

Original Paper

The Impact of Altered Gravity and Vibration on Endothelial Cells During a Parabolic Flight

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Key Words

Endothelial cells • Parabolic flight • Microgravity • Hypergravity • Vibration • Cytoskeleton • Angiogenesis • Extracellular matrix • Apoptosis • Cell cycle

Abstract

Background: Endothelial cells (EC) cultured under altered gravity conditions show a cytoskeletal disorganization and differential gene expression (short-term effects), as well as apoptosis in adherently growing EC or formation of tubular 3D structures (long-term effects).

Methods: Investigating short-term effects of real microgravity, we exposed EC to parabolic flight maneuvers and analysed them on both protein and transcriptional level. The effects of hypergravity and vibration were studied separately. **Results:** Pan-actin and tubulin proteins were elevated by vibration and down-regulated by hypergravity. β -Actin was reduced by vibration. Moesin protein was reduced by both vibration and hypergravity, ezrin protein was strongly elevated under vibration. Gene expression of *ACTB*, *CCND1*, *CDC6*, *CDKN1A*, *VEGFA*, *FLK-1*, *EZR*, *ITBG1*, *OPN*, *CASP3*, *CASP8*, *ANXA2*, and *BIRC5* was reduced under vibration. With the exception of *CCNA2*, *CCND1*, *MSN*, *RDX*, *OPN*, *BIRC5*, and *ACTB* all investigated genes were downregulated by hypergravity. After one parabola (P) *CCNA2*, *CCND1*, *CDC6*, *CDKN1A*, *EZR*, *MSN*, *OPN*, *VEGFA*, *CASP3*, *CASP8*, *ANXA1*, *ANXA2*, and *BIRC5* were up-, while *FLK1* was downregulated. *EZR*, *MSN*, *OPN*, *ANXA2*, and *BIRC5* were upregulated after 31P.

Conclusions: Genes of the cytoskeleton, angiogenesis, extracellular matrix, apoptosis, and cell cycle regulation were affected by parabolic flight maneuvers. We show that the microgravity stimulus is stronger than hypergravity/vibration.

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Introduction

The endothelial cells (EC) form the inner coating of blood vessels and they are key players in human cardiovascular physiology. EC can regulate blood pressure by secreting NO and are very sensitive to external stimuli upon which they may activate different mechanisms of defense [1-5].

Furthermore, when exposed to altered gravity conditions, EC showed strong reactions ranging from alterations in cytoskeletal arrangement and gene expression after only 22 s of real microgravity to long-term effects such as formation of defined 3D structures on the Random Positioning Machine (RPM), a device which aims to simulate microgravity on Earth by randomly rotating the sample around all three axes in space, and thereby cancels out the effect of the gravity vector over time [6-11]. Some of these 3D structures assume tubular shapes resembling vascular intimas and recent studies have shown that these structures are a promising basis toward the systematic tissue engineering of vessels [12, 13].

During and after spaceflights, astronauts suffer from many different health problems, including an impaired immune system, bone and muscle loss, orthostatic intolerance, and cardiovascular problems [14, 15]. The latter are very likely caused by an endothelial dysfunction. There are several studies demonstrating the effects of both simulated and real microgravity on ECs. Cytoskeletal remodelling and actin reduction was a common observation in all the experiments and occurred on a clinostat, on a RPM and in cells cultured in a Rotating Wall Vessel (RWV) bioreactor. In addition to this, it was also found that the expression of surface adhesion molecules and extracellular matrix proteins was changed. Some of these changes resemble those observed in cardiovascular disease [16-20].

After first discovering some altered signalling pathways in ECs cultured on a RPM [10, 13], we have recently analyzed the short-term effects of real microgravity during several parabolic flight campaigns [11]. Our findings were consistent with those described above, but in contrast to the microgravity simulation techniques, there are several additional factors which might have an influence on the EC cultured on board of the aircraft. There will always be a certain amount of vibrations. In addition, there are before and after each phase of microgravity two phases of hypergravity of 1.8 *g*. The influence of hypergravity on endothelial cells has been studied before, but with different *g*-forces (3 *g*) and different durations of exposure (10 min and 48 h, respectively) [21, 22]. Vibrations, however, have not been intensively investigated. Several cell types, among them ECs, have been cultured on devices which transmit vibrations in the nanometer scale into the cells [23], but no effects have been found. Low-frequency fluid vibration at up to 12 Hz revealed an ERK1/2 mediated ET-1 release [24], but these frequencies are not comparable to those on the ZERO-G aircraft.

It is important to investigate and characterize the influence of short-term hypergravity and vibration during a parabolic flight. We therefore aimed to simulate the acceleration profile of one or 31 parabolas as well as the vibrations occurring during the whole flight. Protein as well as gene expression were analyzed and compared to the results obtained from cells exposed to a parabolic flight.

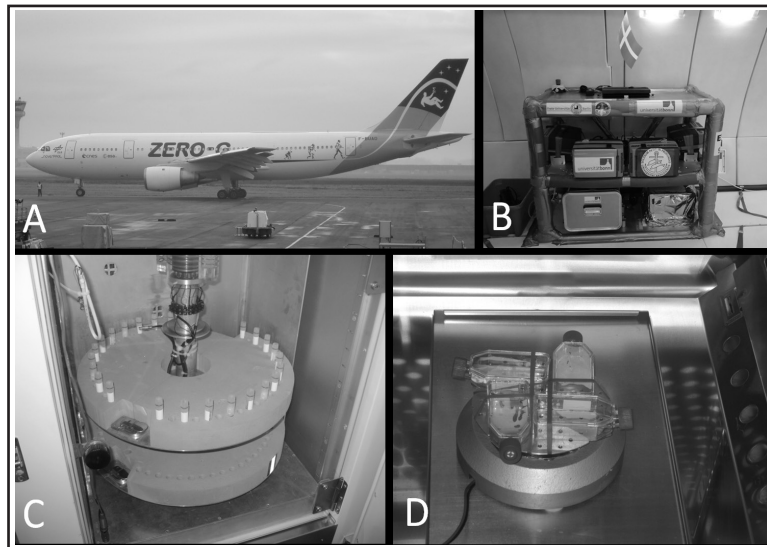
Further insights into the biological processes of ECs in Space will advance the possibilities of both the effective treatment of impaired endothelium in astronauts in Space missions as well as the refinement of tissue engineering techniques towards the generation of a functional blood vessel.

Materials and Methods

Cell culture procedure

Human endothelial EA.hy926 cells [25] were grown in RPMI 1640 medium (Invitrogen, Eggenstein, Germany) supplemented with 10 % fetal bovine serum (Biochrom, Berlin, Germany), 100 units penicillin/mL, and 100 µg streptomycin/mL.

Fig. 1. Platforms to investigate altered gravity and vibration on human endothelial cells. Parabolic flight airplane, the Novespace A300 ZERO-G Airbus (A, Novespace). Overview of the experimental rack (B), detailed views on the MuSIC (Multi Sample Incubator Centrifuge, C) and the Vibraplex vibration platform (D). both DLR, Cologne, Germany.



The cell culture procedure for the parabolic flight campaigns was published recently [26]. Briefly, for the flight experiments we used both T75 cell culture flasks (75 cm², Sarstedt, Nümbrecht, Germany) with subconfluent layers of 10⁶ cells, filled with 10 mL of medium. Syringes containing the appropriate fixative were connected to the flasks via a flexible tube and three-way valve.

Cells for quantitative real-time PCR were fixed with RNAlater (Applied Biosystems, Darmstadt, Germany) at a ratio of 4:1, while samples for Western blot analysis were fixed by addition of ethanol up to a final concentration of 70 %.

One hour before each flight, the cell culture flasks were transported to the aircraft (Fig. 1A) in transportable Cell Trans 4016 incubators (Labotect, Göttingen, Germany) and placed into similar devices which were installed on an experimental rack (Fig. 1B) and pre-heated to 37 °C. Furthermore, in-flight 1 *g* control samples were incubated in a centrifuge which was also mounted on the rack. This centrifuge was controlled by a *g*-sensor and began operation upon reaching microgravity. In addition, corresponding static 1 *g* samples were cultured in the laboratory (1 *g* controls).

Cells were fixed after P1 and P31. All cell samples were transported back to the laboratory for further investigations immediately after landing of the aircraft.

Of both parabolic flight samples (μg) and 1 *g* control groups, we collected N=6 T75 cell culture flasks for Western blot analyses (P31) and N=6 for quantitative real-time PCR (P1, P31).

Parabolic flight

All parabolic flight experiments were conducted aboard the Airbus A300 ZERO-G, which is operated by Novespace and is based in Bordeaux, France. On each of the three days of the campaign, a parabolic flight, which lasts about 3 h, including take-off and landing and encompasses 31 parabolas, takes place. Every parabola started from a steady normal horizontal flight and typically included two hypergravity (1.8 *g*) periods of 20 s, separated on average by a 22 s microgravity period. The first test parabola was followed by six series of five parabolas, separated by breaks of 4 and 8 min, respectively. The microgravity level achieved by parabolic flights is 0 \pm 0.05 *g*. The data presented emerged from the 12th, 13th, 14th and 16th parabolic flight campaigns of the German Space Agency (DLR), representing a total of 12 parabolic flights or 372 parabolas.

Hypergravity experiments

The method was recently published in detail [26]. Hypergravity was achieved by centrifugation on a Multi Sample Incubator Centrifuge (MuSIC, DLR, Cologne, Germany) (Fig. 1C) placed in an incubator at 37 °C and 5% CO₂. Confluently growing cells from T75 cell culture flasks were trypsinized and transferred into 5-mL tubes. The tubes were filled up with cell culture medium and the cells were allowed to equilibrate before centrifugation. Corresponding to the fixation times of the cells on the parabolic flight, the cells were exposed to two 20 s long 1.8 *g* phases interrupted by a 22 s pause (P1) and 2 h lasting 1.8 *g* phases (P31). Control experiments on the Short Arm Human Centrifuge (SAHC) at the DLR with cells growing in T75 cell culture flasks showed no difference to the results from the MuSIC device (data not shown). We collected N=5

Table 1. Primers used for quantitative real-time PCR. All sequences are given in 5'-3' direction

Gene	Primer Name	Sequence
<i>18S rRNA</i>	18S-F	GGAGCCTGCGGCTTAATTT
	18S-R	CAACTAAGAACGGCCATGCA
<i>ACTB</i>	ACTB-F	TGCCGACAGGATGCAGAAG
	ACTB-R	GCCGATCCACACGGAGTACT
<i>ANXA1</i>	ANXA1-F	GCCAAAGACATAACCTCAGACACAT
	ANXA1-R	GAATCAGCCAAGTCTTCATTACA
<i>ANXA2</i>	ANXA2-F	GGTACAAGATTACAGCCCTTATGACA
	ANXA2-R	CATGGAGTCATACAGCCGATCA
<i>BIRC5</i>	BIRC5-F	GCCAGATGACGACCCCATAG
	BIRC5-R	CACCAAGGGTTAATCTTCAAACCTG
<i>CASP3</i>	CASP3-F	GCAGCAAACCTCAGGGAAC
	CASP3-R	AACTGCTCTTTTGTGTGATCT
<i>CASP8</i>	CASP8-F	TGCAAAAGCACGGGAGAAAG
	CASP8-R	CTCTTCAAAGTCTGTGGTCAAG
<i>CCNA2</i>	CCNA2-F	GCAGCAGAGGCCGAAGAC
	CCNA2-R	CCAAGGAGGAACGGTGACA
<i>CCND1</i>	CCND1-F	CCCTGACGGCCGAGAAG
	CCND1-R	AGGTTCCACTTGAGCTTGTTCAC
<i>CDC6</i>	CDC6-F	TGCAGGAGAGCTATTGAAATTGTAGA
	CDC6-R	GGGAATCAGAGGCTCAGAAGGT
<i>CDKN1A</i>	CDKN1A-F	TGGAGACTCTCAGGTCGAAA
	CDKN1A-R	GCGGATTAGGGCTTCCTCTT
<i>EZR</i>	EZR-F	GAAATGCACAAGTCTGGGTACCT
	EZR-R	CTCCCACTGGTCCCTGGTAAG
<i>FLK1</i>	FLK1-F	TCTTCTGGCTACTTCTGTGCATCATC
	FLK1-R	GATGGACAAGTAGCCTGTCTTCAGT
<i>ITGB1</i>	ITGB1-F	GAAAACAGCGCATATCTGGAAATT
	ITGB1-R	CAGCCAATCAGTGATCCACAA
<i>MSN</i>	MSN-F	GAAATTTGTCATCAAGCCCATTTG
	MSN-R	CCATGCACAAGGCCAAGAT
<i>OPN</i>	OPN-F	CGAGGTGATAGTGTGGTTTATGGA
	OPN-R	CGTCTGTAGCATCAGGGTACTG
<i>RDX</i>	RDX-F	GAAAATGCCGAAACCAATCAA
	RDX-R	GTATTGGGCTGAATGGCAAATT
<i>VEGFA</i>	VEGFA-F	GCGCTGATAGACATCCATGAA C
	VEGFA-R	CTACCTCCACCATGCCAAGTG
<i>VEGFD</i>	VEGFD-F	TGCAGGAGGAAAATCCACTTG
	VEGFD-R	CTCGCAACGATCTTCGTCAA

1g controls and N=5 1.8 g hyper-g samples (P1 and P31) for Western blot analyses (N=5) and quantitative real-time PCR (N=5), respectively. The 1g controls were grown in a neighbouring equal incubator.

Vibration experiments

The detailed method was published earlier [26]. The Vibraplex vibration platform (frequency range 0.2 Hz - 14 kHz) was used to create vibrations comparable to those occurring during parabolic flights (Fig. 1D). Corresponding vibrations to the three phases pull up (1.8 g), free fall (μg), and pull out (1.8 g) were recorded and analysed by Schmidt [27]. These data were then used for our simulation experiments with the Vibraplex. For quantitative real-time PCR analyses, we collected N=18 samples of the groups 1 g and 31 parabolas of vibration. The 1g controls were grown separately in a similar incubator.

RNA isolation

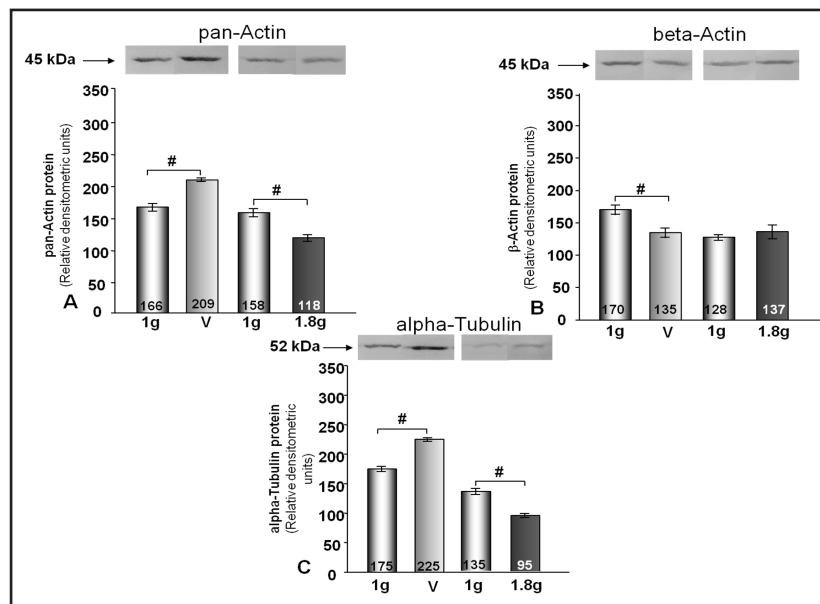
After the flight, the fixative was discarded and replaced by 10 mL of RNeasy Lysis Buffer. Subsequently the flasks were stored at 4 °C and transported to our laboratories in Berlin. The RNeasy Lysis Buffer was replaced by PBS (Invitrogen, Darmstadt, Germany). The cells were scraped off using cell scrapers (Sarstedt, Nümbrecht, Germany), transferred to tubes and pelleted by centrifugation (2500xg, 10 min, 4 °C). The RNeasy Mini Kit (Qiagen, Hilden, Germany) was used according to the manufacturer's instructions to isolate total RNA. RNA concentrations and quality were determined spectrophotometrically at 260 nm using a NanoDrop instrument (Thermo Scientific, Wilmington, DE, USA). The isolated RNA had an A260/280 ratio of >1.5.

cDNA designated for the quantitative real-time PCR was then obtained with the First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany) using 1 μg of total RNA in a 20 μL reverse transcription reaction mixture.

Quantitative real-time PCR

Quantitative real-time PCR was used to determine the expression levels of the genes of interest. The Primer Express® software was utilised to design appropriate primers with a T_m of about 60 °C (Table 1). The primers were synthesised by TIB Molbiol (Berlin, Germany). All assays were run on a StepOnePlus

Fig. 2. The influence of hypergravity and vibration on cytoskeletal proteins. Western Blot analyses are shown for the proteins pan-Actin, β -Actin, and α -Tubulin. 1g: 1g control cells; V: cells exposed to simulated vibration; 1.8g: cells exposed to 31 parabolas of hypergravity according to the parabolic flight profile; #P<0.05 vs. corresponding 1g control. Values are given as mean \pm standard deviation.



Real-Time PCR System using the Power SYBR®Green PCR Master Mix (both Applied Biosystems, Darmstadt, Germany). The reaction volume was 25 μ L including 1 μ L of template cDNA and a final primer concentration of 500 nM. PCR conditions were as follows: 10 min at 95 °C, 40 cycles of 30 s at 95 °C and 1 min at 60 °C, followed by a melting curve analysis step (temperature gradient from 60 °C to 95 °C with +0.3 °C per cycle).

If all amplicons showed one single T_m similar to the one predicted by the Primer Express software the PCR reactions were considered specific. Every sample was measured in triplicate and we utilized the comparative C_T ($\Delta\Delta C_T$) method for the relative quantification of transcription levels. 18S rRNA was used as a housekeeping gene to normalize our expression data.

Western blot analysis

SDS-PAGE, immunoblotting and densitometry were carried out on six replicates following routine protocols [28-30]. Antibodies against the following antigens were used: α -tubulin, pan-actin, and β -actin, moesin and radixin (dilution 1:1000, all antibodies were purchased by Cell Signaling Technology Inc., MA, USA). For the densitometric quantification of the bands, the stained membranes were scanned and analysed using the Image J (<http://rsb.info.nih.gov/ij/>) software [31]. Since no suitable protein was found which could serve as a loading control under the investigated experimental conditions, we carefully loaded equal amounts of protein (40 μ g in 10 μ L) onto each gel lane and normalized the densitometric data to this value.

Statistical analysis

All statistical analyses were performed using the SPSS 16.0 software (SPSS, Inc, Chicago, IL, USA). We employed either one-way ANOVA or the Mann-Whitney-U-test where applicable. Differences were considered significant at the level of $p < 0.05$. All data are represented as means \pm standard deviation.

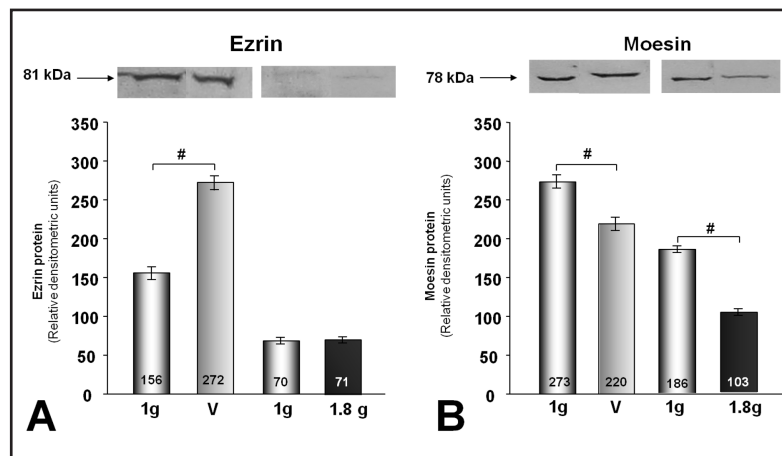
Results

Hypergravity and Vibration

Changes on the Protein Level. In order to investigate the impact of hypergravity and vibration on the protein levels of selected genes of interest, we performed Western Blot analyses on ECs exposed to both conditions as described in the methods section. The Pan-actin and alpha-tubulin contents of the cells were significantly elevated by vibration and significantly down-regulated by hypergravity. In contrast, the beta-actin protein was reduced by vibration, but hypergravity did not change its amount (Fig. 2).

Furthermore, we examined two proteins belonging to the ERM-family of regulators of membrane-cortex interactions and signaling: ezrin and moesin. Ezrin protein was strongly

Fig. 3. The influence of hypergravity and vibration on ERM proteins. Western Blot analyses are shown for the proteins ezrin and moesin. 1g: 1g control cells; V: cells exposed to simulated vibration; 1.8g: cells exposed to 31 parabolas of hypergravity according to the parabolic flight profile, #P<0.05 vs. corresponding 1g control. Values are given as mean \pm standard deviation.



and significantly elevated in EC cultured under conditions of vibration, cultivation under hypergravity of 1.8g did not induce changes. In contrast, moesin protein was significantly reduced by both vibration and hypergravity in ECs after 2 hours (Fig. 3).

Changes on the Transcriptional Level. In addition to the genes belonging to the proteins analyzed in the Western Blots, we choose further transcripts of interest, mainly those which are involved in the regulation of the cell cycle, angiogenesis, (3D) proliferation, cellular signalling, apoptosis and extracellular matrix. The list comprised *ACTB*, *CCNA2*, *CCND1*, *CDC6*, *CDKN1A*, *VEGFA*, *VEGFD*, *FLK1*, *EZR*, *MSN*, *RDX*, *ITGB1*, *SPP1* (*OPN*), *CASP3*, *CASP8*, *ANXA1*, *ANXA2*, and *BIRC5* (Survivin).

ACTB gene expression was significantly diminished after 2 h by hypergravity. Vibration also reduced the expression of *ACTB* significantly (Fig. 4A).

Transcription of *CCNA2* and *CCND1* was not affected by hypergravity, while *CDC6* gene expression was significantly decreased after 2 h and *CDKN1A* transcript levels were significantly suppressed after 20 s of hypergravity. Except for *CCNA2*, the other three transcripts were found to be down-regulated by vibration (Fig. 4B).

Hypergravity had an impact on all three investigated angiogenic genes. Both *VEGFA* and *VEGFD* were significantly down-regulated in the 1P (hypergravity simulation of one parabola) group, but only *VEGFD* remained suppressed in the 31P (hypergravity simulation of 31 parabolas) group. *FLK1*, on the other hand was clearly, although not mathematically significantly, up-regulated in the 1P group and reverted back to control levels after 2h of hypergravity. Vibration decreased *VEGFA*- and *FLK1*-expression significantly (Fig. 4C).

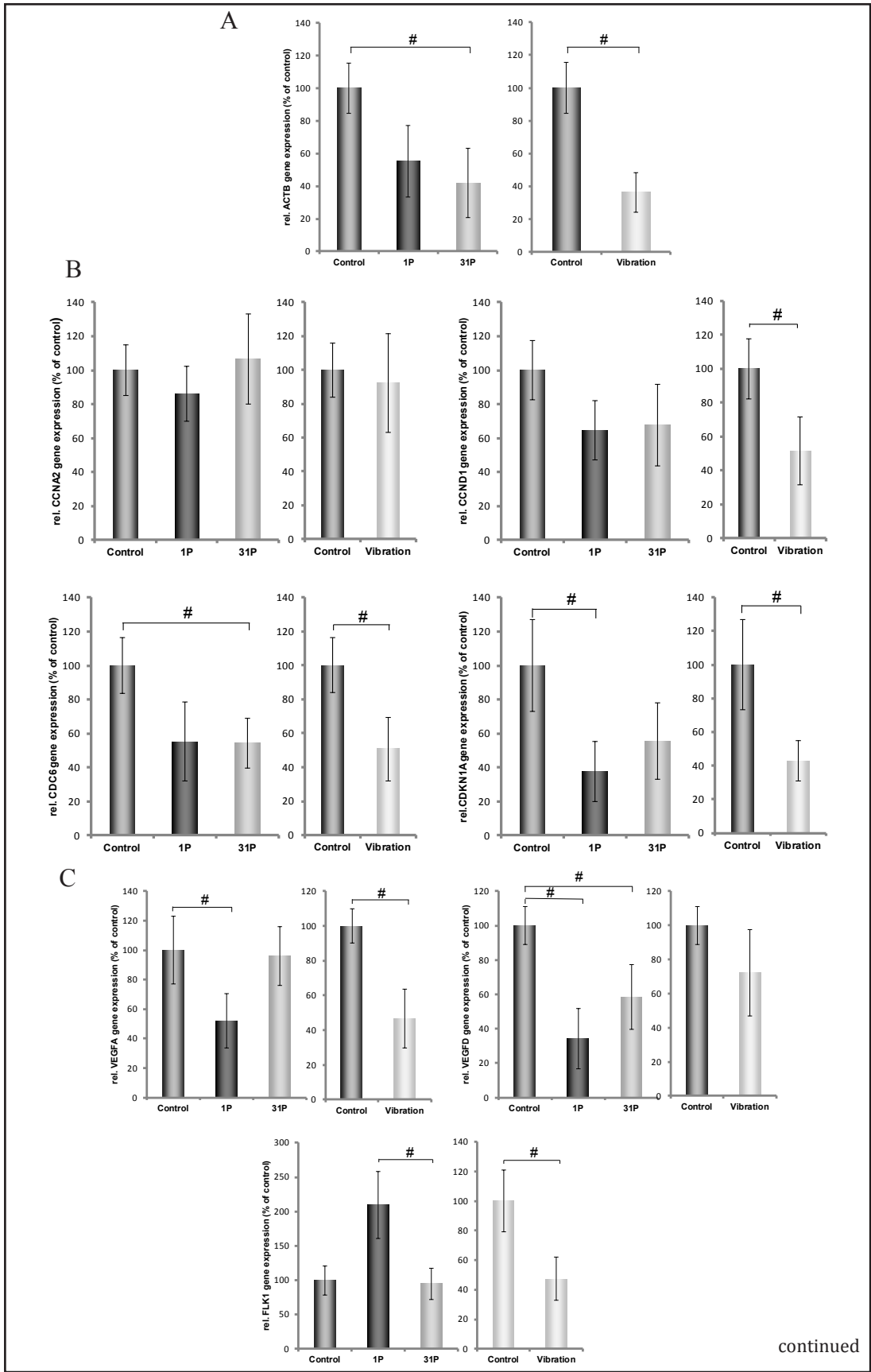
Of the ERM genes, only *EZR* showed a reaction to hypergravity and vibration. In both 1P and 31P groups as well as in cells grown under vibration *EZR* expression was significantly decreased in comparison to the control groups (Fig. 4D).

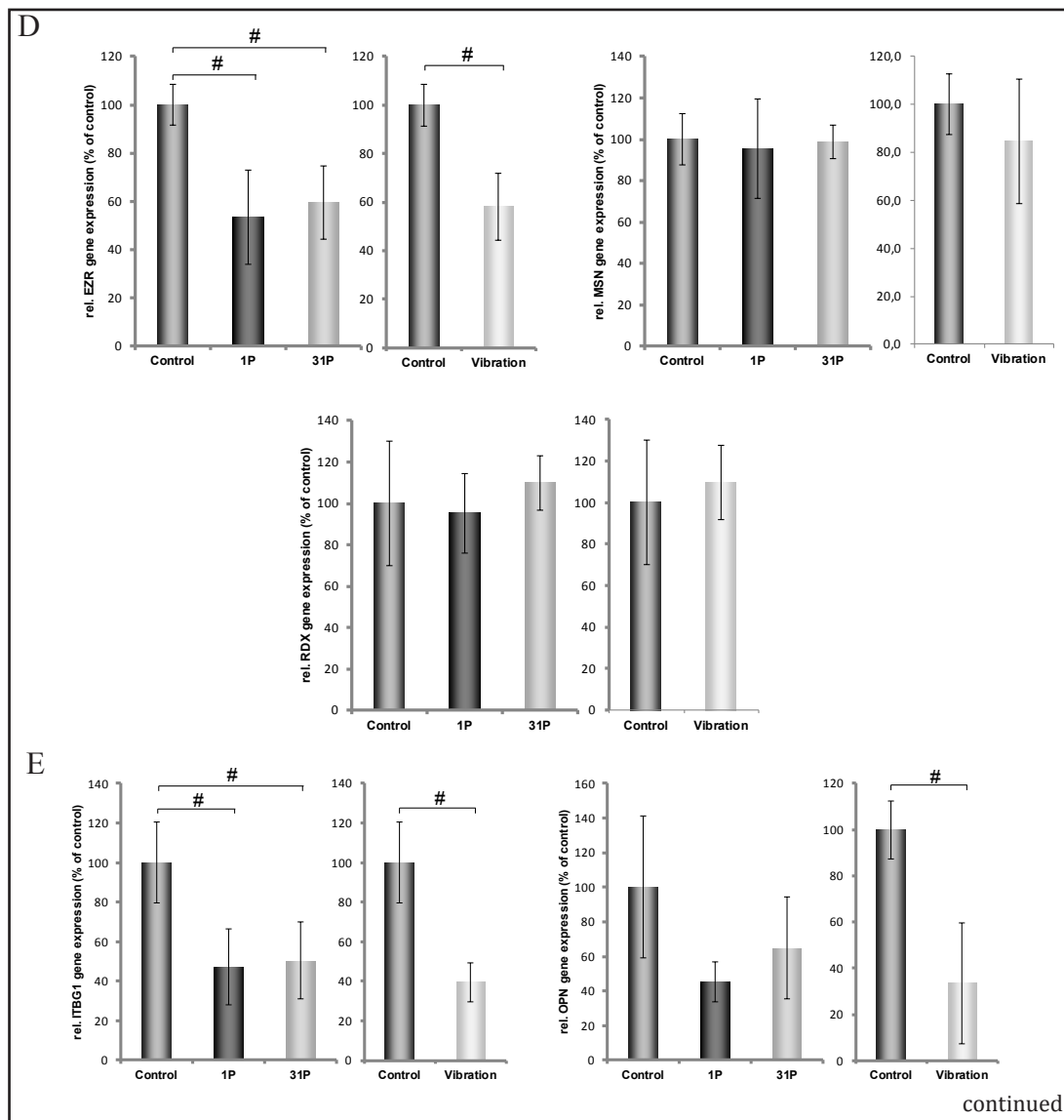
ITGB1 transcript levels were decreased in both hypergravity groups as well as in cells exposed to vibration. *OPN* expression on the other hand, did not change significantly under hypergravity, but also decreased significantly under vibration (Fig. 4E).

CASP3 and -8 gene expression were significantly decreased in the hypergravity 1P group, but while *CASP3* remained suppressed in the 31P cells, *CASP8* rose back to normal levels. Both transcripts were downregulated by vibration. Similarly, *ANXA1* and *ANXA2* expression were significantly reduced in hypergravity 1P cells, but no significant difference was found in 31P cells compared to 1 g control cells. Vibration only decreased *ANXA2* expression significantly but did not affect *ANXA1*. *BIRC5* did not show any significant effects in hypergravity, although a similar tendency for decreased transcription was observed. Vibration reduced *BIRC5* transcript levels significantly (Fig. 4F).

Parabolic Flight

Changes on the Transcriptional Level. To compare the results from the isolated hypergravity and vibration experiments with the effects from a real parabolic flight, we also





measured the gene expression levels of our selected transcripts using the quantitative real-time PCR technique.

Although we observed a general tendency of transcriptional upregulation of *ACTB* during parabolic flight, these changes did not prove to be statistically significant (Fig. 5A).

After one and 31 parabolas of real microgravity *CCNA2*, *CCND1*, *CDC6*, and *CDKN1A* were all strongly and significantly up-regulated in comparison to the 1 *g* control cells (Fig. 5B).

Both *VEGFA* and *VEGFD* expression was significantly elevated after 31 parabolas, while *FLK1* was clearly down-regulated after one parabola and reverted back to control levels after 31 parabolas (Fig. 5C).

Under the conditions of parabolic flight the transcription of *EZR* and *MSN* showed an identical pattern. After one parabola both genes were strongly up-regulated, and transcription decreased after 31 parabolas, but still remained significantly higher than in 1 *g* control cells (Fig. 5D).

Levels of *ITGB1* transcript did not change significantly during the whole parabolic flight, whereas *OPN* gene expression increased over time, reaching a significant peak after the 31st parabola (Fig. 5E).

CASP3, *-8*, and *ANXA1* transcription were significantly upregulated after the first parabola and remained at this level until after the 31st parabola. In contrast to this *ANXA2*

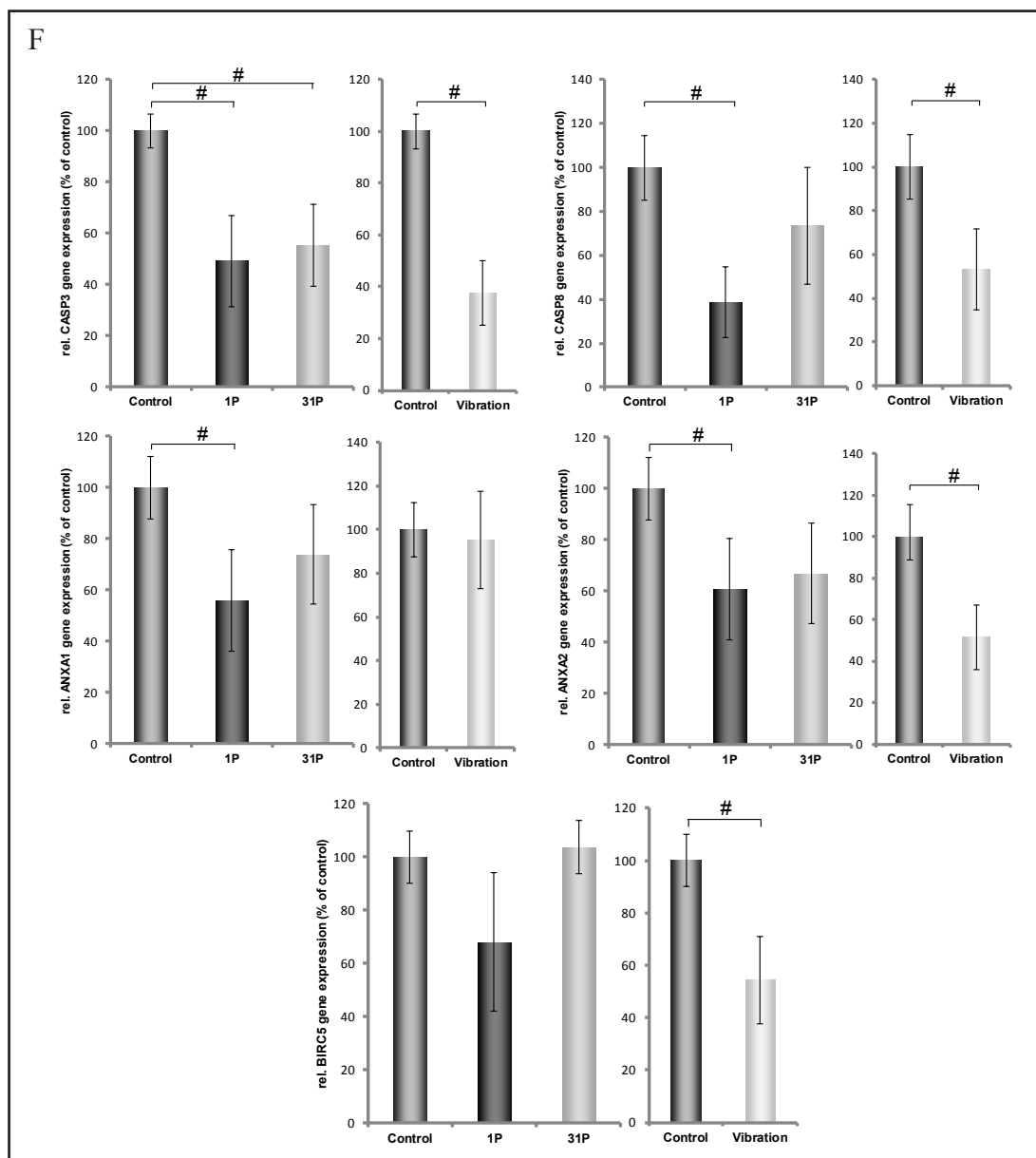
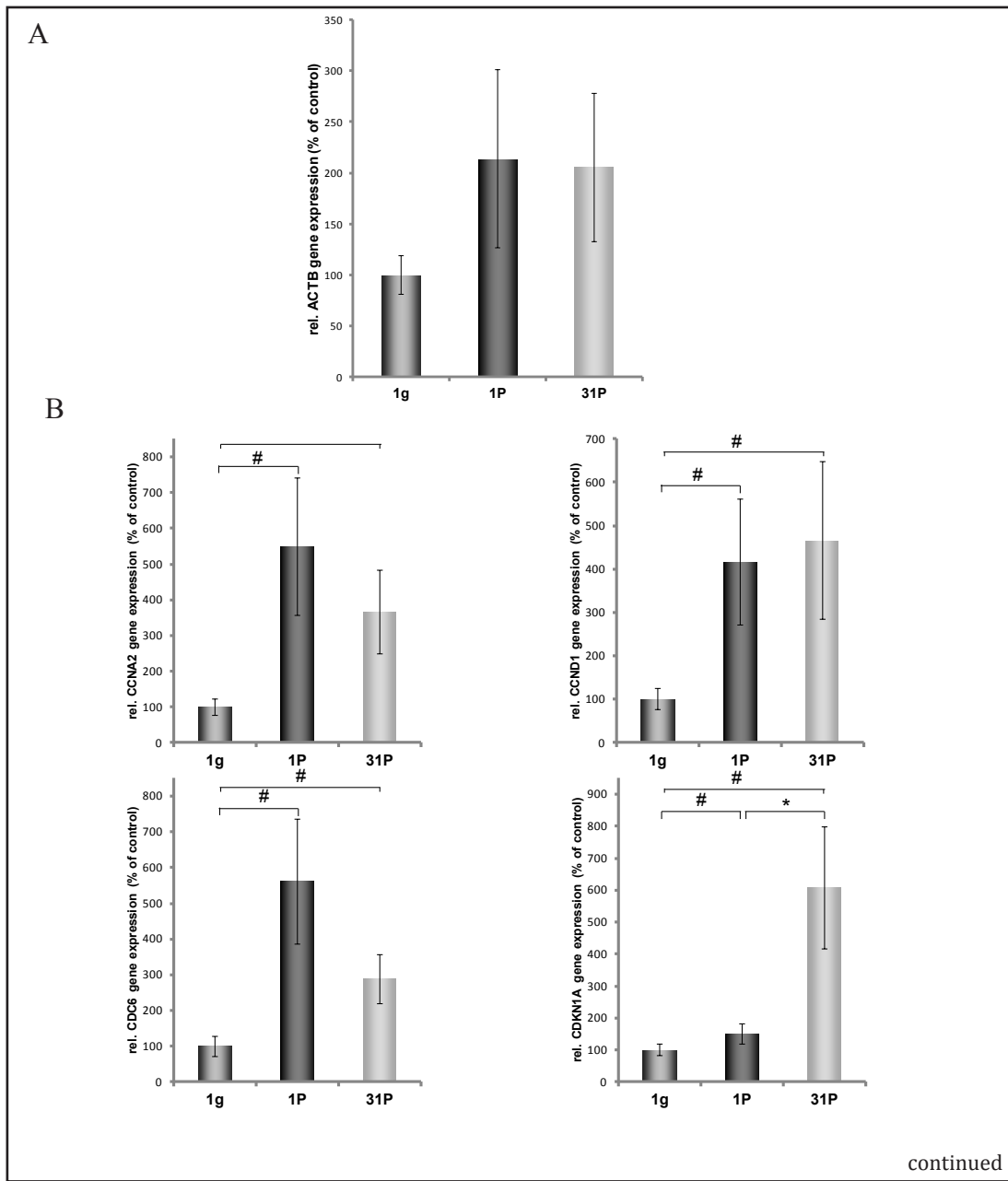


Fig. 4. Quantitative real-time PCR – vibration and hypergravity. Quantitative real-time PCR analyses are shown for A: gene of the cytoskeleton, B: genes involved in cell cycle regulation, C: genes involved in angiogenesis, D: ERM genes, E: extracellular matrix genes, and F: genes involved in apoptosis. 1P: 1 parabola of hypergravity according to the parabolic flight profile; 31P: 31 parabolas of hypergravity according to the parabolic flight profile; #P<0.05 vs. corresponding 1g control; *P<0.05 vs. P1. Values are given as mean \pm standard deviation.

and *BIRC5* gene expressions were strongly elevated after one parabola and decreased after the 31st parabola, while still remaining significantly elevated compared to 1g control cells (Fig. 5F).

Discussion

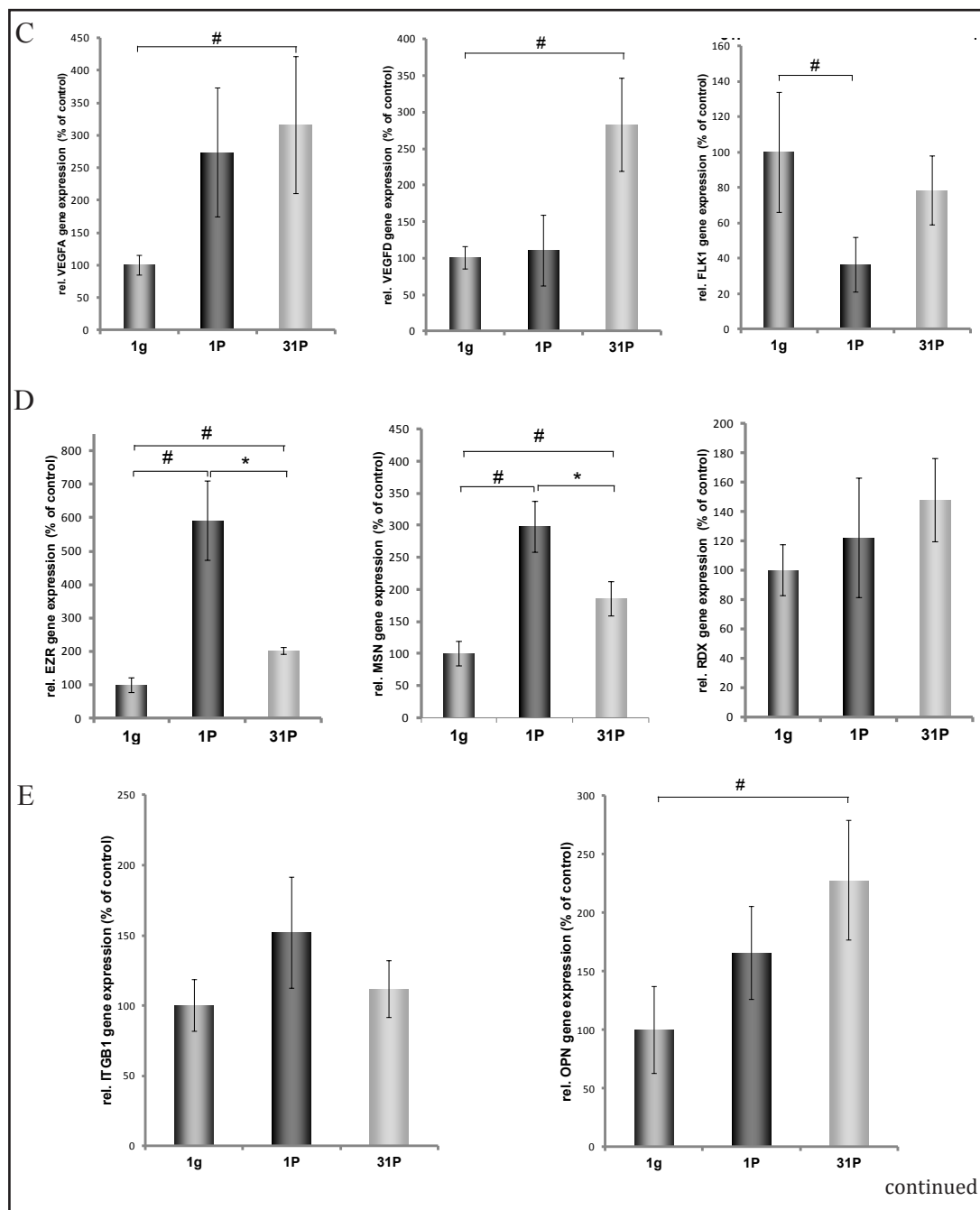
We have shown previously that cells, when exposed to a parabolic flight, undergo severe changes already after the first parabola [11, 26]. During parabolic flights, periods



of micro- and hypergravity alternate and are accompanied by vibrations. In this study, we aimed to investigate to what extent hypergravity and vibrations in addition to microgravity influence the protein- and gene expression of the cell line EA.hy926 during a parabolic flight. Therefore we exposed the cells to the various conditions separately and analyzed genes involved in the cytoskeleton (*ACTB*, *EZR*, *MSN*, *RDX*), cell cycle regulation (*CCNA2*, *CCND1*, *CDC6*, *CDKN1A*), angiogenesis (*VEGFA*, *VEGFD*, *FLK1*), cell adhesion, migration and signalling (*EZR*, *MSN*, *RDX*), apoptosis (*CASP3*, *CASP8*, *ANXA1*, *ANXA2*, *BIRC5*), as well as extracellular matrix (ECM) (*ITGB1*, *OPN*) (Table 2).

Cytoskeleton

ACTB gene and protein expression correlate directly under vibration, with both showing a significant decrease. Interestingly, also hypergravity induced a reduction in *ACTB* gene expression, while *ACTB* protein levels remained unchanged. Tubulin protein was



downregulated in hypergravity and upregulated by vibration. This is in good agreement with earlier studies, where we showed similar tendencies for TUBB gene expression [26]. The results for the parabolic flight correspond with earlier observations [11, 26], which showed that cytoskeletal proteins underwent a drastic rearrangement, but did not change their concentration inside the cells. These results suggest that hypergravity/vibration are severe stress factors for the cells and induce a deterioration of the cytoskeletal network.

The ezrin (EZR), radixin (RDX) and moesin (MSN) proteins are usually referred to as the ERM proteins and play a crucial role in the organization and maintenance of the cell cortex, the interface between the extracellular environment and the cytoskeleton [32]. The three proteins share a high similarity in their amino acid composition, yet there are some differences that hint towards specialized functionality. Ezrin is mostly found in epithelial

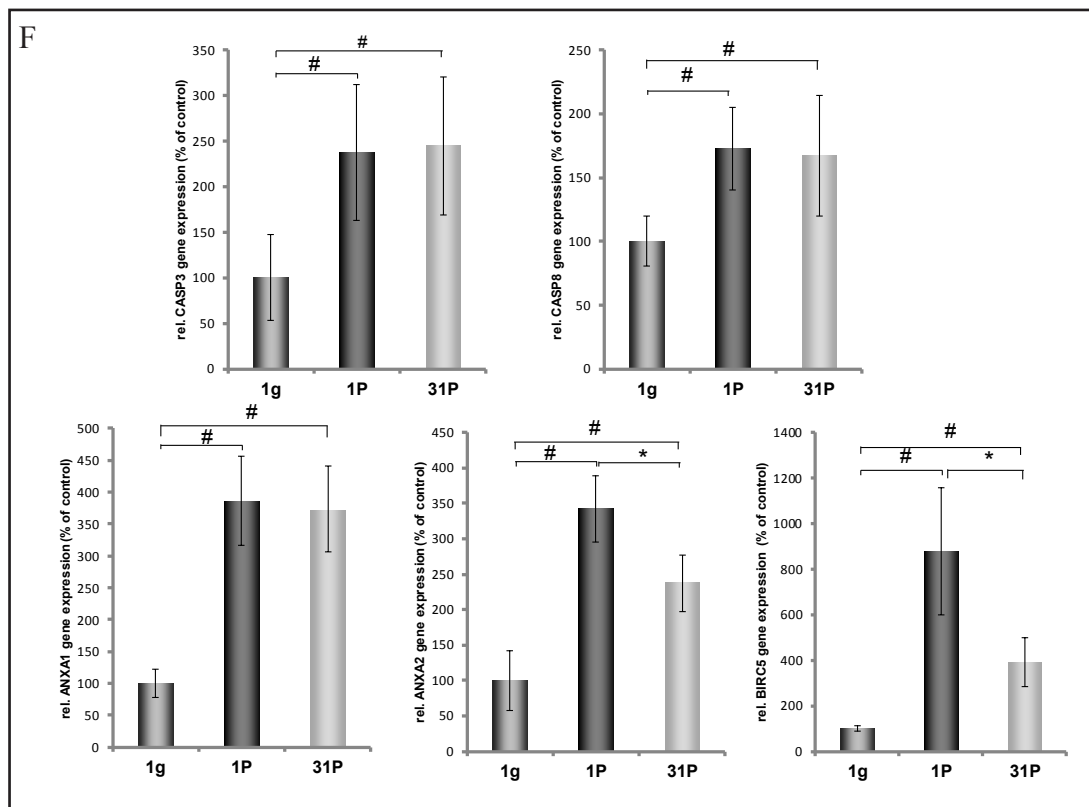


Fig. 5. Quantitative real-time PCR – real microgravity. Quantitative real-time PCR analyses are shown for A: gene of the cytoskeleton, B: genes involved in cell cycle regulation, C: genes involved in angiogenesis, D: ERM genes, E: extracellular matrix genes; and F: genes involved in apoptosis. 1P: cells after one flown parabola; 31P: cells after 31 flown parabolas; #P<0.05 vs. corresponding 1g control; *P<0.05 vs. P1. Values are given as mean \pm standard deviation.

cells, moesin in endothelial cells, and radixin in hepatocytes [33-35]. They all share a common appearance and are composed of an amino-terminal FERM (Four point one, ERM) domain, mediating membrane association, an α -helix rich middle, and an F-actin binding site (C-ERMAD, the C-terminal ERM-association domain) at the carboxy terminus [36]. The best documented and probably most important function of the ERMs is the bundling and arrangement of F-actin filaments parallel to the cell membrane, but numerous studies suggest additional roles for them. ERMs have been implicated in Rho signalling [37-40] and, interestingly, lumen morphogenesis [41-43]. We detected a down-regulation of the corresponding ERM genes by hypergravity/vibration while real microgravity leads to their up-regulation. This might explain the observed actin rearrangement after one parabola and may also be one first step towards tube formation as seen in long-term RPM experiments, whereas under hypergravity or vibrations none such effects were present.

Angiogenesis

The proteins VEGF-A and -D, encoded by *VEGF-A* and *VEGFD* genes, belong to the vascular endothelial growth factor family comprising the five members VEGFA, -B, -C, -D, and F as well as placenta growth factor (PlGF) [44-46]. VEGF-A is the most important type of VEGF for angiogenesis, i.e. the formation of new vessels from existing ones [44]. When VEGF proteins are secreted, they are bound by tyrosine kinase receptors. For VEGF-A and VEGF-D FLK-1, also known as VEGFR-2, encoded by *FLK1*, is the most important receptor and it is mainly expressed in vascular endothelial cells [45]. The VEGF/VEGFR-2 regulatory system plays a central role in angiogenesis and is also involved in various other biological functions, such

Gene expression						
Biological Function	Gene Symbol	Hypergravity 1P	Hypergravity 31P	Vibration 31P	Parabolic Flight 1P	Parabolic Flight 31P
Cytoskeleton	<i>ACTB</i>	(-)	-	-	(+)	(+)
Cell cycle regulation	<i>CCNA2</i>				+	+
	<i>CCND1</i>	(-)	(-)	-	+	+
	<i>CDC6</i>	(-)	-	-	+	+
	<i>CDKN1A</i>	-	(-)	-	+	+
Angiogenesis	<i>VEGFA</i>	-	-	-	(+)	+
	<i>VEGFD</i>	-	-	(-)		+
	<i>FLK1</i>	(+)		-	-	
Cell adhesion, migration, and signalling	<i>EZR</i>	-	-	-	+	+
	<i>MSN</i>				+	+
	<i>RDX</i>					(+)
Extracellular matrix	<i>ITGB1</i>	-	-	-		
	<i>OPN</i>	(-)	(-)	-	(+)	+
Apoptosis	<i>CASP3</i>	-	-	-	+	+
	<i>CASP8</i>	-		-	+	+
	<i>ANXA1</i>	-			+	+
	<i>ANXA2</i>	-		-	+	+
	<i>BIRC5</i>		(-)	-	+	+
Protein Content						
Biological Function	Protein Name	Hypergravity 1P	Hypergravity 31P	Vibration 31P	Parabolic Flight 1P	Parabolic Flight 31P
Cytoskeleton	pan-Actin	n.d.	-	+	n.d.	n.d.
	beta-Actin	n.d.		-	n.d.	n.d.
	alpha-Tubulin	n.d.	-	+	n.d.	n.d.
Cell adhesion, migration, and signalling	Ezrin	n.d.		+	n.d.	n.d.
	Moesin	n.d.	-	-	n.d.	n.d.

P: one parabola; 31P: 31 parabolas; +,-: significant up- or downregulation compared to 1g control; (+,-): suggestive up- or downregulation compared to 1g control; blank: no change compared to 1g control; n.d.: not determined

Table 2. A brief summary of gene-expression and protein content data

as wound healing and burn injury, regulation of endothelial growth or pathophysiological processes such as tumor vascularisation, and is also a survival factor for endothelial cells [47-50].

In our hypergravity experiments, both *VEGFA* and *VEGFD* were down-regulated and, possibly as a reaction to VEGF-A and -D depletion, *FLK1* gene expression was up-regulated. This seems to hint towards the induction of a non-angiogenic phenotype by this kind of treatment. In contrast to this, real microgravity seems to promote angiogenesis, by up-regulating both growth factors and their receptor.

Extracellular matrix

Both the *ITGB1* and the *OPN* gene expression were decreased in hypergravity or vibration. Integrins mediate cell adhesion to ECM and cell-cell contacts. They also play an important role in development and immune responses, and are also vital in the development of many human diseases [51]. Osteopontin, encoded by *OPN*, is secreted by the cells and is a part of the ECM. As such it is also a ligand for different integrins [52, 53]. Besides being implicated in chemotaxis and cell activation it was also shown, that osteopontin is an important anti-apoptotic factor, as it is able to block programmed cell death in stress-exposed endothelial cells [54-56]. The observation, that both *ITGB1* and *OPN* genes are down-regulated under hypergravity and vibration and up-regulated in microgravity shows that alterations in gravity have an influence on cell adhesion and cell survival with microgravity inducing protective effects.

Apoptosis

The *CASP3*, *CASP8*, *ANXA1*, *ANXA2*, and *BIRC5* (also known as Survivin) genes are all implied in the process of programmed cell death, also called apoptosis. The process of apoptosis manifests itself by an internal proteolytic digestion which leads to a breakdown

of cellular function and infrastructure. Possible triggers of apoptosis are manifold and can range from lack of growth factors, DNA damage, ionizing radiation, ischemic injury, and more [57-62].

The majority of apoptotic pathways are mediated by a family of cysteine aspartate-specific proteases, commonly called the "caspases". These caspases can be grouped into the initiator (caspase-8 and -10) and the effector caspases (caspases-3, -6, and -7). CASP8 and CASP3 are involved into the so-called extrinsic pathway. Here, specific death-receptors on the cells surface trigger a cascade which first activates the initiator caspase-8, which in turn cleaves and activates the executioner caspase-3. Caspases disrupt many proteins involved in cytoskeletal integrity and cell-cell communication, they attack the endoplasmatic reticulum, the golgi apparatus and the nucleus itself [62-64].

ANXA1 and ANXA2 belong to the annexin superfamily of calcium or calcium and phospholipid binding ("annexing") proteins, which comprises a total of 13 members [65]. ANXA1 and ANXA2 expression is highly abundant in smooth muscle and endothelial cells [66, 67]. The annexins have multiple physiological roles: they can regulate the plasma membrane architecture, interact with the actin cytoskeleton and are also involved in the regulation of intracellular Ca^{2+} homeostasis [68-70]. Interestingly, ANXA1 in particular was also reported to be a regulator of cell proliferation and apoptosis. It has been shown, that ANXA1, by being a substrate for the EGF rector tyrosine kinase, is an inhibitor of EGF-dependent cell proliferation [71, 72]. Furthermore many, but not all studies indicated that ANXA1 is an inducer of apoptosis. Overexpression of ANXA1 in monocytic cells led to an enhanced TNF- α -induced apoptosis. 5 days after transfection with full-length ANXA1, about 70% of these cells underwent apoptosis and changes in ANXA1 expression had an effect on caspase-3 activity. Exogenous ANXA1 promoted Ca^{2+} influx into human neutrophils and accelerated apoptosis [73-75].

BIRC5 (also known as survivin) is an inhibitor of apoptosis proteins and is involved in cell division and apoptosis suppression [76-78]. The exact mechanism of action of survivin is not completely understood, but studies have shown that it directly interacts with effector caspases-3 and -7, thus preventing their activation. Furthermore, it can also interfere with the caspase-independent AIF pathway of cell death [79, 80].

In this work we have observed, that the proapoptotic genes *CASP3*, *CASP8*, *ANXA1*, and *ANXA2* as well as the antiapoptotic gene *BIRC5* were downregulated in hypergravity/vibration and upregulated in real microgravity during a parabolic flight. It has been observed earlier, that endothelial cells, when exposed to prolonged simulated microgravity on an RPM for up to 72 h, develop apoptosis [10], therefore our results for the parabolic flight might represent the first steps, still contained by the similarly overexpressed *BIRC5*, towards the development of overt programmed cell death at a later stage, which is supported by the fact that EC on a parabolic flight did not show any visible sign of apoptosis [26]. Our results with cells exposed to hypergravity/vibration seem to hint towards the induction of a non-apoptotic cell state, which might have further impact for cell culture or tissue engineering techniques. This is the first time this kind of analysis was performed on endothelial cells and further work is required to study these effects in more detail.

Cell Cycle Regulation

Cyclin A2, encoded by *CCNA2*, and cyclin D1, encoded by *CCND1*, belong to the big family of cyclins. In general, cyclins regulate cell cycle progression. This is mediated by the sequential activation of different cyclins. They all bind, activate, and determine the substrate specificity of their binding partners, the serine-threonin kinases, also called cyclin-dependent kinases (Cdks) [81, 82].

The cyclins can be subdivided into the different calluses A to I, and T, which have different functions. While cyclin A1 is exclusively expressed in testis and germ line, cyclin A2 is found practically ubiquitously in cultured cells and different cancers [83-85]. By interacting either with Cdk2 or Cdk1 it is able to control both S phase and G_2/M transition, respectively.

During the S phase cyclin A2 regulates the DNA synthesis, while it acts as a trigger for cyclin B1-Cdk1 activation at the G₂/M transition [86-89].

The D-type cyclins are important players in the entry into the G1/S phase of the mitotic cell cycle, but they have also been implicated in differentiation, regulation of transcription or apoptosis [90-92].

CDKN1A, also known as p21^{Waf1/Cip1/Sdi1}, functions as an inhibitor of a broad spectrum of Cdks [93, 94] and was first identified as one component of a complex together with cyclin D1, a Cdk, and PCNA [95]. CDKN1A is involved in a variety of biological processes. Most prominently it is a negative regulator of cell cycle progression, which maintains cells in the G0 phase upon accumulation [96, 97]. Furthermore, CDKN1A is also implicated in apoptosis. Generally, downregulation of CDKN1A leads to p53-dependent apoptosis. DNA damage, for example, induces cleavage of CDKN1A by caspase 3, which can promote apoptosis in growth-arrested cancer cells [98]. In addition, it was also shown to be involved in DNA repair and senescence [99, 100].

Interestingly, a recent study investigating T lymphocytes on a 2D clinostat and on a parabolic flight also reported an increase in *CDKN1A* expression under both conditions [101].

CDC6, cell division cycle 6, is a central and essential factor of DNA replication and highly conserved in all eukaryotic organisms. Downregulation of CDC6 levels have been shown to prevent G₁ cells from progression into S phase [102, 103]. Furthermore it was found that lack of CDC6 stops cell proliferation and induces apoptosis [104, 105].

Our results show that these important genes for cell cycle regulation are transcribed in an altered way under both hypergravity/vibration and conditions of a parabolic flight compared to the static 1 g control. The expression patterns indicate that cells cultured under hypergravity or vibration alone undergo changes which lead to a slowed down cell cycle, while parabolic flights (hypergravity + vibration + microgravity) induce proliferation.

Conclusions

In the present study we have shown that *ACTB*, *EZR*, *MSN*, *RDX*, *CCNA2*, *CCND1*, *CDC6*, *CDKN1A*, *VEGFA*, *VEGFD*, *FLK1*, *ITGB1*, *OPN*, *CASP3*, *CASP8*, *ANXA1*, *ANXA2*, and *BIRC5* are expressed in a gravity- and/or vibration-dependent manner in endothelial cells (for a concise overview see Table 2). It is noteworthy, that isolated hypergravity and vibration have diametrically different effects on the expression of these genes compared to the exposure to parabolic flight. Up to now, the exact mode of sensing the three conditions applied is not yet completely elucidated. So far suggested mechanisms can be divided into centralized/direct and decentralized/indirect models. Centralized models comprise the action of mechanosensitive ion channels [106-108], mechanosensitive protein kinases [109, 110], or caveolae [111, 112]. In addition, evidence for indirect mechanisms involving integrins [113-115] or the cytoskeleton [116] have also been reported. We hypothesize that gravisensing is mainly mediated by an indirect mechanism involving the cytoskeleton, because we had observed a rearrangement of various cytoskeletal components after one parabola [11] as well as after prolonged exposure of endothelial cells to simulated microgravity on an RPM for 5 to 7 days [9, 10, 12, 13]. Furthermore, centrifugation and parabolic flights have diametrically different effects on the expression of the genes investigated. This seems to hint towards an ability of the cells to sense different levels of gravity and not only gravitational changes. But the signal exerted by microgravity is able to override the effects of the hypergravity phases and the vibration, because the parabolic flight results resemble more the RPM than the centrifugation results. Therefore, microgravity represents the strongest stimulus during this kind of experiment.

Abbreviations

2D (two-dimensional); 3D (three-dimensional); ACTB (β -actin); ANOVA (analysis of variance); ANXA1 (annexin A1); ANXA2 (annexin A2); BIRC5 (survivin); CASP3 (caspase 3); CASP8 (caspase 8); CCNA2 (cyclin A2); CCND1 (cyclin D1); CDC6 (cell division cycle 6 homolog); CDKN1A (cyclin-dependent kinase inhibitor 1A); cDNA (complementary DNA); C-ERMAD (C-terminal ERM-association domain); C_T (threshold cycle); DLR (Deutsches Zentrum für Luft- und Raumfahrt); EC (endothelial cells); ERK1/2 (extracellular signal-regulated kinase 1/2); ERM (Ezrin-Radixin-Moesin); ET-1 (endothelin 1); EZR (ezrin); FERM (Four point one, ERM); FLK1 (VEGF-receptor 2); Hz (Hertz); ITGB1 (integrin β 1); mg (milligram(s)); mL (millilitre(s)); MSN (moesin); MuSIC (Multi Sample Incubator Centrifuge); OPN (osteopontin); P (parabola); PBS (phosphate buffered saline); PCNA (Proliferating-Cell-Nuclear-Antigen); PCR (polymerase chain reaction); RDX (radixin); RNA (ribonucleic acid); RPM (Random Positioning Machine); RWV (Rotating Wall Vessel); s (seconds); SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis); T_m (annealing temperature); TUBB (tubulin β); VEGFA (vascular endothelial growth factor A); VEGFD (vascular endothelial growth factor D).

Competing Interests

The authors declare that they have no competing interests.

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