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Gravitational Unloading Induces an Anti-Angiogenic Phenotype in Human Microvascular Endothelial Cells

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Abstract Creating conditions similar to those occurring during exposure of cells to microgravity modulates endothelial functions. We have previously demonstrated that human macrovascular endothelial cells in simulated hypogravity proliferate faster than controls, partly because they downregulate interleukin 1α. On the contrary, murine microvascular endothelial cells are growth inhibited in simulated hypogravity, and this is due, at least in part, to the decrease of interleukin 6. Since endothelial cells are very heterogeneous and differences between various species have been reported, we exposed human microvascular cells to gravitational unloading and found that it retards cell growth without affecting cell migration. Interestingly, we detected the induction of Tissue Inhibitor of Metalloprotease-2, which inhibits endothelial growth in vitro and angiogenesis in vivo. Together with the finding that hypogravity stimulates the synthesis of nitric oxide, involved also in neovascularization, our results underscore a modulation of the angiogenic properties of microvascular human endothelial cells. We also show that hypogravity inhibits proteasome activity, thus suggesting that post-translational mechanisms are involved in the adaptations of these cells to hypogravity. These results underscore that hypogravity differently impacts on micro- and macro-vascular human endothelial cells. In particular, these results may shed some light on the molecular mechanisms contributing to the impairment of angiogenesis observed in different models in space. Our data might also explain why bioengineered tissues to be used for regenerative medicine fail to neovascularize when assembled in simulated hypogravity. J. Cell. Biochem. 104: 129–135, 2008.

Key words: microvasculature; endothelial cell; gravity; TIMP-2

Vascular endothelial cells form a thin layer on the interior surface of all vessels and are known to function in a variety of important physiological processes. Indeed, the endothelium is involved in a network of interactions between cells, cellular and humoral factors and matrix components [Cines et al., 1998]. Therefore, it is not surprising that endothelial malfunctions are the cause of a great variety of pathophysiological states [Cines et al., 1998]. Several factors modulate endothelial cell behavior and eventually promote disease. Among others, it has recently become evident that alteration of gravity influences endothelial cell function. Indeed, both hypogravity and hypergravity have been shown to modulate growth, gene expression and enzymatic activities [Carlsson et al., 2002; Sanford et al., 2002; Cotrupi et al., 2005; Spisni et al., 2003]. Hypogravity downregulates interleukin (IL)-1α and induces the growth of human umbilical vein endothelial cells (HUVEC) [Carlsson et al., 2002]. Similar results were obtained in bovine aortic endothelial cells [Sanford et al., 2002].

Endothelial cells are very heterogeneous [Ribatti et al., 2002]. Morphological and functional differences as well as different responses to growth factors have been observed in endothelial cells derived from large and small vessels. In addition, microvascular endothelial cells synthesize a different range of mediators, display distinct adhesion molecules, activate unique sets of genes and direct innate and adaptive immunity [Danese et al., 2007]. It is also noteworthy that the microvascular bed represents the bulk of the total endothelial surface, lining an area 50 times greater than that of all large vessels together [Danese et al., 2007]. Therefore, the modulation of some of microvascular endothelial functions may have

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important systemic implications. In particular, microvascular endothelial cells are crucial players in inflammation, since their activation leads to increase of vascular permeability, leukocyte adherence with cell clumping, and microthrombi formation [Danese et al., 2007]. Microvascular endothelial cells are also the protagonists of angiogenesis, that is, the outgrowth of new capillaries from the pre-existing primary plexus, an event induced in response to tissue demands both in physiological and pathological conditions [Carmeliet, 2005]. An impairment of angiogenesis in low gravity is suggested by several evidences. For instance, wound healing, in which neovascularization is an early and fundamental step, is retarded in space-flown animal models [Davidson et al., 1999], and the development of vascular channels in a rat fibular osteotomy model is inhibited after a shuttle flight [Kirchen et al., 1995]. In addition, any attempt to neovascularize reassembled bioengineered tissue equivalents under gravitational unloading has failed [Unsworth and Lelkes, 1998]. These results point to an alteration of microvascular endothelial cell functions in hypogravity. Indeed, in murine microvascular cells we have demonstrated an impairment of cell growth, partly due to the inhibition of interleukin 6 synthesis [Cotrupi et al., 2005].

While space flight studies are limited, the development of different bioreactors, such as the rotating wall vessel (RWV) and the random positioning machine (RPM), has facilitated ground-based studies of the effects of hypogravity on different cell types. It is noteworthy that experiments performed on U937 cells cultured in the RWV or in the RPM yielded results which are very similar to those obtained in space [Hatton et al., 1999; Maier, 2006], suggesting that these bioreactors are reliable systems to study cell proliferation and cytokine synthesis in reduced gravity. Similar conclusions were reached for studies on immune cells [Ritz et al., 2006].

Since endothelial cells are very heterogeneous and differences between various species have been reported, we have evaluated the impact of gravitational unloading obtained by the use of both RWV and RPM on human microvascular endothelial cells (HMEC). We found that in hypogravity the growth of HMEC is retarded and this correlates with a p53-independent upregulation of p21. By protein array, we detected the overexpression of tissue inhibitor of metalloproteases (TIMP)-2 and the downregulation of IL-8. In addition, nitric oxide synthesis is increased in parallel with the upregulation of endothelial nitric oxide synthase (eNOS).

MATERIALS AND METHODS

Cell Culture, Proliferation and Migration

Human dermal microvascular cells (HMEC) were obtained from CDC (Atlanta) and grown in MCDB131 containing epidermal growth factor (10 ng/ml), hydrocortisone (1 μg/ml), and 10% FBS. All culture reagents were from Gibco. To reduce gravity, we utilized the RWV (Synthecom, Inc., Houston, TX) and, in some experiments, the RPM (Dutch Space, Leiden, NL). To be used in the RWV, the cells were seeded on beads (Cytodex 3, Sigma); as controls, HMEC grown on beads were cultured in Petri dish or in the vessels not undergoing rotation [Carlsson et al., 2002]. In the RWV, 10 ml vessel rotates around a horizontal axis (28 rpm) and allows diffusion of oxygen and carbon dioxide across a semipermeable membrane. Such a rotation reduces gravity to approximately 3×10^{-2} g [Rucci et al., 2007].

We utilized the RPM facility at the Dutch Experiment Support Center (DESC) which is allocated in a dedicated temperature controlled incubator capable of supplying a 5% CO_2/air mixture. The used speed of rotation is 60°/s ($\approx$1 rad/s), which reduces gravity to approximately 6×10^{-2} g [Loesberg et al., 2007]. To be cultured on the RPM, HMEC cells were grown in Petri or multiwell dishes. To study proliferation, cells were cultured for various times in the RWV or in the RPM, trypsinized, stained with trypan blue solution (0.4%) and counted the viable cells using a Burker chamber [Carlsson et al., 2002]. Migration of HMEC cells cultured in the RPM for 48 h was determined using an in vitro model of wound repair as previously described [Manganini and Maier, 2000]. Briefly, confluent endothelial cells were wounded and treated with hepatocyte growth factor (HGF) (Tebu-Bio) (20 ng/ml) for 18 h. The number of cells migrating from the wound origin was counted with a light microscope using a grid. All experiments were performed in triplicate at least three times. Data are expressed as the mean $\pm$ standard deviation. Statistical analysis was performed using the

GraphPad InStat software, and differences were considered significant when $P < 0.05$.

Nitric Oxide Synthase (NOS) Activity

NOS activity was measured in the conditioned media of HMEC by using the Griess method for nitrate quantification according to the manufacturer's instructions. Briefly, 1:5 diluted media were exposed to nitrate reductase (250 mU/ml) and NADPH (100 mM) for 30 min at 37°C to reduce nitrate to nitrite. The samples were then treated with L-glutamine dehydrogenase and mixed with an equal volume of freshly prepared Griess reagent. The absorbance was measured at 540 nm. The concentration of NO in the samples was determined using a calibration curve generated with standard $NaNO_2$ solutions. The experiments were performed in triplicate at least three times. Data are expressed as the mean ± standard deviation. Statistical analysis was performed using the GraphPad InStat software.

Western Blot

HMEC cells were lysed in 10 mM Tris–HCl (pH 7.4) containing 3 mM $MgCl_2$, 10 mM NaCl, 0.1% SDS, 0.1% Triton X-100, 0.5 mM EDTA and protein inhibitors, separated on SDS–PAGE and transferred to nitrocellulose sheets. Western analysis was performed using antibodies against eNOS, iNOS and GAPDH (Tebu Bio-Santa Cruz). Secondary antibodies were labeled with horseradish peroxidase (Amersham Pharmacia Biotech). The SuperSignal chemiluminescence kit (Pierce) was used to detect immunoreactive proteins [Carlsson et al., 2002].

Measurements of TIMP-2

TIMP-2 was measured in 1:2 diluted medium using a double-antibody sandwich ELISA (GE Healthcare) according to the manufacturer's instructions. The concentrations of TIMP-2 were determined by interpolation from a standard curve.

Determination of Proteasome Activity

HMEC cells were cultured in the RWV for 48 and 96 h and lysed in 20 mM Tris–HCl containing 10% glycerol, 5 mM ATP and 0.2% NP-40. After centrifugation, the proteasome activity was determined according to the manufacturers' instructions (20S Proteasome Activity Assay kit, Chemicon) as described [Bernardini et al., 2005]. Statistical analysis was performed using the GraphPad InStat software.

RESULTS

Endothelial Proliferation and Migration in Hypogravity

No experiment conducted on earth can model the conditions in space. However, different tools are available to isolate specific individual components of the collective effects induced by gravity. These devices are valuable to develop hypothesis concerning gravitational cell biology and to direct the design and scope of orbital flight studies. On these bases, HMEC were grown both in the RWV and in the RPM for different times. As shown in Figure 1A, culture in the RWV inhibited HMEC proliferation at all the time points tested, as previously demonstrated in murine microvascular endothelial cells [Cotrupi et al., 2005]. Similar results were obtained when the cells were cultured in the RPM, which creates a condition in which the weight vector is continually reoriented as in traditional clinorotation, but with increased directional randomization (Fig. 1B). Accordingly, we found that the growth inhibition correlated with the upregulation of p21 (WAF1), an inhibitor of cyclin-dependent kinases in HMEC both in the RWV and in the RPM (not shown). Like murine cells [Cotrupi et al., 2005], also HMEC synthesize higher amounts of nitric oxide (NO) as measured by the Griess method (Fig. 1C). This event correlates with the upregulation of eNOS after 48 and 96 h in the RWV, while iNOS is only slightly down-regulated after 96 h in the RWV (Fig. 1D). These results demonstrate that human and murine microvascular endothelial cells respond to hypogravity by inducing NO production. They also suggest that eNOS is responsible for the increased synthesis of NO.

Endothelial migration is an early pivotal event in angiogenesis. It is also necessary to repair damages to the vascular wall. The culture system of RPM allowed to investigate whether hypogravity affected endothelial migration, whereas this is not feasible in the RWV because the cells are cultured on beads. Therefore, HMEC were cultured in the RPM for 48 h, wounded and exposed to HGF (20 ng/ml) for 18 additional h in the RPM. Figure 2 shows that

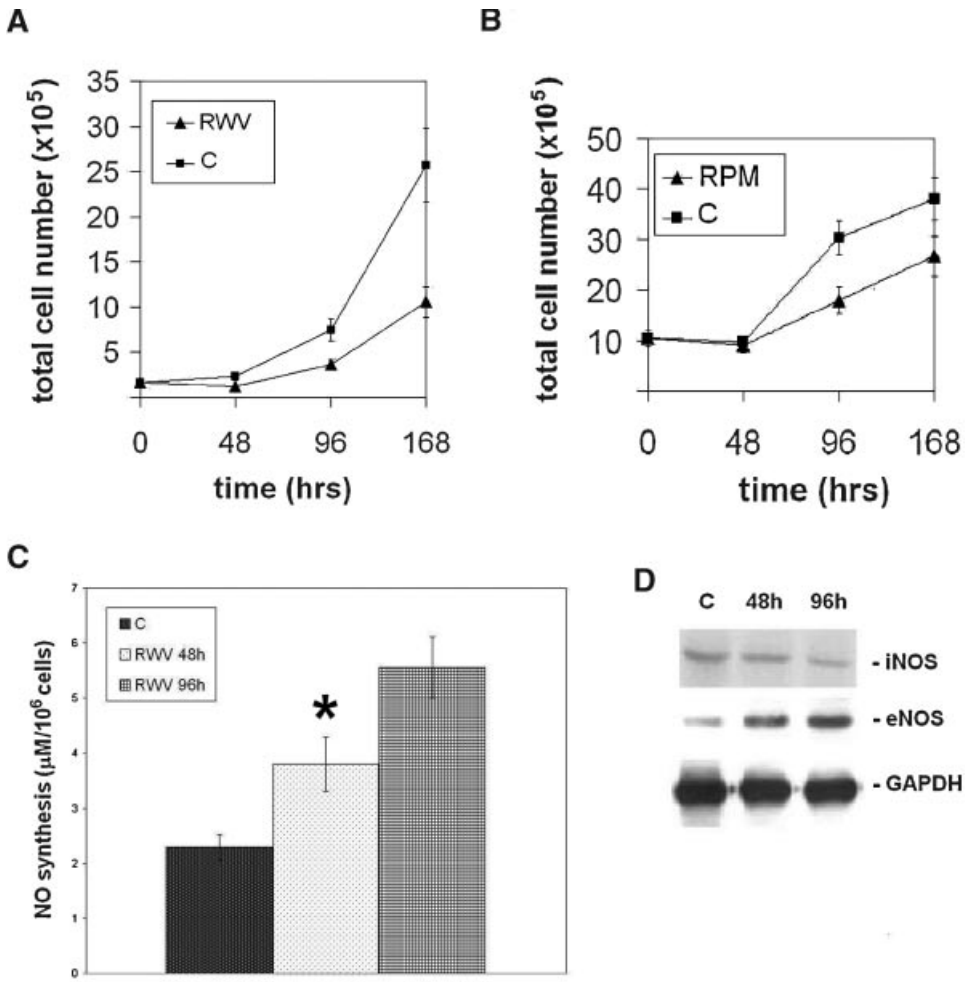

Fig. 1. Hypogravity inhibits HMEC growth and stimulates NO synthesis. **A**,**B**: HMEC were cultured for different times in the RWV or in the RPM, trypsinized and viable cells counted using a Burker chamber. C: control. **C**: Nitric oxide synthesis was measured by the Griess method after 48 and 96 h culture in the RWV. Data are expressed as the mean of three different experiments in triplicate ± standard deviation. $^*P < 0.05$ versus control. **D**: Western analysis was performed using specific antibodies against eNOS and iNOS on 60 μg of lysates from HMEC cultured in the RWV for 48 and 96 h. Incubation with an anti-GAPDH antibody shows that comparable amounts of protein were loaded.

hypogravity did not alter endothelial migration in response to HGF.

Modulation of Molecules Involved in Angiogenesis by Hypogravity

Because an impairment of angiogenesis is described in microgravity [Kirchen et al., 1995; Davidson et al., 1999], we evaluated whether culture in the RWV modulated the levels of different molecules involved in angiogenesis. To this purpose we utilized a protein array approach specifically tailored for proteins involved in inflammation/angiogenesis (purchased from RayBiotech-TebuBio). HMEC were cultured for 72 h in the RWV. The culture medium was then utilized for the proteomic analysis. Out of 44 proteins tested, we found a slight reduction of the release of IL-8 and a marked increase of secreted TIMP-2 in cells in hypogravity versus controls. The increased amounts of TIMP-2 in HMEC cultured in hypogravity for different times were confirmed by ELISA (Fig. 3).

Proteasome Activity in HMEC in Hypogravity

Evidence has been provided that the proteasome might contribute to the modulation of the total amounts of some proteins in hypogravity [Ikemoto et al., 2001; Maier, 2006]. On these bases, the proteasome activity on crude extracts of HMEC in the RWV for 48 and 96 h was measured. Figure 4 shows that hypogravity gradually decreased the total activity of the proteasome in HMEC.

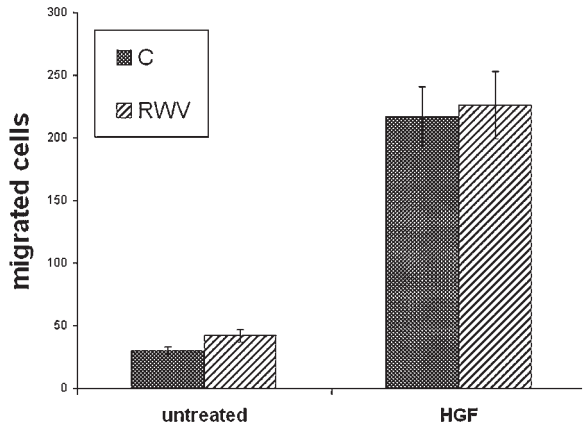

Fig. 2. Hypogravity does not affect cell migration. HMEC were grown in the RPM for 48 h, exposed to HGF (20 ng/ml) for 18 h and evaluated for migration using a wound assay as described in the methods.

DISCUSSION

In the adult, the formation of new vascular sprouts from pre-existing capillaries occurs in response to a variety of pathologic and pathophysiologic stimuli. Angiogenesis is a multistep process in which microvascular endothelial cell are induced to migrate and proliferate. Several lines of evidence suggest an impairment of angiogenesis in space. Indeed, a significant decrease of the density of small vessels was reported in the quail chorioallanthoic membranes after space flight [Henry et al., 1998]. In addition, the development of vascular channels in a rat fibular osteotomy model was inhibited after a shuttle flight [Kirchen et al., 1995]. Orbital spaceflight also retards the capacity of

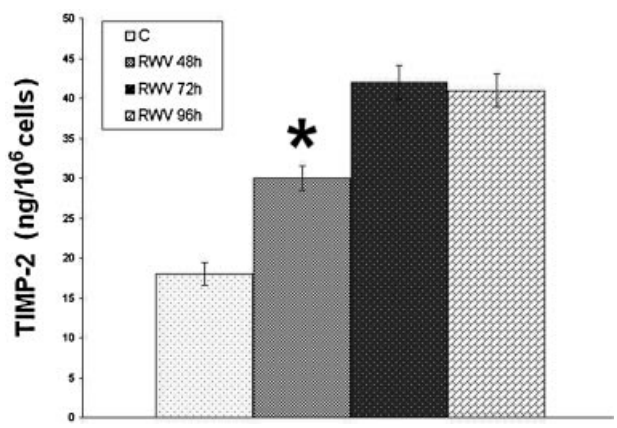

Fig. 3. Hypogravity induces TIMP-2. TIMP-2 was measured by ELISA in media collected after different times of culture in the RWV. Data are expressed as the mean of three different experiments in triplicate ± standard deviation. $*P < 0.05$ versus control.

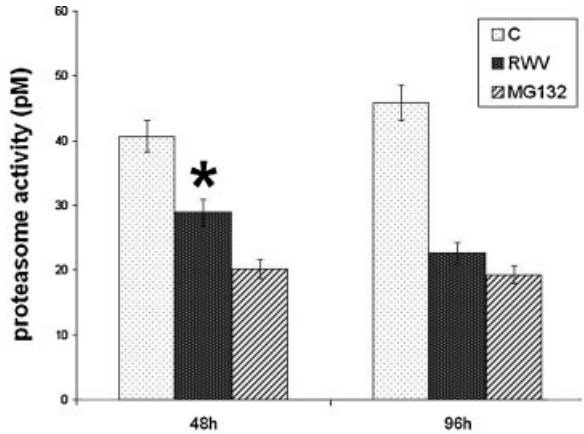

Fig. 4. Hypogravity reduces the proteasome activity. Proteasome activity was determined using the 20S proteasome activity assay kit according to the manufacturer's instructions. The proteasome activity was calculated on the standard activity curve of the 20S proteasome positive control. The results are the mean ± standard deviation of two separate experiments in triplicate. $*P < 0.05$ versus control.

cutaneous wounds to heal in the rat [Davidson et al., 1999]. Therefore, our results showing that hypogravity impairs HMEC proliferation and alters the synthesis of proteins involved in neovascularization may shed some light on the molecular mechanisms contributing to impaired angiogenesis in space. In addition, our data may offer insights to overcome crucial issues in generating artificial tissues for transplantation. Indeed, RWV bioreactors have been used to promote the differentiation of preassembled bioengineered tissue equivalents, such as skin, cartilage and fetal neuronal cells [Unsworth and Lelkes, 1998], but any attempt to neovascularize these prototissues by inoculating endothelial cells has failed.

Here we describe that gravitational unloading retards HMEC cell growth and this correlates with the upregulation of p21 (not shown), an inhibitor of the activity of the cyclin/CDK2 complexes necessary for the transition from the G1 to the S phase. It is noteworthy that murine microvascular endothelial cells behave similarly when grown in the RWV [Cotrupi et al., 2005]. While hypogravity induces increased apoptosis in porcine aortic endothelial cells engineered to overexpress vascular endothelial growth factor-receptor 2 [Morbidelli et al., 2005], we never detected apoptosis in HMEC cultured both in the RWV and in the RPM (data not shown).

An early and pivotal event in angiogenesis is the migration of endothelial cells [Carmeliet,

2005]. We did not detect any relevant difference in the motility of HMEC both under basal condition and after HGF-treatment in hypogravity, suggesting that this environment does not impact on pathways involved in cell motility.

We also report that in hypogravity HMEC overexpress TIMP-2 and downregulate IL-8. IL-8 has been shown to induce proliferation, survival, migration and metalloprotease (MMP) production in endothelial cells. It also induces tube formation in vitro and is angiogenic in vivo [Li et al., 2003]. Since IL-8 is an autocrine growth factor for HUVEC and HMEC [Li et al., 2005], it is feasible that its downregulation in hypogravity may contribute to HMEC growth retardation.

In parallel, we observed a marked increase of TIMP-2. Interestingly, elevated levels of TIMP-2 modulate decreased angiogenesis in aged tissues, most likely via TIMP-2-mediated inhibition of MMP-2 and MT1-MMP [Koike et al., 2003]. Recently, TIMP2 has been shown to abrogate angiogenic factor-induced endothelial cell proliferation in vitro and angiogenesis in vivo by a MMP-independent mechanism [Stetler-Stevenson and Seo, 2005]. Indeed, TIMP-2 binds to the surface of human microvascular endothelial cells through interaction with the integrin $\alpha 3\beta 1$, and this interaction mediates suppression of fibroblast growth factor-2- or vascular endothelial growth factor-a-induced endothelial cell proliferation in vitro and angiogenesis in vivo. On these bases, we hypothesize that hypogravity induces pathways that function to resist endothelial cell "activation" and preserve endothelial cell homeostasis.

We also show that nitric oxide synthesis is increased by hypogravity. These data are in agreement with the results obtained in bovine aortic and in murine microvascular endothelial cells cultured in the RWV [Sanford et al., 2002; Cotrupi et al., 2005] as well as with the increased NO synthesis observed in rodents subjected to hindlimb unloading, a model which simulates reduced gravity [Vaziri et al., 2000]. In particular, differential alterations of NOS expression and nitric oxide synthesis of different arteries after hindlimb unweighting were reported, and this represents, at least in part, a localized adaptation to body fluid redistribution. Indeed, NO acts in a paracrine fashion to relax smooth muscle cells, thus participating to the regulation of vascular tone [Moncada et al., 1988]. Because of this, increased NO synthesis by microvascular endothelial cells, which are much more abundant than macrovascular cells, may contribute to the altered control of blood pressure after space flight in humans. NO has also role in angiogenesis because it upregulates VEGF, a potent paracrine angiogenic molecule implicated both in normal and pathological neovascularization [Fukumura et al., 2006]. Since HMEC do not express VEGF (not shown), more studies are required to evaluate whether NO might induce VEGF in simulated microgravity both in vivo and in in vitro co-culture models.

Upon exposure to cytokines or to mechanical stress, endothelial cells undergo profound alterations of function that involve gene expression and modulation of protein synthesis and degradation. Gene expression profiles have shown that real and simulated hypogravity alter the levels of transcripts of various genes some of which involved in the regulation of cell growth [Semov et al., 2002; Boonyaratanakornkit et al., 2005]. In addition, evidence is provided that post-transcriptional events may contribute to cell adaptation to altered gravity. Space shuttle flight induces the degradation of rat myosin by the proteasome [Ikemoto et al., 2001]. In addition, it was recently shown by a proteomic approach that human T lymphocytes in conditions similar to those occurring in microgravity downregulate the 26S proteasome subunit 6 and the proteasome activator complex subunit 3, thus suggesting an impairment of the proteasome machinery [Risso et al., 2005]. Accordingly, hypogravity reduces the activity of the proteasome in U937 cells after culture in the RWV [Maier, 2006]. Similarly, HMEC show a reduced activity of the proteasome when grown in the RWV. We conclude that altered gravity can modulate endothelial functions by transcriptional and post-transcriptional mechanisms.

Briefly, we conclude that human and murine microvascular cells respond to hypogravity in a similar fashion in terms of proliferation and nitric oxide production, while important differences are detected when we evaluate the profile of cytokine and growth factors expression. Overall, our results demonstrate that simulated hypogravity inhibits crucial steps of the angiogenic program in HMEC.

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