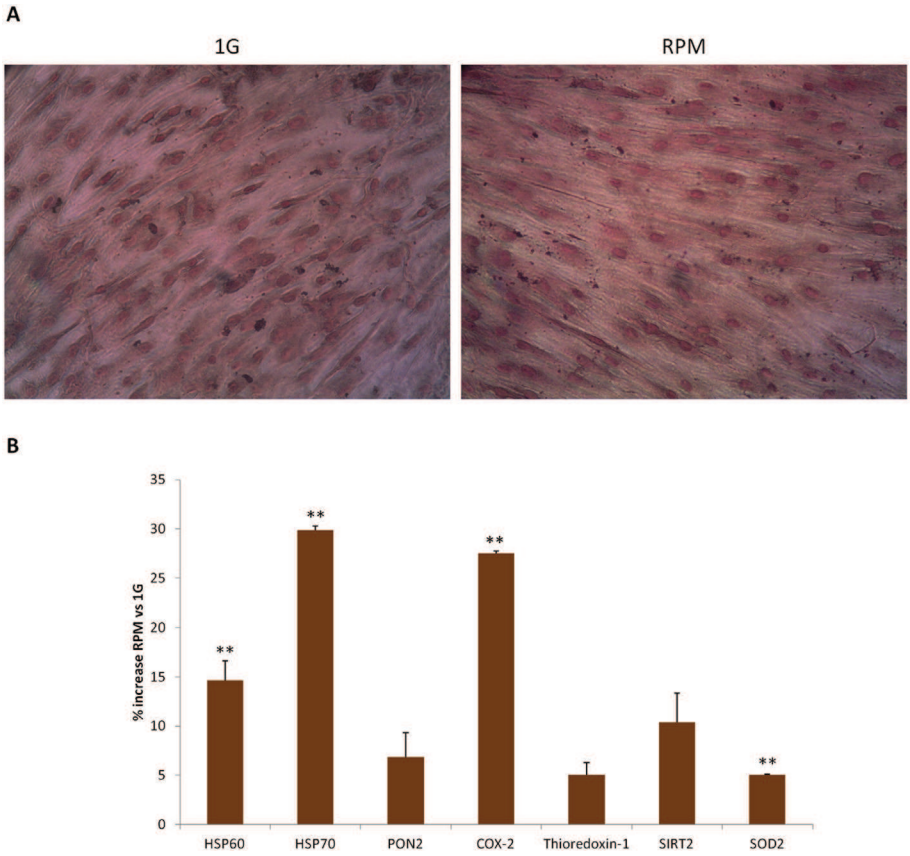

Fig. 2. bMSC from donor 1 were cultured in the RPM or in static 1G-conditions for 24 h. (A) The cells were photographed at 10x magnification. (B) Protein array performed on cell extracts are reported. Densitometric analysis on array spots was performed and data are expressed as % of the variation in the signal intensity of RPM *vs* static 1G-conditions.

4. Discussion

In agreement with previous studies indicating that simulated microgravity activates osteogenesis in bMSC [10,11], we found that culturing bMSC in the RPM induces the overexpression of several markers of osteogenesis, such as *RUNX2*, *OSX*, *OSP* and *OSC* [15]. Differently from Zayzafoon [8], we did not observe the overexpression of *PPARγ*. However, it is noteworthy that in Ref. [8] bMSC were cultured on beads and the device used for simulating microgravity was the Rotating Wall Vessel. On the other hand, we found an overexpression of *RUNX2* and it known that *RUNX2* inhibits MSC differentiation into adipocytes, as shown in *RUNX2*−/− calvarial cells, which spontaneously differentiate into adipocytes [20].

Gravitational unloading in the RPM is sufficient to overexpress several osteogenic genes, but not *COL1A1*. This finding might explain why culture in the RPM does not lead to the acquisition of a full osteogenic phenotype, an event which is reached only after the addition of OM. Indeed, collagen 1A1 represents more than 90% of the organic material in the bone matrix and is the fundamental for calcium deposition [21]. We suggest that culture in the RPM without OM does not result in full differentiation because of the failure to induce *COL1A1* expression. It is also possible that the activation of the other osteogenic genes by culture in the RPM does not reach the threshold to sustain the full differentiation program. Only bMSC exposed to OM showed gene expression levels adequate for stimulating their differentiation towards an osteoblastic phenotype with deposition of calcified matrix both in the RPM and under static 1G-conditions. It is noteworthy that the calcium deposition is similar in bMSC in the RPM and static 1G-conditions. Our results also suggest that, among the genes tested, *COL1A1* is the only one that seems not to be involved in the mechano-sensing response pathway.

We anticipated that the stimulatory effect of culture in the RPM could be ascribed to the capability of bMSC to sense alterations of the mechanical forces and, consequently, to activate an adaptive response. Both spaceflight and simulated microgravity have been shown to activate stress proteins in plant and animal cells as well as in Drosophila and Daphnia [22–25]. Stress proteins, which are overexpressed in response to various strains, are chaperons that help the correct folding of newly synthesized proteins or correct misfolded proteins after various stresses. We found the increase of HSP60 and HSP70 after 24 h in the RPM. Interestingly, HSP70 promotes osteogenesis of bMSC by inducing the expression of *RUNX2* and *OSX* [26], while HSP60 maintains the cells viable [27]. Also COX-2 increases when bMSC are cultured in the RPM. COX-2 contributes to the osteogenic differentiation of bMSC and to the repair of bone fracture [28]. We hypothesize that the increase of HSP70 and COX-2 might be involved in initiating the response of bMSC to simulated microgravity. The increased amounts of SOD2 suggest that the cells activate defense mechanisms against oxidative stress. Some evidence has accumulate indicating that alterations of the balance between free radicals and anti-oxidants occur in microgravity [29].

It is noteworthy that cell morphology is not altered after 24 h in the RPM. On the contrary, bMSC were recently reported to respond to ground based simulated microgravity by adopting more rounded, less-spread shapes which correlates with the inhibition of cell migration [30]. The results from these two studies are only apparently discordant, since we seeded the cells 72 h before culture

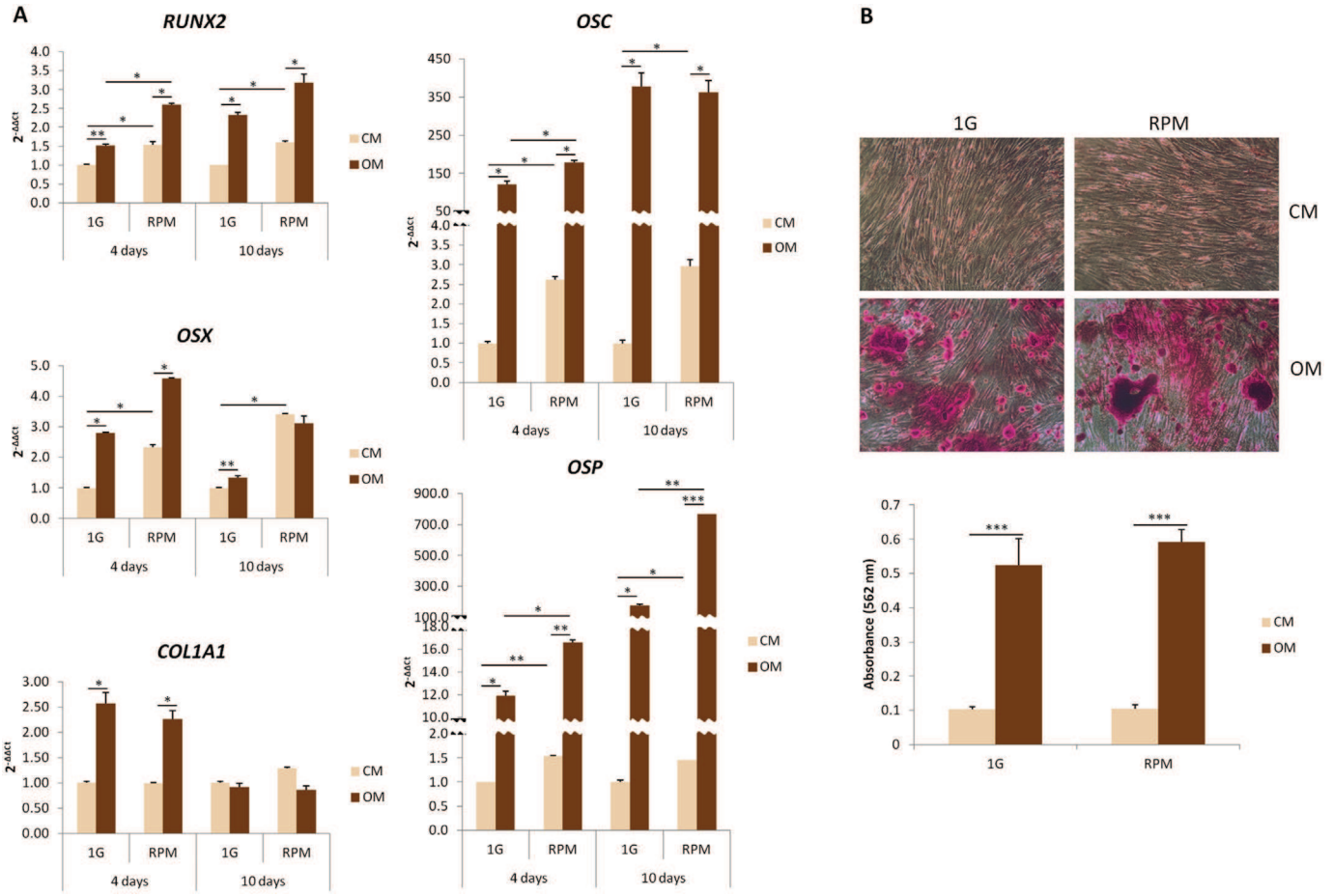

Fig. 3. bMSC from donor 1 were cultured in the RPM or in static 1G-conditions for 4 and 10 days. (A) Real-Time PCR was performed three times in triplicate on RNA extracted using primers designed on *RUNX2*, *OSX*, *COL1A1*, *OSC* and *OSP* sequence. (B) Alizarin Red staining was performed after exposure to OM for 10 days. Photographs were taken at 10x magnification (upper panel). After acid extraction the absorbance was measured at 562 nm (lower panel).

in the RPM thus allowing the cells to spread, while Luna et al. [30] exposed bMSC to simulated microgravity 10 min after seeding.

We conclude that simulated microgravity does not inhibit bMSC osteogenic differentiation in the presence of an osteogenic cocktail. We therefore hypothesize that the impaired function of other bone cells, resulting in an imbalance between osteoblasts and osteoclasts, is involved in space-associated osteopenia. Interesting insights will derive from the results obtained in space. Indeed, bMSC from donor 1 were flown to the ISS in 2015 (Soyuz 42S spaceflight mission) and when experimental data from samples in space are available it will be possible to compare the effects of real and simulated microgravity on these cells, even though in addition to microgravity other spaceflight environmental factors such as launch associated stresses, vibrations and cosmic radiations, might impact on cell behavior.

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Original Research (DRAFT)

Culture of human cells in experimental units for spaceflight impacts on their behavior

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Impact statement

Cell cultures represent valuable preclinical models to decipher pathogenic circuitries. This is true also for biomedical research in space. A lot has been learnt about cell adaptation and reaction from the experiments performed on many different cell types flown to space. Obviously, cell culture in space has to meet specific requirements for the safety of the crew and to comply with the unique environmental challenges. For these reasons, specific devices for cell culture in space have been developed. It is important to clarify whether these alternative culture systems impact on cell performances to allow a correct interpretation of the data.

Abstract

Because space missions produce pathophysiological alterations such as cardiovascular disorders and bone demineralization which are very common on Earth, biomedical research in space is a frontier that holds important promises not only to counterbalance space-associated disorders in astronauts but also to ameliorate the health of Earth-bound population. Experiments in space are complex to design. Cells must be cultured in closed cell culture systems (from now defined experimental units (EUs)), which are biocompatible, functional, safe to minimize any potential hazard to the crew, and with a high degree of automation. Therefore, to perform experiments in orbit, it is relevant to know how closely culture in the EUs reflects cellular behavior under normal growth conditions. We compared the performances in these units of three different human cell types, which were recently space flown, i.e. bone mesenchymal stem cells, micro- and macrovascular endothelial cells. Endothelial cells are only slightly and transiently affected by culture in the EUs, whereas these devices accelerate mesenchymal stem cell reprogramming toward osteogenic differentiation, in part by increasing the amounts of reactive oxygen species. We conclude that cell culture conditions in the EUs do not exactly mimic what happens in a culture dish and that more efforts are necessary to optimize these devices for biomedical experiments in space.

Keywords: Human bone mesenchymal stem cells, human endothelial cells, cell culture experimental unit, reactive oxygen species, osteogenic differentiation

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Introduction

Gravity has shaped life on earth. This concept became evident when the space race started in the 1950s. After hundreds of space missions it is clear that spaceflight and, in particular, long-duration missions activate adaptive responses that might result in potential hazard for the astronauts. Indeed, several pathophysiological alterations are reported that lead to cardiovascular deconditioning, spatial disorientation because of vestibular impairment, immune deficiency, muscle atrophy, and bone demineralization, among others.[1]

We are interested in investigating the alterations of the bone and of the endothelium in microgravity.

As mentioned above, astronauts experience serious, weightlessness-induced bone loss and this is due to an unbalanced process of bone remodeling that involves osteoblasts, osteocytes, and osteoclasts.[2] The effects of microgravity on the cells of the bone have been studied in simulated and real microgravity,[3] but it is only recently that consideration has been given to the role of bone mesenchymal stem cells (bMSCs), which can differentiate into osteoblasts, chondrocytes, and adipocytes depending on the stimuli they receive.[4] bMSCs also produce several factors that regulate bone homeostasis and hematopoietic progenitors.[5] The effects of simulated microgravity on these cells are controversial.[6–9] We have recently demonstrated the culture of bMSC in the random positioning machine, a bioreactor which simulates microgravity, activates stress response, and accelerates the expression of several osteogenic genes.[10] Experiments on bMSC in real microgravity might help to better understand how these cells respond to mechanical unloading.

Also endothelial cells are particularly puzzling to study, not only because they are responsible for maintaining the integrity of the vessels, but also because they influence the neighboring tissues by secreting various molecules.[11]

Endothelial cells are highly heterogeneous in structure and function, gene expression, and antigen composition. [12] Accordingly, heterogeneous responses are described in endothelial cells from different vascular beds and even in different sections of the same vascular bed. Simulated microgravity affects endothelial function and different responses to gravitational unloading have been described in micro- and macrovascular human endothelial cells. [13–16] We have also shown that microvascular endothelial cells in simulated microgravity release proteins that affect osteoblast behavior in a co-culture system. [17] Recently, both macrovascular[18] and microvascular endothelial cells were flown to the International Space Station (ISS), which offers unique opportunities to study the effect of space on the cells.

For biological experiments in space, several requirements need to be met and specific closed cell culture systems (from now defined experimental units (EU)), which must be safe, light, and functional, have been developed.[19] For manned space flight there are rigorous safety requisites to minimize any potential hazard to the crew. In addition, because the daily schedule of astronauts is very tight, the EU should possess a high degree of automation. Moreover, to reduce the cost of space missions, it is important that the EUs for cell culture are light. They must be biocompatible and provide the optimal conditions for the cells including the possibility of supplying fresh medium or specific reagents to collect and preserve the samples at the end of the experiment.

We have been involved in the preparation of some experiments on cells which were flown to the ISS. In particular, bMSC, human umbilical vein endothelial cells (HUVEC), and dermal microvascular endothelial cells (HMEC) were space flown in the EU used in this study.

We investigated two main issues: (i) whether culture in the EU, which is a close system, induces oxidative stress. To find an answer, we cultured the cells in the EU and measured the production of reactive oxygen species (ROS); (ii) whether culture in the EU alters the behavior of the cells. In the case of bMSC, we exposed the cells cultured in the EU to an osteogenic medium (OM) and evaluated the expression of Runt-related transcription factor 2 (RUNX2) and osterix (OSX), crucial transcription factors in osteogenesis. For endothelial cells, we evaluated cell growth at 24 and 96 h after assembling the EU.

Methods

EUs

The EUs, electromechanical devices for the autonomous execution of a scientific protocol in microgravity, were developed by Kayser Italia[18] (Figure 1). Briefly, each experimental EU is composed of a brick of biological compatible plastic (PEEK©) with a cell culture chamber, five cylindrical reservoirs to store media and chemicals, and a fluidic path for exchanging fluids. The culture chamber is designed to accommodate cells cultured in monolayer on 2.3 cm^2 Thermanox coverslips (Nunc, Roskilde, Denmark) with 1.3 mL of culture medium (CM). Each reservoir has a piston, which injects fresh fluids into the culture chamber so that the wasted fluids are collected in the empty cylinder. In these experiments, three reservoirs were filled as following: 1–2 contained phosphate buffered saline (PBS) to rapidly wash the monolayer, three contained various compounds, i.e. RNAlater to extract RNA, 4%

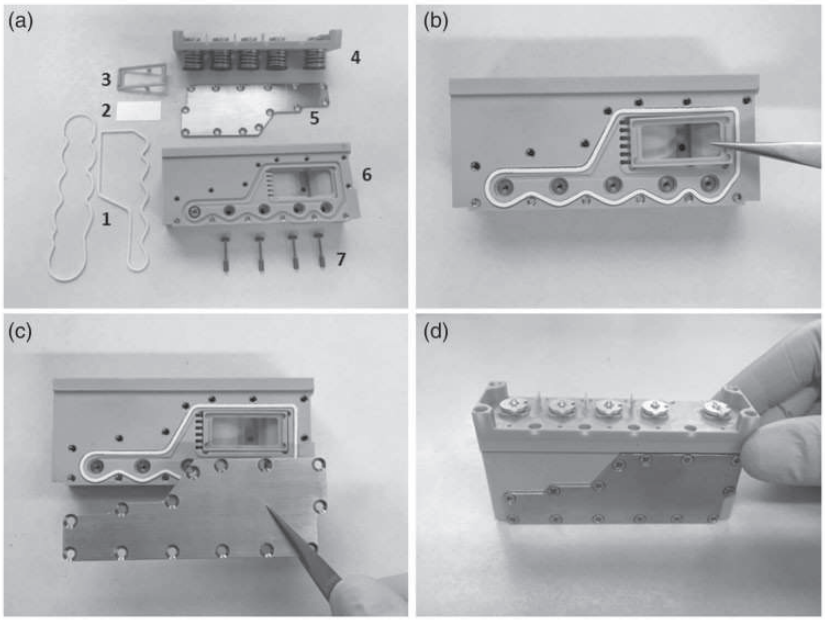

Figure 1 Experimental unit. EU and its components are shown before (a), during (b, c), and after (d) the assembling. (a) Gaskets (1) prevent contamination and liquid leakage; Thermanox coverslip (2) are used to culture the cells and are inserted into the Thermanox coverslip support (3) which is then assembled into the main body (6) and covered with the lateral cover (5); pistons (4) compress the fluids and valves (7) regulate the exchange of fluids. The cylindrical reservoirs are indicated with an arrow. EU: experimental unit

paraformaldehyde (PFA) to fix the cells for microscopy, or PBS for determining ROS and counting the cells (Figure 2).

Cell culture

HMECs were obtained from CDC (Atlanta) and grown in MCDB131 (Gibco, Thermo Fisher Scientific, Waltham, Massachusetts, USA) containing epidermal growth factor (10 ng/mL), hydrocortisone (1 mg/mL), 10% fetal bovine serum (FBS), and glutamine (2 mM).[14] HUVECs (American Type Culture Collection) were cultured in M199 containing 10% FBS, glutamine (2 mM), endothelial cell growth factor (150 mg/mL), sodium pyruvate (1 mM), and heparin (5 units/mL). [20] Both HMEC and HUVEC were cultured on gelatin-coated dishes (2% in water). bMSCs from adult human bone marrow of a male healthy volunteer were donated by Prof. Berti (Policlinico, Milan).[10] These cells, which flew to the ISS in 2015, were cultured in Dulbecco's Modified Eagle's Medium with 1000 mg/L glucose and containing 10% FBS and 2 mM glutamine. In some experiments, bMSCs were cultured in the presence of the antioxidant N-acetylcysteine (NAC) (1 mM). All the cells were maintained at 37 C and 5% CO_2. All culture reagents were from Sigma-Aldrich, Saint Louis, Missouri, USA. To be cultured in the EU, all these cells were seeded on Thermanox coverslips in their own CM supplemented with 12.5 mM HEPES to buffer culture media and kept overnight at 37 C before being loaded in the EU. For endothelial cells, the coverslips were coated with 2% gelatin. Control cells on Thermanox were maintained in culture dishes with 1.3 mL of CM. All the cells were cultured at 37 C in the presence of HEPES. Endothelial cells were trypsinized, stained with trypan blue solution (0.4%), and the viable cells were counted using a cell counter just before assembling the EU (Time 0) and after 24 or 96 h. The experiments were performed at least three times. To study in vitro osteogenic differentiation of bMSC, part of the samples were exposed to an OM containing 10^{8} M1a,25-dihydroxyvitamin D_3,10mMb-glycerolphosphate, and 0.05 mM ascorbic acid (Sigma-Aldrich). [10]

For morphology evaluation, hematoxylin–eosin staining was performed. Briefly, the cells were fixed with 4% PFA for 10 min and washed with PBS for three times. After adding hematoxylin for 3 min, the cells were dyed with eosin, dehydrated with gradient ethanol, soaked with xylene, and mounted with neutral balsam. Stained cells were photographed with a Zeiss Imager M1 microscope equipped with the AxioCam MRc5 camera using AxioVision 4.6 software (Carl Zeiss Microimaging GmbH, Gottingen, Germany).

ROS production

For ROS quantification, the cells were detached from the Thermanox by trypsinization and resuspended in a 20 mM 2^{0}-7^{0}-dichlorofluorescein diacetate (DCFH) solution. After 30' of incubation in a 96-well black plate, the DCFH dye emission was monitored at 529 nm using Promega Glomax Multi Detection System. ROS production was normalized on the basis of cell number. The results are the mean of three independent experiments performed in triplicate. Data are shown as the fold increase in ROS levels of the samples compared to 24 h control cells cultured in dish.

Real-time PCR

Total RNA from bMSC was extracted by the PureLink RNA Mini kit (Ambion, Thermo Fisher Scientific). Single-stranded cDNA was synthesized from 0.2 mg RNA in a

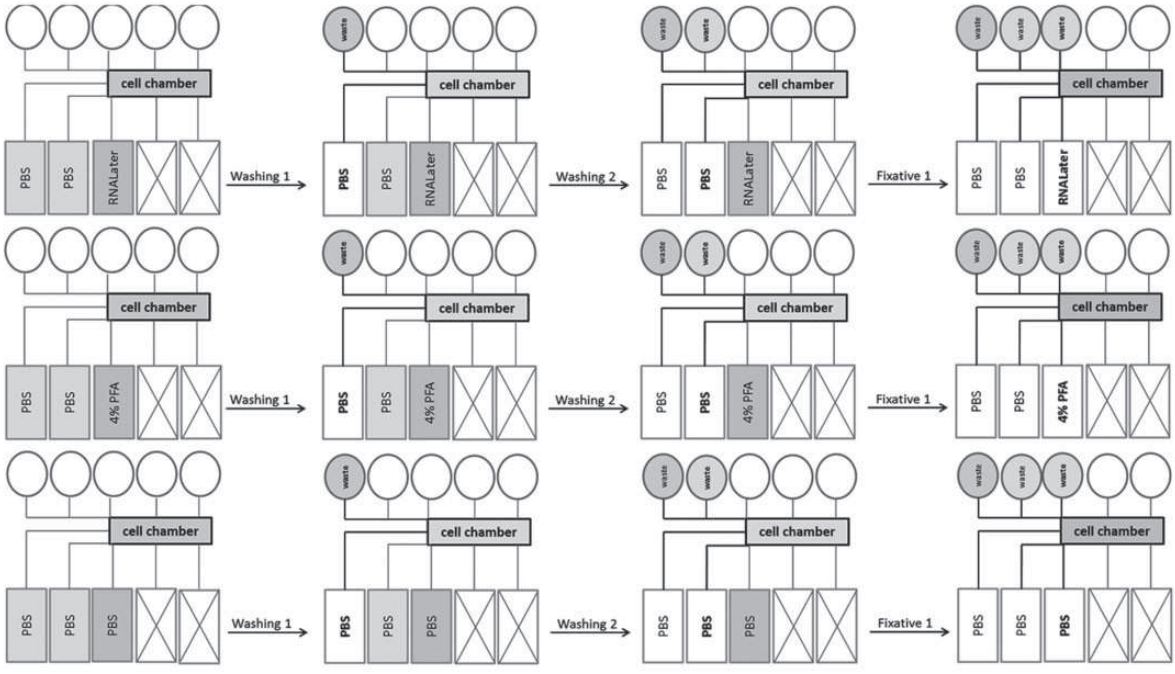

Figure 2 Schematic representation of fluid exchanges in the EU. This protocol was used for all the cells tested. EU: experimental unit

20 mL final volume using High Capacity cDNA Reverse Transcription Kit, with RNase inhibitor (Applied Biosystems, Thermo Fisher Scientific) according to the manufacturer's instructions. Real-time PCR was performed in triplicate on the 7500 FAST Real-Time PCR System instrument using TaqMan Gene Expression Assays (Life Technologies, Carlsbad, California, USA): Hs00231692_m1 (RUNX2) and Hs01866874_s1 (OSX). The housekeeping gene GAPDH (Hs99999905_m1) was used as an internal reference gene. Relative changes in gene expression were analyzed by the 2^{Ct} method.[10,21]

Statistical analysis

Statistical significance was determined using Student's t-test and set as following: *P < 0.05, **P < 0.01, ***P < 0.001.

Results

Culture of bMSC in the EU

Confluent bMSCs on Thermanox were utilized. We measured ROS production by DCFH 24 and 96 h after assembling the EU and found a significant increase of ROS generation in bMSC in the EU (Figure 3(a)). To investigate whether this overproduction of ROS impacted on the response of the cells, we cultured bMSC in the EU in OM or CM. After 96 h the EUs were disassembled and cell morphology as well as the expression of osteogenic markers was investigated. As shown in Figure 3(b), the cells were elongated with a round nucleus and a prominent nucleolus. We did not detect any significant morphological difference in cells grown in CM versus OM in the culture dish. However, in cells cultured in the EU and exposed to the OM, the cells are less than in the controls and tend to align and organize in bundles.

Then we evaluated the expression of two markers of osteogenic differentiation, RUNX2 and OSX, by real-time PCR after culturing bMSC in the EU or in a dish in OM or CM for 96 h. Figure 3(c) shows that the induction of RUNX2 and OSX is higher in cells in the EU than in the corresponding controls in a culture dish. To investigate whether this difference could be ascribed to the overproduction of ROS by bMSC in the EU, we exposed the cells to the synthetic

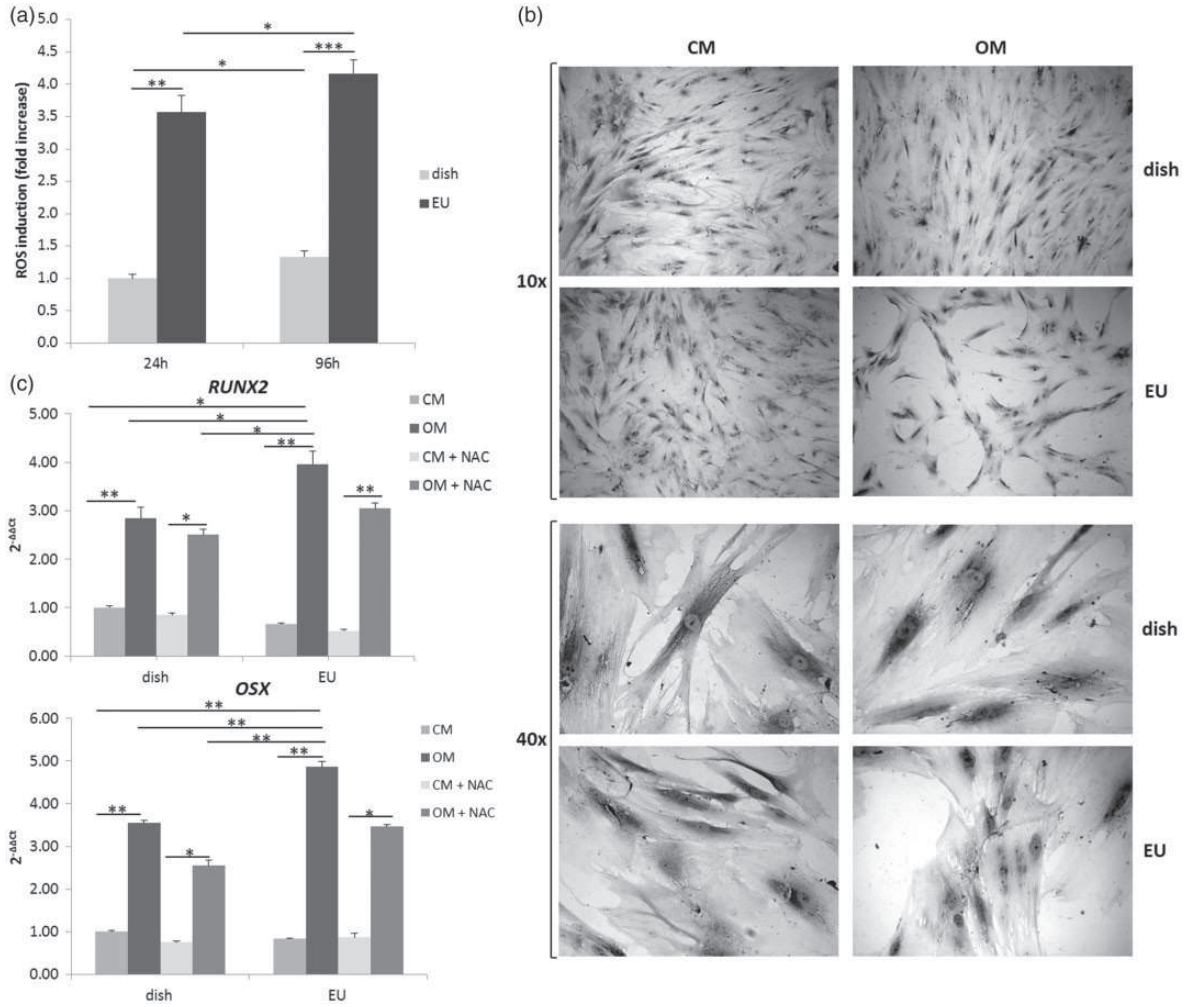

Figure 3 Effects of culture in the EU on bMSC. (a) ROS generation was measured by DCFH 24 and 96 h after assembling the EU. Data are shown as the fold increase in ROS levels of the samples compared to 24 h control cells cultured in dish. Each bar is the mean of three separate experiments standard deviation. (b) Microphotographs of cells stained with hematoxylin–eosin were taken 96 h after culture in the EU or in control dish. Scale bar: 100 mm (c) RNAs were extracted and a real-time PCR was performed using primers designed on RUNX2 and OSX sequence. bMSC: bone mesenchymal stem cell; DCFH: 2',7'-dichlorofluorescein diacetate; EU: experimental unit; OSX: osterix; PCR: ; ROS: reactive oxygen species; RUNX2: Runt-related transcription factor 2

antioxidant NAC (1 mM) for the duration of the experiment. We found that NAC decreased the expression of RUNX2 and OSX in bMSC cultured in the EU in OM to levels comparable with the controls cultured in a dish (Figure 3(c)), thus indicating that the increased production of ROS is involved in the accelerated expression of osteogenic markers.

Culture of HMEC in the EU

10^4 HMEC were seeded on Thermanox coated with 2% gelatin. Twenty-four and 96 h after culture in the EU, we measured ROS production by DCFH and did not detect any significant difference (Figure 4(a)). It is noteworthy that ROS production dropped after 96 h when the cells reach confluence, indicating that ROS production is higher in sparse versus confluent cells. Indeed, while after 24 h proliferation in the EU was retarded, no significant differences of cell number were observed after 96 h (Figure 4(b)).

Culture of HUVEC in the EU

A total of 5 10 ^{3} HUVEC were seeded on gelatin-coated Thermanox. We measured ROS production by DCFH 24 and 96 h after assembling the EU. As shown in Figure 5(a), after 24 h we found decreased amounts of ROS in cells in the EU, while no differences were detected between the two experimental conditions after 96 h. It should be pointed out that the reduced generation of ROS after 96 h is a common feature in HUVEC and HMEC (Figure 4(a)). When we counted the cells, we found more cells in the EU after 24 h, while after 96 h the number of cells was comparable in the EU and in the controls (Figure 5(b)).

Discussion

Our data show that when cultured in the EU the behavior of three different cell types is different from controls grown under normal condition. It is noteworthy that HUVEC, HMEC, and bMSC were all cultured in these EUs to be flown to space for experiments onboard the ISS. For HUVEC the data of the experiment onboard the ISS have been published,[18] while the postflight analyses on HMEC and bMSC are in progress.

bMSCs are a rare population in bone marrow and show a broad spectrum of differentiation potential. [4] In response to osteogenic stimuli, they activate a genetic program leading

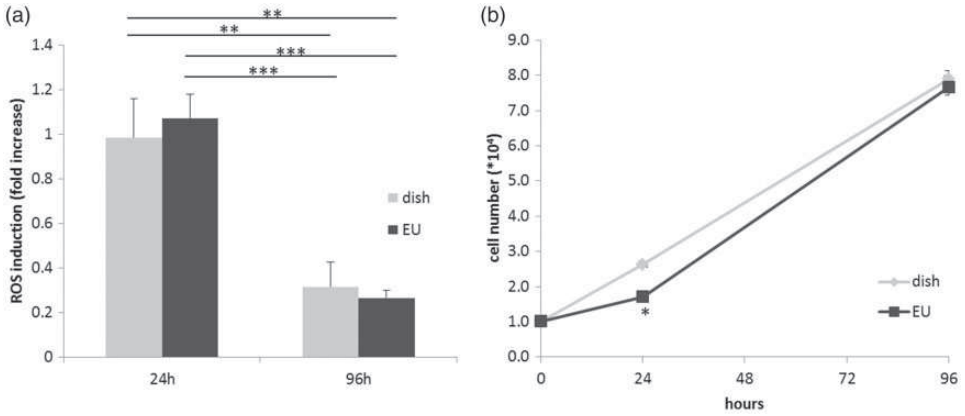

Figure 4 Effects of culture in the EU on HMEC. (a) ROS generation was measured 24 and 96 h after assembling the EU. Data are shown as the fold increase in ROS levels of EU cultured cells compared to controls cultured in dish. Each bar is the mean of three separate experiments standard deviation. (b) After 24 and 96 h, viable cells were counted. EU: experimental unit; ROS: reactive oxygen species

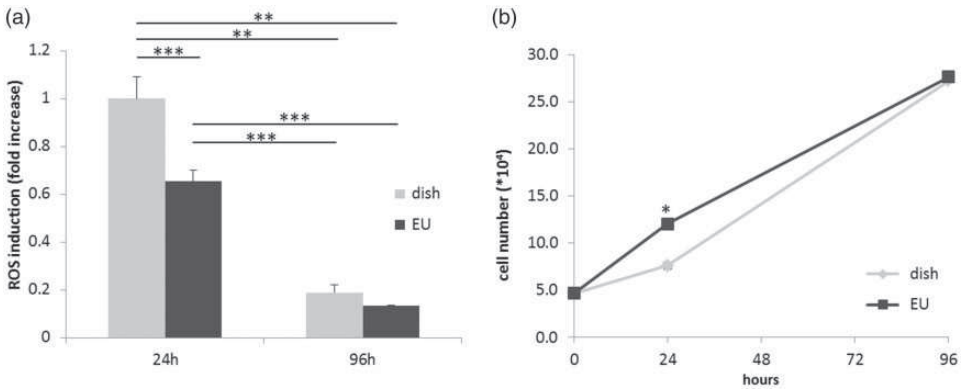

Figure 5 Effects of culture in the EU on HUVEC. (a) ROS generation was measured 24 and 96 h after assembling the EU. Data are shown as the fold increase in ROS levels of EU cultured cells compared to controls cultured in dish. Each bar is the mean of three separate experiments standard deviation. (b) After 24 and 96 h, viable cells were counted. EU: experimental unit; HUVEC: human umbilical vein endothelial cell; ROS: reactive oxygen species

to differentiation into osteoblasts, the cells in charge of bone formation. Since no ossification occurs in RUNX2 knockout mice,[22] RUNX2 is considered the master regulator of osteogenesis. OSX is necessary to promote the early stages of osteogenesis,but it is not sufficient to reach complete differentiation.[22] Here we show that culture in the EU accelerates bMSC genetic reprogramming by overexpressing RUNX2 and OSX in response to OM. This finding might be of interest not only for scientists preparing experiments for space biology but also for professionals involved in the growing field of regenerative medicine in orthopedics, with the aim of replacing or repairing diseased or injured skeletal tissue. We also found an increased generation (about threefold induction) of ROS in the EU. In general, while excess of ROS is cytotoxic, an adequate amount of ROS is required to maintain cell proliferation, self-renewal, and regulation of differentiation.[23] In bMSC cells and in preosteoblasts, the generation of ROS is needed for osteogenic differentiation.[24,25] Indeed, since the antioxidant NAC reduces the expression of RUNX2 and OSX in bMSC in the EU exposed to OM, we propose that the induction of osteogenic differentiation markers is mediated, in part, by ROS. It should also be noted that increased amounts of ROS might activate an adaptive response that enhances differentiation. Indeed, some stress proteins are important in regulating bMSC performances. In particular, HSP70 induces the expression of RUNX2 and OSX, thus promoting osteogenesis.[26]

HUVECs are widely used as a model of macrovascular endothelial cells. These cells were studied in simulated microgravity generated by the random positioning machine and the rotating wall vessels.[13,16,27]In 2010, these cells were flown to the ISS after being assembled into the EU used in this study.[18] Space modulates the expression of more than 1000 genes.[18] Here we show that cell number is increased in the EU after 24 h, suggesting that the microenvironment generated in the closed system of the EU favors HUVEC growth. At 96 h both EU cultured and control cells cultured in dish reach confluence. It is interesting to note that ROS production is lower in HUVEC in the EU after 24 h. In addition, ROS are markedly decreased after 96 h in HUVEC in the EU and in the dish, when the cells are confluent. These data are in agreement with the findings that cell number is inversely related to the production of ROS in HUVEC (data not shown). In space-flown HUVEC, on the basis of gene expression studies, an increase of oxidative stress has been postulated.[18] The results presented here suggest that oxidative stress is not generated by culture in the EU, but it is possible that space environmental factors, such as vibrations, microgravity, and radiations, might induce the production of ROS.

HMEC in the EU behave differently from HUVEC. We found an initial and transient growth retardation, while after four days the cells in the EU and their controls in the dish are confluent. We hypothesize that HMEC might require some adaptive response early after culturing in the EU, but they rapidly rescue their normal proliferation rate and reach confluence within four days like the controls. Differently from HUVEC and bMSC, these cells do not alter ROS production in the EU. The differences between HUVEC and HMEC might be ascribed to the fact that endothelial cells from different vascular beds are heterogeneous in terms of function, structure, and responses to in vitro stimulation. Accordingly, we demonstrated the different response to simulated microgravity of these cells.[14,16] There is one interesting similarity in the behavior of these cells, i.e. the production of ROS dramatically drops when HUVEC and HMEC are confluent, thus indicating an inverse correlation between cell number and ROS generation.

We conclude that culturing cells in the EU influences cell behavior and this is particularly evident in bMSC, while both micro- and macrovascular endothelial cells seem to rapidly adapt to culture in these devices. To this purpose, it is well known that endothelial cells show a remarkable capacity to suit local requirements and to comply with different humoral, neural, and mechanical stimuli.[28]

Several spaceflight hardware are available and, when planning experiments with cells in space, it would be interesting to evaluate whether and how these different devices affect cell performances. This will help to design proper protocols to ensure the optimization of the experimental conditions so that culture in the EU can reflect as closely as possible what happens in a culture dish. This approach will facilitate the interpretation of the results and the comparison with data obtained on earth under normal culture conditions.

Authors' contribution: AC, CM, JAMM, and SC conceived and designed the experiments. SC and AC performed the following experiments: cell culture, ROS quantification, real-time PCR; CM performed the hematoxylin–eosin staining and took the microphotographs of the cells; AC, CM, JAMM, and SC analyzed the data. JAMM wrote the paper. All authors reviewed the manuscript.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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