Original Paper

Reduced Expression of Cytoskeletal and Extracellular Matrix Genes in Human Adult Retinal Pigment Epithelium Cells Exposed to Simulated Microgravity

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Key Words

Simulated microgravity • Retinal pigment epithelium cells • 3D growth • Spheroids • Gene expression • Random Positioning Machine • Cytoskeleton • Vascular endothelial growth factor

Abstract

Background/Aims: Microgravity (μg) has adverse effects on the eye of humans in space. The risk of visual impairment is therefore one of the leading health concerns for NASA. The impact of μg on human adult retinal epithelium (ARPE-19) cells is unknown. **Methods:** In this study we investigated the influence of simulated μg (s- μg ; 5 and 10 days (d)), using a Random Positioning Machine (RPM), on ARPE-19 cells. We performed phase-contrast/fluorescent microscopy, qRT-PCR, Western blotting and pathway analysis. **Results:** Following RPM-exposure a subset of ARPE-19 cells formed multicellular spheroids (MCS), whereas the majority of the cells remained adherent (AD). After 5d, alterations of F-actin and fibronectin were observed which reverted after 10d-exposure, suggesting a time-dependent adaptation to s- μg . Gene expression analysis of 12 genes involved in cell structure, shape, adhesion, migration, and angiogenesis suggested significant changes after a 10d-RPM-exposure. 11 genes were down-regulated in AD and MCS 10d-RPM-samples compared to 1g, whereas *FLK1* was up-regulated in 5d- and 10d-RPM-MCS-samples. Similarly, *TIMP1* was up-regulated in 5d-RPM-samples, whereas the remaining genes were down-regulated in 5d-RPM-samples. Western blotting revealed similar changes in VEGF, β -actin, laminin and fibronectin of 5d-RPM-samples compared to 10d, whereas different alterations of β -tubulin and vimentin were observed. The pathway analysis showed complementing effects of VEGF and integrin β -1. **Conclusions:** These findings clearly show that s- μg induces significant alterations in the

F-actin-cytoskeleton and cytoskeleton-related proteins of ARPE-19, in addition to changes in cell growth behavior and gene expression patterns involved in cell structure, growth, shape, migration, adhesion and angiogenesis.

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Introduction

Disturbance of visual function is considered to be a major complication of spaceflights. An identification of the mechanisms underlying *e.g.* impairment of visual acuity is therefore an important focus area for the microgravity society as well as Space agencies, including the National Aeronautics and Space Administration (NASA). Studies of astronauts participating in space missions on the International Space Station (ISS) have demonstrated spaceflight-induced ocular changes such as choroidal folds, optic disk edema, globe flattening, and hyperoptic shifts [1]. The adverse outcomes were observed in astronauts after a long-term spaceflight and it has been hypothesized that these visual changes are connected to cephalad fluid shifts, intracranial pressure and optic nerve sheath compartment syndrome, as a consequence of prolonged microgravity exposure [1-3].

Simulated microgravity (μg) on Earth can be obtained by a number of different techniques including the RPM [4-6], the Horizontally Rotating Bioreactor (HRB) [7-9] and the rotating wall vessel (RWV) [4]. By using a HRB device, Dutt et al. have studied the impact of altered microgravity in the human retinal (HRet) cell line 301-SV-40T [10, 11]. Following incubation in the HRB vascular endothelial growth factor (VEGF) and basic fibroblast growth factor was found to be up-regulated. Moreover, it was shown that HRB promotes three-dimensional (3D) assembly when HRet was co-cultured with bovine endothelial cells (ECs) [11]. Also Tombran-Tink and Barnstable found evidence showing that a space shuttle flight microgravity environment disturbs normal retinal development including loss of retinal pigment epithelium (RPE) cells in a rodent model [12]. Furthermore, Roberts and coworkers demonstrated that simulated microgravity using a RWV bioreactor might induce an inflammatory response in human RPE (hRPE [13]) cells [14]. RPE cells constitute the pigmented layer of retina outside the neurosensory retina that nourishes retinal visual cells. Hence, the RPE cells are involved in several important processes including autophagy [15, 16] and protection against oxidative damage [17].

In order to investigate the effect of annulling gravity in RPE cells we exposed human adult retinal epithelium cells (ARPE-19) to simulated microgravity using a RPM. The cells were cultured on the RPM for 5d and 10d, respectively. ARPE-19 cells that remained adherent to the bottom of the culture flask (AD) and cells forming multicellular 3D aggregates (MCS) were isolated individually. The cytoskeleton and gene expression in AD and MCS cells were scrutinized and compared to ARPE-19 cells, cultivated under normal gravity (1g). Our study revealed microgravity-provoked cytoskeletal alterations of ARPE-19, in addition to changes in cell growth and expression pattern of selected genes involved in cell structure, shape, adhesion, extracellular matrix, migration, and angiogenesis.

Materials and Methods

Cell culture

ARPE-19 human adult retinal pigment epithelium cells (catalog number CRL-2302; American Type Culture Collection, Boras, Sweden) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Lonza, Verviers, Belgium), supplemented with 10% fetal bovine serum (Biocrom AG, Berlin, Germany), 100 IU penicillin/mL and 100 μg streptomycin/mL (Life Technologies, Naerum, Denmark) in 75 cm² ventilated cell culture flasks (Sarstedt AG, Nümbrecht, Germany) under standard conditions of 37°C and 5% CO₂ as previously described [18-20]. ARPE-19 cells form stable monolayers.

For RPM experiments, ARPE-19 cells were seeded into 25 cm² ventilated cell culture flasks (Sarstedt AG), and given at least 24h to properly attach to the bottom of the flasks, before the flasks were filled with

complete medium and fixed on the RPM or used as controls [21]. For 10d-experiments, it was necessary to exchange 50% of the medium with fresh medium after 5d, to ensure proper concentrations of nutrients and waste products. To assure that any MCS were not removed in the process, the flasks were placed upright, to allow the MCS to sediment before carefully aspirating the old medium and refilling with fresh medium.

Random Positioning Machine

Microgravity conditions were simulated using a desktop RPM (Airbus Defense and Space, former Dutch Space, Leiden, Netherlands). The RPM rotates a central frame around two perpendicular axes, randomly changing the direction of rotation and the angular velocity anywhere between 60°/s and 75°/s around both axes. Thus, the direction of the gravitational acceleration affecting the samples is continuously randomized, and the magnitude of the net gravitational vector will over time approach zero, producing a simulated state of microgravity. The RPM was placed inside a commercial incubator under standard conditions at 37°C and 5% CO₂ with up to fifteen 25 cm² flasks fixed to the central frame. The 25 cm² flasks were all completely filled with medium without air bubbles, to prevent sloshing of the medium and shear stress on the cells. Samples were run on the RPM for 5d and 10d, while an equivalent number of 25 cm² flasks, also filled with medium, were placed in the same incubator, to serve as 1g-controls to the RPM samples.

Immunofluorescence of fibronectin and F-actin-staining

Fibronectin and F-actin were visualized by seeding 5×10^5 cells into a SlideFlask chamber (Nunc, Roskilde, Denmark). The cells were then given 24h to adhere to the surface of the chamber before being filled with medium and run on the RPM for 5d or 10d, or acting as 1g-controls in the same incubator. After 5d in the 10d-experiment 50% of the medium in the chamber was carefully aspirated and exchanged with fresh medium. After 5d or 10d of cultivation the chambers were washed twice with DPBS (Life Technologies) and fixed with 4% paraformaldehyde for 30 minutes. After fixation, the cells were permeabilized with Triton-X-100 (Sigma, Taufkirchen, Germany) and subsequently washed twice with DPBS. Fibronectin was visualized by immunofluorescence using a primary antibody against fibronectin (1:200, F3648, Sigma). After incubation with the primary antibody, the cells were washed twice with DPBS and afterwards incubated with a secondary antibody conjugated to Alexa Fluor 488 antibody (1:400, 4412S and 4408S, both Cell Signaling Technology, Danvers, MA, USA). F-actin was visualized by incubating with phalloidin conjugated to TRITC for 30 minutes (Merck Life Sciences, Hellerup, Denmark) before washing away unbound phalloidin. For both fibronectin and F-actin staining, Fluoroshield with 4', 6-diamidino-2-phenylindole (DAPI) (Sigma) was used for nuclear counterstaining and mounting before subsequent imaging by confocal laser scanning microscopy (CLSM) (see below).

Microscopy

Phase contrast microscopy was performed before and after any RPM experiment, to ensure viability and to determine morphological changes of the cell cultures. Pictures were taken using a Canon EOS 550D camera (Canon GmbH, Krefeld, Germany) through a Leica DM IL LED inverted microscope (Leica Microsystems, Wetzlar, Germany).

Fluorescent staining was analyzed using a Zeiss LSM 710 CLSM (Zeiss, Jena, Germany) fitted with a Plan-Apochromat 63x1.4 objective as previously described [18, 22]. Excitation and emission wavelengths for Alexa Fluor 488 were $\lambda_{\text{ex}} = 488 \text{ nm}$ and $\lambda_{\text{em}} = 525 \text{ nm}$. Correspondingly for TRITC: $\lambda_{\text{ex}} = 532 \text{ nm}$ and $\lambda_{\text{em}} = 576 \text{ nm}$.

RNA isolation

Cells from RPM experiments were detached from the culture flasks with scrapers and transferred to tubes for isolation by centrifugation at 3400g for 10 minutes at 4°C. Any MCS were isolated by centrifuging the culture medium, in which the MCS were suspended. Total RNA was extracted from the samples with an AllPrep RNA/Protein Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA concentrations and quality were determined spectrophotometrically at 260 nm with a SpectraMax M2 Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). The isolated RNA had an A260/A280 ratio of >1.5. cDNA for quantitative PCR was obtained using a First-Strand cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer's instructions.

Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was used to determine the expression levels of the genes of interest [23, 24]. Appropriate primers were designed using Primer express software (Applied Biosystems, Darmstadt, Germany) with a T_m of 60°C (Table 1). All primers were synthesized by TIB Molbiol (Berlin, Germany). cDNA analysis was performed using a 7500 Fast Real-Time PCR System with Fast SYBR Master Mix (both Applied Biosystems). Final reaction volume was 25 μ l, including 1 μ l template cDNA and a primer concentration of 500 nM. PCR conditions were as follows: 20 s at 95°C, 40 cycles of 3 s at 95°C, 1 min at 60°C, followed by a melting curve analysis, with a temperature gradient from 60°C to 95°C in 0.3°C increments. If all amplicons showed a T_m similar to the one predicted by the Primer Express software, the PCR reaction was considered specific. Each sample was measured in triplicate. Relative transcription levels were quantified using the comparative C_t ($\Delta\Delta C_t$) method. The transcription level of the housekeeping gene *18S rRNA* was used as a reference gene for data normalization.

Table 1. Primers used for qRT-PCR

Gene	Primer name	Sequence (5'-3' direction)
<i>18S rRNA</i>	18S-F	GGAGCCTGCGGCTTAATTT
	18S-R	CAACTAAGAACGGCCATGCA
<i>ACTB</i>	ACTB-F	TGCCGACAGGATGCAGAAG
	ACTB-R	GCCGATCCACACGGAGTACT
<i>TUBB</i>	TUBB-F	CTGGACCGCATCTCTGTACTAC
	TUBB-R	GACCTGAGCGAACAGAGTCCAT
<i>LAMB2</i>	LAMB2-F	TGCTCATGGTCAATGCTAATCTG
	LAMB2-R	TCTATCAATCCTCTTCTTGGACAA
<i>FN1</i>	FN1-F	AGATCTACCTGTACACCTTGAATGACA
	FN1-R	CATGATACCAGCAAGGAATTGG
<i>VEGFA</i>	VEGFA-F	GCGCTGATAGACATCCATGAAC
	VEGFA-R	CTACCTCCACCATGCCAAGTG
<i>COL4</i>	COL4A5-F	GGTACCTGTAAGTACTATGCCAACTCCTA
	COL4A5-R	CGGCTAATTCGTGCTCCTCAAG
<i>FLK1</i>	FLK1-F	TCTTCTGGCTACTTCTGTGTCATC
	FLK1-R	GATGGACAAGTAGCCTGTCTTCAGT
<i>ITGB1</i>	ITGB1-F	GAAAACAGCGCATATCTGGAAATT
	ITGB1-R	CAGCCAATCAGTGATCCACAA
<i>ITGB3</i>	ITGB3-F	CGACCGAAAAGAATTGCTAA
	ITGB3-R	CGGTACGTGATATTGGTGAAGGTA
<i>KRT8</i>	KRT8-F	GATCTCTGAGATGAACCGGAACA
	KRT8-R	GCTCGGCATCTGCAATGG
<i>VIM</i>	VIM-F	TTCAGAGAGAGGAAGCCGAAAAC
	VIM-R	AGATTCCACTTTGCGTTCAAGGT
<i>TIMP1</i>	TIMP1-F	GCCATCGCCGAGATC
	TIMP1-R	GCTATCAGCCACAGCAACAACA

Western blot analysis

SDS-PAGE, immunoblotting and densitometry were carried out as previously described [25, 26]. Antibodies against the following antigens were used: Fibronectin (F3648), laminin (L9393), β -tubulin (T5293), vimentin (V5255) (all from Sigma, Taufkirchen, Germany), VEGF (ab46154, Abcam, Cambridge, UK), and GAPDH (5174s, Cell Signaling Technology, Danvers, MA, USA) all used at a dilution of 1:1000, and β -actin (A5316, Sigma) was used at a dilution of 1:4000. Secondary HRP-conjugated antibodies were used at a dilution of 1:4000 (anti-mouse, P0260, Dako, Glostrup, Denmark) and 1:2000 (anti-rabbit, P0399, Dako). Bound antibodies were visualized with Clarity Western ECL substrate (Bio-Rad, Hercules, CA, USA) on an ImageQuant LAS 4000 digital imaging system (GE Healthcare, Brøndby, Denmark) as previously described [18]. Densitometric quantification of the bands was carried out using ImageJ (<http://rsb.info.nih.gov/ij/>).

Pathway analysis

To investigate and visualize interactions between proteins and genes selected, we entered relevant UniProtKB entry numbers in the Pathway Studio v.11 software (Elsevier Research Solutions, Amsterdam, the Netherlands). The genes identified were analysed according to their mutual regulation. The proteins were evaluated in regard to their cellular localization and their interaction.

Assessment of released VEGF protein by ELISA

Released VEGF levels were measured using in-house time resolved immunofluorometric assays (TRIFMA) according to previously described methods [21, 23, 27]. In brief, the supernatant samples were diluted 1:2 and 96-well plates were read using a VICTOR 2030 instrument (Perkin Elmer, Inc.). Standard curves were used to calculate the concentrations using the standard software implemented in the VICTOR 2030.

Fig. 1. Morphologic examination of the ARPE-19 cells. Phase-contrast microscopy images of ARPE-19 cells after establishment before start of the experiment (T0) (A), after 3d at 1g (B), and after they have been cultured on the RPM for 5d (D) and 10d (F). Control samples of 5d (C) and 10d (E) formed no MCS. The samples cultured for 5d (D) and 10d (F) on the RPM formed two distinct populations represented by cells that remained adherently as a monolayer or MCS. Generally, the MCS build after 10d (green arrowheads in F) increased in size compared to the MCS formed after 5d (green arrowheads in D). In both cases less-adherent cells, probably representing cells that have the potential to form MCS are observed (purple arrowheads in D and F). Scale bar: 100 μ m.

Statistical analysis

All statistical analyses were performed using SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA). Data are presented as the mean $\pm$ SD. Statistical differences between two groups were evaluated using Mann-Whitney-U. $P < 0.05$ was considered statistically significant.

Results

Growth pattern of ARPE-19 cells reveals several phenotypes during RPM-exposure

We used freshly established ARPE-19 cells and investigated them under conditions of $s\text{-}\mu$ g using a RPM, a so-called ESA recommended ground-based facility. The 1g-control-sample was placed next to the machine for two selected time points (5 and 10d). The cells were subjected randomly to both conditions at time point 0 (T0). As shown in Fig. 1A and B the ARPE-19 cells were growing in a monolayer at T0 and 3d after the experiment was started. Following incubation at the two conditions the cells were then collected for imaging or harvested for further processing including gene expression analysis and protein amount assessments.

We first analyzed the morphology of ARPE-19 cells exposed to microgravity (μ g) conditions by standard phase-contrast imaging. The experiment revealed ARPE-19 cells growing in adherent (AD) monolayers during exposure to static 1g-conditions (Fig. 1C and E). The 1g-control cells showed no 3D growth and only the AD phenotype was observed. In contrast the ARPE-19 cells formed small compact round 3D MCS following exposure to simulated μ g conditions for 5d and 10d on the RPM. The MCS were floating in the cultivation medium or found to be partly attached to the adherent monolayer. In addition to the MCS partly detached unicellular aggregates (preMCS) were also observed in the μ g-samples, resembling cells in a precursor stage prior to MCS formation. As early as after 5d-exposure

Fig. 2. Cytoskeletal staining of F-actin microfilaments and quantitative alterations of genes of cytoskeletal proteins in ARPE-19 cells exposed to simulated microgravity. (A-H) CLSM after a 5d-exposure (C, D) and a 10d-exposure (G, H) on the RPM and their corresponding 1g-control cells (A, B, E, F). Inserts of 2A and B show freshly established ARPE-19 cells with normal cytoskeleton at T0 (1g) and 3d (1g), respectively. Red staining: TRITC-phalloidin to visualize the F-actin; blue staining: DAPI labeling of nucleus. White arrowheads indicate region of interest. Scale bar: 20 μm. (I-L) Gene expression analyses of cytoskeletal genes assessed by qRT-PCR. After 5d and 10d 1g-control, RPM-adherent (AD) cells, and RPM-multicellular spheroids (MCS) were analyzed for their relative expression levels of (I) *ACTB*; (J) *TUBB*; (K) *VIM*; (L) *KRT8* correlated to *18S rRNA*. The 5d and 10d 1g-controls were set to 100% * = p < 0.05.

preMCS and MCS were observed in the μg-sample, but not in the 1g-control-samples (Fig. 1C and D). The numbers of MCS increased from 5d to 10d, and a concomitant decline in the numbers of preMCS was observed in the μg-sample (Fig. 1D and F). From the presented data it is also seen that MCS grow in size as increased numbers of cells in the spheroids were observed in 10d-MCS compared to the MCS at 5d. Thus, the AD and MCS phenotypes were observed at both 5d and 10d.

RPM-induced alterations of the cytoskeleton

We performed immunostaining and confocal laser scanning microscopy (CLSM) of fixed and F-actin-stained ARPE-19 cells to investigate whether simulated microgravity introduces further changes of the cell shape and in the cytoskeleton after cultivation for 5d and 10d on the RPM. As expected well-organized intracellular F-actin filaments were observed under static 1g-conditions (Fig. 2A, B, E and F). The inserts of Fig. 2A and B show freshly established ARPE-19 cells with normal cytoskeleton at T0 and 3d. Following RPM-exposure for 5d, the intracellular F-actin filaments had almost disappeared in favor of development of F-actin structures accumulated at the cell boundaries (Fig. 2C and D). Notably, further exposure to μg-conditions for another 5d resulted in a F-actin staining pattern similar to the

1g-control (Fig. 2G and H). In addition, F-actin filaments appeared to be located on the cell boundaries of MCS formed after a 10d-exposure. As shown in Fig. 1 relatively few MCS were formed during the RPM-experiment. Consequently, only a modest number of MCS could be analyzed by CLSM. However, the result shown in Fig. 2H is a representative example of the investigated MCS.

Simulated microgravity changes the gene expression of both cytoskeletal and cytoskeletal binding proteins

Changes in gene expression levels of four cytoskeletal genes – β -actin (*ACTB*), β -tubulin (*TUBB*), vimentin (*VIM*) and cytokeratin 8 (*KRT8*) – as a response to altered gravity conditions facilitated by the RPM were investigated by quantitative real-time PCR (qRT-PCR). As shown in Fig. 2I the expression of *ACTB* was slightly reduced in both AD and MCS after 5d-RPM-exposure. This tendency further developed in the samples experiencing simulated μg for 10d resulting in a significantly ($p < 0.05$) reduced expression of *ACTB* in AD and MCS amounting to 52% and 45% compared to 1g-control-samples, respectively (Fig. 2I).

For the other three investigated cytoskeletal genes similar expression patterns were obtained. The expression of *TUBB* and *VIM* was only markedly reduced in AD- and MCS-samples following incubation at simulated μg conditions for 5d (Fig. 2J and K). After a 10d-exposure the expression level of *TUBB* was significantly reduced to 50% and 29% compared to 1g-samples (Fig. 2J). Similarly, qRT-PCR analysis showed that expression of *VIM* in AD and MCS was significantly reduced to 69% and 24% compared to the 1g-samples (Fig. 2K). In the case of *KRT8* expression levels following both 5d- and 10d-RPM-exposure to simulated microgravity were significantly decreased amounting to 67% and 60% in AD and MCS compared to the corresponding 1g-samples at 5d, and 52% and 41% in AD and MCS compared to the corresponding 1g-samples at 10d (Fig. 2L).

Changes in extracellular matrix and related proteins

We next investigated whether reduced gravity influenced the cellular distribution of fibronectin, which mediates a wide variety of cellular interactions with the extracellular matrix (ECM) and plays important roles in cell adhesion, migration and growth, as well as introduce changes in the expression of ECM genes.

Immunostaining and CLSM analysis showed that the majority of the anti-fibronectin positive ECM material was accumulated in the extracellular space around the cells at 1g-conditions for 5d (Fig. 3A and B). The amount of fibronectin was clearly reduced at simulated μg -conditions (Fig. 3C and D). Extension of the period of simulated μg to 10d revealed a reduced amount of fibronectin protein compared with 1g (Fig. 3E-H). These findings indicated an overall reduction of anti-fibronectin positive signals in samples experiencing simulated μg compared to the 1g-controls (compare Fig. 3A with Fig. 3C, and Fig. 3E with Fig. 3G).

A representative example of anti-fibronectin staining of a MCS is shown in Fig. 3D. The image suggests that fibronectin, similar to F-actin, is localized at the cell boundaries and in the extracellular space around the MCS.

Simulated microgravity alters the gene expression of ECM proteins

Simulated microgravity conditions also altered the gene expression of fibronectin and other investigated ECM proteins in the ARPE-19 cells (Fig. 3I-N). Hence, at 5d the expression of *FN1* (fibronectin) was significantly decreased to 51% and 31% of the 1g-controls as determined by qRT-PCR (Fig. 3I). The expression of *FN1* was further reduced at 10d amounting to 32% and 24% of that observed in the 1g-control cells (Fig. 3I). For *LAMB2* (laminin subunit β -2) the expression was markedly reduced to 49% in the AD-sample after 5d, whereas *LAMB2* expression in the 5d MCS-sample was only decreased to 83% of that in the 1g-control (Fig. 3J). However, in both the AD- and MCS-samples subjected to simulated microgravity for 10d *LAMB2* expression was significantly reduced to 38% and 44% compared to 1g-controls (Fig. 3J).

Fig. 3. Cytoskeletal staining of fibronectin and quantitative alterations of extracellular matrix genes in ARPE-19 cells exposed to simulated microgravity. (A-H) CLSM after a 5d-exposure (C, D) and a 10d-exposure (G, H) on the RPM and their corresponding 1g-control cells (A, B, E, F). Green staining: Alexa Fluor 488-conjugated antibodies to visualize fibronectin filaments; blue staining: DAPI labeling of nucleus. White arrowheads indicate region of interest. Scale bar: 20 μm. (I-N) Gene expression analyses of ECM genes assessed by qRT-PCR. After 5d and 10d 1g-control, RPM-adherent (AD) cells and RPM-MCS were analyzed for their relative expression levels of (I) *FN1*; (J) *LAMB2*; (K) *COL4*; (L) *ITGB1*; (M) *ITGB3*; (N) *TIMP1* correlated to *18s rRNA*. The 5d and 10d 1g-controls were set to 100% * = $p < 0.05$.

As shown in Fig. 3K simulated μg only has a minor impact on the expression of *COL4* (type IV collagen) at 5d in both AD- and MCS-samples. At 10d more pronounced declines were observed amounting to 63% of the 1g-control samples (Fig. 3K). For both *ITGB1* (integrin- β -1) and *TIMP1* (tissue inhibitor of metalloproteinase 1) expression levels in the 5d-RPM-AD-samples increased to 120% and 136%, respectively, compared to the 1g-controls (Fig. 3L and N). In case of, *ITGB1* the expression level observed in the MCS-sample was reduced to 57%. Analysis of the 10d samples showed non-significant down-regulation of the *ITGB1* expression in both AD and MCS (Fig. 3L). In the 5d-MCS-sample the expression of *TIMP1* increased to 132% of the 1g-control and was comparable to that observed in the 5d-RPM-AD-sample (Fig. 3N). However, the gene expression levels of *TIMP1* were diminished in both 10d-RPM-AD- and 10d-RPM-MCS-samples (Fig. 3N).

Finally, the analysis of *ITGB3* (integrin- β -3) showed that the expression of this gene in the 5d-RPM-AD-sample was significantly decreased in 5d-RPM-AD-samples to 57% of the

Fig. 4. Alterations of cytoskeletal and extracellular matrix protein content in ARPE-19 cells exposed to simulated microgravity. (A-E) Assessment of the relative protein content of (A) β -actin, (B) β -tubulin, (C) fibronectin, (D) laminin and (E) vimentin in 5d- and 10d-samples correlated to GAPDH. The 5d and 10d 1g-controls were set to 100% (F-M) Protein content of 5 and 10d-experiments. 1g-control (1g), RPM-adherent (AD) and RPM-MCS (MCS) cells were analyzed by Western blotting for their protein content of (F) β -actin, (G) β -tubulin, (I) fibronectin, (K) laminin and (L) vimentin. As a loading control for the cell samples, an antibody against GAPDH was used (H, J and M). The images shown in F-H, I-J and K-M were obtained from three separate SDS-gels loaded with identical samples. Following SDS-PAGE and Western blotting membranes were incubated with different primary antibodies as indicated. * = $p < 0.05$. The positions of β -actin (β -Act), β -tubulin (β -Tub), fibronectin (Fn), laminin (Ln), vimentin (Vn), and GAPDH (GAPDH) are indicated on the right.

1g-control level (Fig. 3M). In contrast to this finding the expression of *ITGB3* in the 5d-RPM-MCS-sample was almost unaltered compared to the 1g-control. However at 10d, the *ITGB3* expression in both AD- and MCS-samples was significantly declined to approx. 37% and 45% of that of the control samples, respectively (Fig. 3M).

The content of cytoskeletal and ECM proteins is altered by RPM-induced microgravity

In the next step, we investigated whether these changes were also reflected in the amount of detectable proteins. Therefore we assessed the abundance of three cytoskeletal proteins (β -actin, β -tubulin and vimentin) and two ECM proteins (laminin and fibronectin). GAPDH was used as loading control.

As shown in Fig. 4A and F the amount of β -actin was significantly decreased in AD-cells after 5d. A similar decline was observed in the AD-cells after 10d-exposure. Likewise, decreased levels of β -actin were seen in the MCS samples after both 5- and 10d-RPM-exposure. Even though the level of β -actin in these samples was reduced to 40-70% compared to the 1g-controls it was not significant. This was also the case for any of the other densitometric assessments of MCS, probably reflecting the modest numbers of MCS samples obtained from the RPM experiment.

The level of β -tubulin was static in 5d-RPM-AD- and 5d-RPM-MCS-samples as well as in the 10d-RPM-AD-samples. In contrast, the amount of β -tubulin was increased in the 10d-RPM-MCS-samples (Fig. 4B and G). After 5d-RPM-exposure both AD and MCS cells exhibited increased levels of vimentin. However, after 10d both AD and MCS showed reduced levels of vimentin compared to 1g-controls (Fig. 4C and L). The amount of laminin was

Fig. 5. Quantitative alterations of angiogenic genes and protein content in ARPE-19 cells exposed to simulated microgravity. (A-B) Gene expression analyses of *VEGFA* and *FLK1* (VEGFA receptor) examined by qRT-PCR. After 5d and 10d 1g-control, RPM-AD cells and RPM-MCS were analyzed for their relative expression levels of (A) *VEGFA* and (B) *FLK1* correlated to *18S rRNA*. The 5d and 10d 1g-controls were set to 100% (C) Released VEGF protein in 1g- and RPM-samples determined by a time resolved immunofluorometric assay (TRIFMA). (D) Assessment of the relative protein content of VEGF of samples from 5d- and 10d-samples correlated to GAPDH. (E) Intracellular VEGF protein content of 5d- and 10d-experiments. 1g-control (1g), RPM-adherent (AD) and RPM-MCS (MCS) cells were analyzed by Western blotting. (F) As a loading control for the cell samples, an antibody against GAPDH was used. * = $p < 0.05$. The positions of VEGF and GAPDH are indicated on the right.

significantly decreased in AD-samples after 5d and 10d (Fig. 4D and K). Similarly, reduced amounts of laminin were observed in the MCS samples where as the level of fibronectin was static in all samples after a 5d- and 10d-RPM-exposure (Fig. 4E and I).

Molecules of the VEGF pathway are changed by simulated microgravity

In addition to the analysis of the cytoskeleton, gene expression, protein content, and release of factors playing a central role in angiogenesis were scrutinized. Expression of VEGF, an important driver of angiogenesis, was analyzed on the transcriptional as well as on the protein level for alterations during reduced gravity conditions.

The *VEGF* gene expression in 5d-samples revealed a significant down-regulation in RPM-AD- and RPM-MCS-ARPE-19 cells compared to the 1g-controls (Fig. 5A). After a 10d-exposure ARPE-19 cells exhibited a similar reduced *VEGF* expression in all groups (Fig. 5A).

Vascular endothelial growth factor receptor 2 (VEGFR2), also known as fetal liver kinase 1 (FLK1) or kinase insert domain receptor (KDR) is one of the VEGF-receptors. The expression of *FLK1* in 5d-RPM-AD-samples revealed a mostly unchanged expression and a significant up-regulation in 5d-RPM-MCS-samples compared to the 1g-controls (Fig. 5B). After a 10d-exposure the ARPE-19 cells exhibited a similar *FLK1* expression pattern in all groups compared to 5d (Fig. 5B).

Assessing the VEGF release into the medium after 5d revealed a significantly lower content in RPM-samples of the ARPE-19 cells compared to the corresponding controls. As shown in Fig. 5C the content was more than 2-fold reduced from 550 ng/L in RPM-samples to 245 ng/L in the 1g-control cells.

Finally, we evaluated the intracellular content of VEGF protein by means of Western blotting analysis. The results presented in Fig. 5D-F show a decrease in the VEGF amount in 5d- and 10d-samples exposed to simulated μg compared to the 1g-control cells, thereby supporting the findings from the expression analysis as well as the investigation of released VEGF. Notably, the amount of VEGF was markedly reduced in the MCS-samples exposed to simulated microgravity (Fig. 5D).

Fig. 6. Mutual interaction of selected genes at gene expression level. 12 selected genes, whose up- or down-regulation were analyzed by qRT-PCR after 5d (A) and 10d (B) of RPM-exposure and shown in Figs. 2, 3 and 5. Blue background indicates down-regulation and red background shows up-regulation. The yellow background refers to non-regulated genes. The lower part of each icon indicates the gene status in MCS cells, whereas the upper part indicates the status of the gene in the AD cells. The green arrows indicate activating and the red arrows inhibiting effects. The grey lines tell that interactions take place between the proteins, whose effects have not been clarified yet. The interaction network was built up using Elsevier Pathway Studio v.11.

Fig. 7. Mutual interaction and localization of proteins expressed in adherent cells after exposure to simulated microgravity. Interaction and localization of proteins coded by 12 selected genes found in AD cells after 5d (upper part) and 10d (lower part) on the RPM. The green arrows indicate activating and the red arrows inhibiting effects. The grey lines tell that interactions take place between the proteins, whose effects have not been clarified yet. Full lines indicate direct interaction, and dotted lines indicate regulation via intermediates. The icons on colored background indicate the proteins' increase (red) and decrease (blue) as compared to controls. The yellow background refers to unchanged protein content. The interaction network was built up using Elsevier Pathway Studio v.11.

Investigation of the underlying mechanisms of the phenotypically change of the cells

In order to uncover the underlying mechanisms for the observed 2D to 3D growth behavior transition in ARPE-19 cells, 12 genes, encoding proteins known to be involved in the maintenance and regulation of cell structure and shape or in cell adhesion, ECM, migration and angiogenesis, were selected (listed in Table 1). The Swissprot numbers of the proteins encoded by these genes were entered in the Elsevier Pathway Studio in order to gain knowledge about their interactions.

The pathway analysis shown in Fig. 6 provides an impression of the regulation of the 12 genes determined by qPCR analysis after 5d (Fig. 6A) and 10d (Fig. 6B) of exposure to μg as depicted in Figs. 2, 3 and 5. It shows that apart from β -tubulin (*TUBB*), laminin (*LAMB2*) and keratin 8 (*KRT8*) the expression of the other 9 genes is mutually controlled within the frame of a network. These genes encode 9 individual products: 4 extracellular proteins, 3 membrane proteins and 2 cytoplasmic proteins (Fig. 7). The representation suggests that the *VEGF* gene influences the majority of the neighboring genes and thus, may play a central role within this network of regulation. As determined by the PCR technique, the *VEGF* gene like the *FN1* gene is down-regulated in MCS and AD cells after five days and remains down-

regulated until the tenth day of incubation. In parallel, the *FLK1/KDR* gene is up-regulated in MCS but unchanged in AD cells during the whole period of incubation. Also *TIMP1*, *COL4A5* in MCS and *VIM* and *ITGB1* in AD cells remain unchanged during the whole period of incubation.

Focusing on the proteins, the interaction analysis presented in Fig. 7 envisions a network, which stretches from extracellular space (upper part) via the membrane (drafted bilayer in the middle) to the cytoplasm (lower). It indicates that VEGFA together with ITGB1 is activating the KDR (FLK1), while TIMP1 supports enrichment of ITGB1, which together with LAMB2 has positive influence on fibronectin. The VIM content is enhanced after 5d and affects the integrins β -1 and β -3 as well as β -actin and fibronectin.

Discussion

In the present study we investigated the influence of simulated microgravity on human RPE cells. For this purpose ARPE-19 cells were exposed to conditions of microgravity (μg) using a RPM for 5d and 10d, respectively. In addition, we also cultivated the cells under static conditions (1g).

Simulated microgravity (also termed functional weightlessness) is based on the assumption that a lack of weight load is perceived similarly to neutralization of sedimentation during weightlessness (0g) [28]. Even though the latter condition is never entirely reached under simulated circumstances, where weak residual acceleration forces are still active, ground based devices like RPM, RWV and fast-rotating clinostats have successfully reproduced microgravity responses [4]. However, these devices generally seem to underestimate the effects observed in real microgravity during spaceflights [29].

It is well known that a long-term spaceflight has immense impact on the health of humans in space and ocular changes are considered as a high human health risk of spaceflight [1-3]. Studies of astronauts participating in missions on the ISS have shown prominent adverse outcomes in visual function [1]. These spaceflight-induced vision changes include choroidal folds, optic disc edema, globe flattening and hyperopic shifts.

In the eye *in vivo* as well as under *in vitro* conditions RPE cells grow as a monolayer and 3D growth behavior of RPE cells is usually not observed. This feature is important for the RPE cells that constitutes the outer blood-retinal barrier and hence for the maintenance of the retinal homeostasis required for visual transduction. During simulated microgravity 3D assembly-structures, probably consisting of aggregated photoreceptor (PR) cells, can be observed when co-incubating HRet cells and ECs in the HRB [11]. It has been suggested that the microgravity-stimulated 3D assembly is promoted by cell-to-cell interaction together with secretion of growth factors like VEGF [11]. Likewise, multilayered RPE cells capable of proliferating and differentiating were observed following isolation [30, 31]. However, to our knowledge MCS derived from RPE cells exposed to microgravity has not yet been observed.

We used a RPM to explore whether simulated microgravity can stimulate ARPE-19 cells to form MCS, scrutinized changes in the F-actin cytoskeleton as well as the ECM, and investigated the expression of genes coding for proteins which are involved in cell structure, shape, migration, adhesion, or angiogenesis, and hence might play a role in the cellular capacity to sense gravitational alterations and development of MCS [21, 32]. Interestingly we observed that a minor fraction of the ARPE-19 cells produced MCS after a 5d-RPM-exposure. We repeatedly detected that ARPE-19 cells developed into two phenotypically different populations; one subpopulation remaining adherent to the bottom of the cultivation flask, and another subpopulation that formed MCS freely floating in the cultivation medium (Fig. 1). The number and size of MCS increased after a 10d-RPM-exposure suggesting that reduced gravity conditions provided by the RPM promotes development of MCS and 3D growth.

Based on the nature of the presented study, it is not possible to conclude whether the observed MCS have pathologic effects *in vivo*. This would of course require further analysis *in vivo* in rodents. The background for the relatively small number of MCS detected in the RPM-samples could be excessive cell confluence. For instance, no or very low numbers of

MCS are observed in confluent FTC-133 cell cultures incubated on the RPM compared to semi-confluent samples [33, 34].

Tightly regulated expression of VEGF is mandatory for proper function of the eye and increased intra-ocular levels of this growth factor, may stimulate to neovascularization as observed in age-related macular degeneration (AMD) and proliferative diabetic retinopathy (DR) [35]. VEGF is part of a signaling pathway controlling important biological processes including proliferation, survival, migration, and actin reorganization [36]. The gene expression of *VEGFA* as well as synthesis and release of VEGF following incubation for 5d and 10d on the RPM was significantly decreased compared to the corresponding 1g-control samples. In particular, the decline in *VEGFA* and VEGF protein was more pronounced in the MCS-samples. This is in line with our recent findings using breast cancer cells (MCF-7) [21] and thyroid cancer cells (FTC-133) [37, 38] showing reduced VEGF levels. In addition, endothelial cells cultured on the RPM revealed an increased synthesis of VEGF [26, 39]. Similarly, retinal cells exposed to microgravity by using the HRB device displayed an up-regulation of VEGF [11].

In response to the decreased VEGF levels observed in the present study the expression of VEGF receptor *FLK1* at both time points increased significantly in RPM-MCS-samples, whereas a static expression was observed in the RPM-AD-samples. The elevated expression of *FLK1* in MCS exposed to simulated microgravity is similar to the findings in MCS produced by MCF-7 cells cultivated on the RPM [21], suggesting that MCS originating from ARPE-19 and MCF-7 behave similarly in key regulatory aspects of angiogenesis.

Actin, tubulin, and cytokeratin are key components of the cytoskeleton and have various functions [40]. They play an important role in the perception of exterior signals including the gravity force [40]. In this study we found down-regulated *ACTB*, *TUBB*, and *KRT8*, and *VIM* in AD and MCS samples after 5d-RPM-exposure. This trend was further developed after a 10d-RPM-experiment. After this time period, we observed a significant down-regulation in all samples with a clear tendency of MCSs displaying the lowest values (Fig. 2). In addition, *KRT8*, which is a luminal epithelial marker [41], was significantly down-regulated in AD and MCS after 5d and 10d of cultivation on the RPM compared to the corresponding 1g-controls.

The presented densitometric analyses demonstrated a decreased amount of β -actin in 5d- and 10d-RPM samples. Even though the decline was only significant in the 5d-RPM-AD sample, the general trend of reduced protein levels in the RPM samples mirrored the observed reduction in *ACTB* expression. In contrast, the amount of β -tubulin seemed to be static with a tendency of increased levels in the 10d-RPM-MCS-samples, despite a significant reduction of *TUBB* expression in RPM-samples. The impact of simulated microgravity on the cytoskeleton of ARPE-19 cells seems to be different compared to *e.g.* FTC-133 human follicular thyroid cells and Nthy-ori 3-1 primary human thyroid follicular epithelial cells after a 7d- and 14d-exposure on the RPM in which the expression of *ACTB* and *TUBB* are up-regulated [23]. Similarly, increased levels of β -actin were observed in human MCF-7 breast cancer cells exposed to simulated microgravity for 5d [21]. Using human chondrocytes Aleshcheva et al. showed that *ACTB* and *TUBB* mRNAs were up-regulated after a 24h-incubation on the RPM [42]. However, no significant effect on the corresponding protein levels was observed [42]. The expression of *VIM* was unchanged compared to 1g-controls, whereas the protein level of vimentin was 3-fold increased [42]. In case of *KRT8*, this gene was down-regulated in ML-1 thyroid cancer cells, whereas it was found to be significantly up-regulated in follicular thyroid carcinoma cells (UCLA R082-W-1) [43].

As the conditions of the experiments regarding *ACTB* and *TUBB* are similar, the observed differences in the cellular perception and handling of simulated microgravity most likely reflect the different origins of the investigated cells.

In addition to these findings, we observed that the F-actin filaments almost disappeared in favor of F-actin structures accumulating at the cell boundaries following RPM-exposure for 5d. However, F-actin staining pattern similar to the one observed in the 1g-control reappeared following after a 10d-exposure, advocating for a time-dependent adaptation to altered microgravity. Notably, no stress fibers were observed, probably due to the time point

of analysis. This is in contrast to other studies showing stress fiber formation in *e.g.* FTC-133 and endothelial cells at shorter time points [24, 39]. Another reason explaining why no stress fibers were observed might be the decrease in the expression levels of the integrins *ITGB1* and *ITGB3*. Integrins are known to co-localize with moesin in microvilli in endothelial cells resulting in RhoA activation and hence induction of stress fiber production [44]. In addition, studies of *e.g.* mesenchymal stem cells exposed to simulated microgravity also revealed a decreased integrin signaling [45].

The ECM is composed of extracellular proteins secreted by the cells, thereby providing important structural, mechanical, and biochemical support to surrounding cells. We observed several alterations in components of the ECM. *FN1* and *LAMB2* were all found to be significantly down-regulated in all RPM-samples. We found *COL4* to be similarly regulated after 5d in RPM-samples as detected in 1g-samples, but significantly down-regulated after 10d in AD-RPM-samples. A profound reduction was also observed in the MCS cell population harvested from the RPM, however it was not significant.

Simultaneously, the amount of laminin protein was decreased in AD and MCS cells after 5d- and 10d-RPM-exposure as compared to the 1g-control cells, while equal amounts of fibronectin were found in all measurements. The observed reduction of the laminin protein content in cells thereby correlates to the observed decrease in *LAMB2* expression. In case of fibronectin our data suggest that the amount of this protein is static during simulated microgravity even though the expression of *FN1* is significantly decreased at both time points. These results are partly in line with the findings in MCF-7 breast cancer cells showing a down-regulation of *FN1* as well as reduced levels of fibronectin in AD samples [21].

In concert with these findings, the CLSM analysis revealed an apparent reduction of fibronectin in cells exposed to simulated microgravity compared to 1g-controls. Even though CLSM is not a quantitative method, the obtained images shown in Fig. 3 indicated an overall reduction of anti-fibronectin-positive signals in samples experiencing simulated μg compared to the 1g-controls (Fig. 3). The images resulting from the immunostaining and CLSM analysis also demonstrate that F-actin and fibronectin are localized at the cell boundaries and in the extracellular space around the MCS. This finding was also observed when endothelial cells were cultured on the RPM [39, 46]. Fibronectin has been shown to play a key role in the 3D cell cohesion of FTC-133 thyroid cancer cells [47].

Components of the ECM play an important role in cell adhesion. In focal adhesion in which actin filaments are connected to the ECM or hemidesmosomes connecting intermediate filaments (such as cytokeratin) to the ECM. We detected a significant reduction of *ITGB3* after 10d in both cell populations harvested from the RPM. Similar, a decreased expression of *ITGB1*, however not significant, was observed in AD and MSC samples after 10d. Integrin- β -1 and β -3 are membrane proteins, involved in focal adhesion [44, 48]. Both integrins are described to be crucial for the activation of signaling pathways controlling important cellular processes including cytoskeleton rearrangements, angiogenesis, differentiation, and proliferation [44]. Similar to the situation of MCF-7 cells, the present findings indicate that integrin- β -1 is involved in the detachment of cells from the bottom of the culture flask. Moreover, *COL4* was down-regulated. Therefore, we may suggest that simulated microgravity has a general impact on cell adhesion as both focal adhesion- and hemidesmosome-mediated processes are affected.

This impact is obviously not due to a one way event, but is exerted by a complex system of up- and down-regulation of gene expression (Fig. 6) as well as an increase or decrease of proteins (Fig. 7) [33]. Key players at protein and gene levels are VEGF and the *ITGB1*, which together act on the VEGF receptor. Although the VEGF content is lowered and the VEGF gene is down-regulated, the action of *ITGB1* on the Flk1 receptor (also known as KDR or VEGFR-2) may keep the angiogenic signaling on an effective level [49].

The presented data suggest that simulated microgravity created by the RPM has a clear impact on the ARPE-19 cells. Taken together, we observed significant cytoskeletal alterations as well as a down-regulation of cytoskeletal genes following exposure to microgravity. Simultaneously, massive changes in the ECM were detected and a number of key ECM genes

were down-regulated. Finally, we observed a significant decrease in genes involved in cellular adhesion (*ITGB1* and *ITGB3*) and tissue remodeling (*TIMP1*). These results suggest that weakening of cytoskeletal structures together with diminished capability to retain adhesion and stiffness of exterior structures in the RPE cells may contribute to the explanation of why reduced microgravity causes ocular changes as an outcome of compromised barrier to and abridged nursing of the PR layer. This notion may also explain why MCS are observed following exposure to reduced microgravity.

Conclusion

Our findings clearly show that simulated microgravity induces significant alterations in the F-actin-cytoskeleton and cytoskeleton-related proteins of ARPE-19, in addition to changes in cell growth behavior and gene expression patterns involved in cell structure, growth, shape, migration, adhesion and angiogenesis. Surprisingly, a minor fraction of the ARPE-19 cells produced MCS following incubation on the RPM suggesting that reduced gravity conditions promote 3D growth of ARPE-19 cells *in vitro*. With attention to these findings, we may suggest that weakening of interior and exterior cellular structures of the RPE cells following exposure to simulated microgravity may affect function of the retinal layer. However, further studies are required in order to investigate whether these changes contribute to the ocular changes observed in astronauts participating in prolonged spaceflights. A first step in this process might include analysis of RPE cells in real microgravity during parabolic flights as recently studied in primary human T lymphocytes [50] and thyroid cancer cells (FTC-133) [24].

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Disclosure Statement

The authors declare no competing financial interests.

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