Spheroid formation of human thyroid cancer cells under simulated microgravity: a possible role of CTGF and CAV1

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Abstract

Background: Multicellular tumor spheroids (MCTS) formed scaffold-free under microgravity are of high interest for research and medicine. Their formation mechanism can be studied in space in real microgravity or on Earth using ground-based facilities (GBF), which simulate microgravity. On Earth, these experiments are more cost-efficient and easily performable. However, each GBF might exert device-specific and altered superimposingly gravity-dependent effects on the cells.

Results: FTC-133 human thyroid cancer cells were cultivated on a 2D clinostat (CN) and a random positioning machine (RPM) and compared with corresponding 1 g control cells. Harvested cell samples were investigated by microscopy, quantitative realtime-PCR and Multi-Analyte Profiling. Spheroid formation and growth occurred during 72 h of cultivation on both devices. Cytokine secretion and gene activation patterns frequently altered in different ways, when the cells were cultured either on the RPM or the CN. A decreased expression of CAV1 and CTGF in MCTS compared to adherent cells was observed after cultivation on both machines.

Conclusion: The development of MCTS proceeds similarly on the RPM and the CN resembling the situation observed under real microgravity conditions, while no MCTS formation was observed at 1 g under identical experimental conditions. Simultaneously, changes in the regulation of CTGF and CAV1 appeared in a comparable manner on both machines. A relationship between these molecules and MCTS formation is discussed.

Keywords: Adherent growth, Three-dimensional growth, Cytokine, Microgravity, MCTS, RPM, 2D clinostat

Background

During a recent flight on board the Shenzhou-8 spacecraft human follicular thyroid cancer cells (FTC-133) were exposed to real microgravity for 10 days [1,2]. The returned samples revealed that scaffold-free formation of multicellular tumor cell spheroids (MCTS) occurred while the cells had been exposed to real microgravity. The spheroids obtained after landing of the space ship return capsule showed a similar shape but larger diameters (5–10 mm) than those usually induced on Earth with the help of a random positioning machine (RPM) [1-4]. In contrast, 1 g controls kept under static conditions remained adherent and formed no spheroids. From these results we concluded that microgravity could be a major cause of transition from 2- to 3-dimensional cellular growth. However, involved molecules and signaling pathways responsible for this change of growth behavior remained unknown [5].

In order to understand and explain the effects of altered gravity on spheroid formation, we complemented our studies using two different ground-based facilities in order to simulate microgravity conditions – the 2D clinostat (CN) and the RPM. Both devices are cost-efficient and enable a sufficient number of experiments, which is rarely achieved under real microgravity conditions [6]. Each of these ground-based approaches prevents cell sedimenta-
tion, however, in a device-specific manner. On the clino-
stat, sedimentation is prevented by a fast and constant ro-
tation of the samples around one horizontal axis, assuming
that the sample does no longer perceive the gravity stimu-
lus [7]. In contrast, the RPM consists of two independently
rotating frames enabling a 3D rotation with random speed
and random direction of the samples aiming to alter the in-
fluence of the gravity vector [8,9].

Considering the construction of both machines, we con-
cluded that a permanent change of the direction of the
gravity vector and thus prevention of sedimentation is a
common capacity of both machines, while their particular
modes of operations are rather different. Therefore, we
aimed to analyze whether biological processes triggered by
altered gravity may show identical or different results after
exposure on these two kinds of devices.

In order to prove ground-based microgravity simula-
tion approaches, we investigated human follicular thy-
roid cancer cells (FTC-133) cultivated either on the
CN or the RPM in a parallel manner focusing on the
formation of spheroids as well as on alterations of gene
expression and protein secretion. We learned that
spheroids are formed on both devices and concluded
that caveolin-1 (CAV1) and connective tissue growth
factor (CTGF) could be directly involved in the initi-
ation of 3D cell growth.

Results
Spheroid formation on the RPM and the CN
Subconfluent monolayers of human follicular thyroid car-
cinoma cells (FTC-133) were cultivated either on the RPM
or on the CN and in parallel to the 1 g controls located in
the same incubator, respectively. On both devices spher-
oid formation progressed like shown in Figure 1 for
cells harvested from the CN. While under static 1 g
conditions (1 g controls), the cells remained adherent
(Figure 1 A-C), two cell populations developed within the
culture flasks mounted on each of the two machines,
respectively (Figure 1 D-I). Of these populations, one
continued to grow adherently (AD cells) (Figure 1 D-F), the
other one detached from the bottom of the culture flask
and assembled to MCTS (Figure 1 H-I). The separation of
the two cell populations is delayed, as 4 h after exposure
to the devices only adherent growth was observed in each
sample (Figure 1 A, D, G). After approximately 24 h early
spheroids became visible on each of both the devices in
addition to the adherently growing cells (Figure 1 E, H).
During the subsequent 48 h, spheroids became more nu-
numerous and larger, while adherently growing cells were still
present (Figure 1 F, I). Spheroid size can be assumed to be
around 100 μm on the clino stat, as shown in Figure 1, but
also reaching up to 1 mm on the RPM as previously pub-
lished by Pietsch et al. [4].

Differential gene expression in FTC-133 cells after
exposure to CN and RPM
After spheroid formation was observed on the RPM and
the CN, we were interested to see whether similar alter-
ations of mRNA expression occurred on both machines.
Therefore, qPCR on several types of mRNA was per-
formed, which had been recognized in former experiments
on FTC-133 cells exposed to the RPM for 24 h to be im-
portant for MCTS formation [10]. The selected genes be-
long to several biological categories including cytoskeletal
proteins, and factors of growth, apoptosis, angiogenesis
and signal transduction (Table 1).

As shown in Figure 1, after a 4 h-incubation on the RPM
and on the CN the whole FTC-133 population grew adher-
ently like in 1 g-control samples. Nevertheless, some mRNA
changes were already found in cells harvested from either
machine as compared to control cells. An up-regulation in
the CTGF gene expression was observed in cell cultures on
both devices. However, an up-regulation of CAV2 and a
down-regulation of ERK1 gene expression were only evi-
dent after culturing on the CN (Figure 2).

After 72 h of exposure to the CN or the RPM, cells had
parted into adherent cells (AD) and cells forming multici-
ellular tumor spheroids (MCTS), which floated in the super-
natant (Figure 1 I). At this time, two fractions were
harvested from the CN and the RPM, respectively. Subse-
quently, it was investigated how the expression of the se-
lected genes (Table 1) had been regulated within the 4 cell
samples indicated in Figure 3 as compared to the corre-
sponding static ground controls (1 g). Interestingly, the
regulation of CTGF and CAV1 split on both machines
equally. In adherent cells, CTGF remained up-regulated
and CAV1 unchanged, while in MCTS the expression of
these two genes was decreased (Figure 3 E-F). Other genes
have also changed their expression behavior, but differ-
ently, when incubated on the two machines. Exposure of
FTC-133 cells to the RPM led to an up-regulation of ERK1
and EGF gene-expression in MCTS, but did not affect one
of the selected genes in AD cells. In contrast, exposure of
the same type of cells to CN triggered down-regulation of
ERK1, ITGB1, and PRKCA gene-expression in MCTS only
(Figure 3 I, M, O), but decreased expression of CAV2 and
IL8 in both, AD and MCTS cells (Figure 3 C, K).

The translation from mRNA to protein is a complex
process, and results obtained on mRNA level do not ne-
necessarily reflect the situation on the protein level. There-
fore, investigations of CN- and RPM- related effects on
cytokine release were performed additionally using Multi-
Analyte Profiling Technology (MAP).

Cytokine release of FTC-133 cells after 72 h exposure to
RPM and CN
Concentrations of selected cytokines within the various
culture supernatants were determined by MAP in order to
estimate the secretion activities of the cells (Table 2). As compared to the relevant 1\text{g} ground controls, significantly higher amounts of GM-CSF, IL-6, IL-8, BDNF, Eotaxin-1, ICAM1, IL-1α, IL-1β, IL-1Ra, IL-12p40, IL-15, IL-17, IL-23, MMP-3 and SCF were detected in the supernatants of FTC-133 cells cultured on the RPM for 72 h. In contrast, significantly lower quantities of GM-CSF, IL-6, IL-8, MIP-1\text{α}, MIP-1\text{β} and BDNF were detected in cell supernatants of CN samples than in 1\text{g} control supernatants (Table 2) after 72 h cell culturing. After cultivation on both machines, however, a tendency of the cells to increase eotaxin-1 and to decrease VEGF secretion was observed in a comparable manner (Table 2).

Discussion

Spheroid formation occurs on both machines
The main result of this study is that FTC-133 cells form spheroids on the CN as well as on the RPM like they did during the Shenzhou-8 spaceflight in real microgravity [1,2]. During the MCTS formation process, the cells were separated into two populations. One continued adherent cell growth, while the other one detached and formed MCTS. Cultivation on the CN and the RPM resulted in spheroid formation like it was observed in real microgravity. Hence, both machines equally influence the cells in this respect. However, different device-specific alterations of gene-expression and cytokine secretion were found after 4 and 72 h cultivation, indicating device-specific characteristics. Common effects could be due to the prevention of sedimentation. Nevertheless, data from real microgravity are needed to clarify the situation and to control for any possibly confounding variables in the GBF and to choose the appropriate microgravity approach. A review on the suitability of diverse ground-based facilities was given by Herranz et al., indicating that both, CN and RPM are generally suitable for adherent mammalian cells [6]. Nevertheless, it is also clearly stated, that although some similar results in s-\text{μg} and real \text{μg} were achieved, extensive studies have to be performed for each biological system and thus, individually for each type of the cell culture of interest [6]. For future studies, equal time frames, hardware and procedures should be used in space and ground-based studies in order to exclude an impact of external factors influencing the results.

Figure 1 Phase-contrast microscopy of FTC-133 after culturing on the CN or as corresponding 1\text{g} control. The cells grew exclusively as adherent monolayers (AD) in 1\text{g} controls (A-C). On the CN, the FTC-133 cell population began to split in adherent cells (E, F) and MCTS cells (H, I) after 24 h clinorotation. 1\text{g} control, adherent cells, and MCTS after 4 h (A, D, G), 24 h (B, E, H), and 72 h (C, F, I) of clinorotation.

Spheroids formed scaffold-free under the condition of microgravity are valuable models for tumor research [1,3,11], as they resemble the in vivo situation much better than 2D monolayer cultures or spheroids grown on Earth using liquid overlay or spinner flask techniques [12,13], which consist mainly of concentric layers surrounding central necrotic cells by a thin shell [14]. The importance of studies on tumor cells exposed to microgravity was recently reviewed by Becker and Souza [15]. However, MCTS generated under simulated microgravity conditions show viable cells throughout the whole body and lack necrotic centers, even if the thyroid cancer cells (ML-1) had formed spheroids with diameters of up to 300 \text{μm} [3]. The similarity between in vivo tumors and RPM- or CN-derived MCTS is explained by the following hypothesis:
On the RPM or the CN, cells can undergo a transition from a 2- to a 3-dimensional growth, with the adherent layer serving as a starting point. Therefore, cell-cell contacts are established by forces of biochemical components expressed on the cell surfaces in a cell type specific manner under low shear forces [3]. Results of former RPM experiments with the two human follicular thyroid carcinoma cell lines FTC-133 and CGTH W-1 showed differences in the size of spheroids formed which were correlated to their capability to bind to fibronectin [4].

Table 1 Primers used for quantitative real-time PCR

| Gene   | Primer name | Sequence            |
|--------|-------------|---------------------|
| 18S rRNA | 18S-F       | GAGGCTCGCGCTAATT    |
|         | 18S-R       | CAACTAGAAGCGGCAAG    |
| CAV1    | CAV1-F      | GTACGACGGCAACACCA    |
|         | CAV1-R      | TCCCTTGGTTCTGCAAT    |
| CAV2    | CAV2-F      | GATCCCACCAGCTCACA    |
|         | CAV2-R      | CACCGGCTCGAGGATCA    |
| CD44    | CD44-F      | ACCCTCCCTATCTCCAC    |
|         | CD44-R      | GTTGTGACTAAGGTTMGCTGAT    |
| CTGF    | CTGF-F      | ACAAAGCCCTTCTGTGACTT  |
|         | CTGF-R      | GTGACCGCTCCACCAAGAT    |
| EGF     | EGF-F       | TGCCAGCTGCAAAATACAGA  |
|         | EGF-R       | TCTACGAGAAATGTGAGTCTAC    |
| ERK1    | ERK1-F      | ACCCTCCCTAAAGATGGTCTGTA    |
|         | ERK1-R      | AGCCCACTACTCCCTGCGAA    |
| IL8     | IL8-F       | TGGCAGCTCTCCTGATTCT    |
|         | IL8-R       | GGTTGGAAAGGTGAGGAT   |
| ITGB1   | ITGB1-F     | GAAAAACGCGCATCTCTGGAAAT    |
|         | ITGB1-R     | CAGCCAACTACGTGACCAA    |
| NFKBP65 | NFKBP65-F   | CGCTTTCCCTACACACTGATTC    |
|         | NFKBP65-R   | ACTGCCGGGATGCTCTCT    |
| OPN     | OPN-F       | CGAGGTGATAGTGTTGTTTGAGATGA    |
|         | OPN-R       | CTCGTTGAATCTGACAGGTTCT    |
| PKCα    | PKCα-F      | CATTCAACAGCTGGGCAAGT    |
|         | PKCα-R      | GTAGATGATGCCTGATGGAAG    |
| TLN1    | TLN1-F      | CATGCGCTTTCTACTGACAGC    |
|         | TLN1-R      | CATAGTGACTCCTCTCCTTCCA    |
| VEGF    | VEGF-F      | GGCTCTGACACATCTCAAG    |
|         | VEGF-R      | CTCCTCCACATGGCAAGTG    |
| VEGFD   | VEGFD-F     | TGCGAGGAAATCTCCATG    |
|         | VEGFD-R     | CCTCAGCGTTCTGGCTCA    |
| NGAL    | NGAL-F      | AGGGATCTCTCAAGATCCCTCTA    |
|         | NGAL-R      | AGAGTTGAGGAGGGAGAGTGA    |
| MSN     | MSN-F       | GAAATTGGTGATCAAGCCATTG    |
|         | MSN-R       | CAGAGGCGAGGTGCAAGAT    |

All sequences are given in 5’-3’ direction.

Genes and proteins playing a possible role in spheroid formation

As compared to the corresponding Ig controls, higher amounts of the selected cytokines were frequently detected in supernatants obtained from RPM cultures and lower quantities in those harvested from CN cultures, respectively (Table 2), clearly indicating device-specific differences. Interestingly, VEGF secretion indicated a tendency of down-regulation, while eotaxin-1 suggested up-regulation on the CN as well as on the RPM after 72 h (Table 2). Former investigations of culture supernatants of samples cultured on the RPM or returned from the Shenzhou-8 spacecraft experiment showed a different picture. No changes of EGF and VEGF secretion were observed after real microgravity exposure, while both cytokines were significantly down-regulated after a 10d-RPM-exposure [2]. The different cytokine concentrations could be due to a change of the secretion activity during prolonged incubation under microgravity when the transition from a 2- to a 3-dimensional growth is completed as it has been shown for human endothelial cells [16,17]. A comparison of cytokines in the supernatant appears only possible during incubation within a few days, as investigation of IL-6 after 1 and 3 days performed in this and a former study [10] suggested. VEGF prevents apoptosis in thyroid carcinomas in an autocrine manner [18]. Its reduction may contribute to an enhanced apoptotic rate in thyroid cells cultured on the RPM [3]. Eotaxin-1 induces changes in the cytoketoskeleton and cell morphology and thus could favor the transition from a 2- to a 3-dimensional kind of growth [19].

Although spheroids were not seen in the cultures on both machines after 4 h, a change in gene expression activity was already expected based on earlier parabolic flight experiments [20-22]. Indeed, three of the selected genes were found to be changed. Most interesting of them is CTGF’s significantly enhanced expression as compared to the corresponding Ig controls. It remained up-regulated for another 68 h in AD cells on both machines, while the MCTS cells showed CTGF mRNA concentrations even lower than the control cells after 72 h of culturing. The split of CTGF gene expression between adherent and MCTS cells corresponds nicely to earlier data from 10 d RPM and spaceflight experiments, which indicated more mRNA in AD than in MCTS cells [2] and could therefore be a microgravity-dependent process involved in spheroid formation. CTGF was found to be over-expressed in papillary thyroid carcinoma correlating with metastasis, size and clinical stage [23]. It was also suggested to play an important role in angiogenesis and tumorigenesis of prostate cancer [24]. Its reduction in MCTS cells could hint to a diminished aggressiveness of cancer cells incorporated in MCTS (manuscript in preparation).
Besides CTGF, genes of adherent cells remained unaffected until 72 h on the RPM, while expression of CAV1, EGF and ERK1 had been altered in the MCTS. In contrast, after 72 h on the CN, CAV2 and IL8 expression were changed in AD cells and CAV1, CAV2, CTGF, ERK1 and IL8 in the MCTS. Most interesting of these observations was a down-regulation of CAV1 and an up-regulation of ERK1 in MCTS cells after three days following an at least one-day-lasting stability of these genes [10,25], which differ from the CAV2 gene that is stable only for 4 h (Figure 2) but down-regulated after 24 h and 72 h [10]. Caveolin-1 is an integral membrane protein and plays crucial roles in the regulation of cellular proliferation, differentiation and apoptosis [26]. The down-regulation of caveolin-1 appears to enhance the capacity of the cell to incorporate in a tissue [27]. Furthermore, the expression of this protein seems to be gravisensitive, because an overexpression in healthy mice staying in space had been observed [28]. Since CAV1 is an obviously gravisensitive gene and its product influences the incorporation of cells in tissue, we conclude from our observations that CAV1 may play a role in microgravity-dependent MCTS formation.

**Device-dependent cell modification**

Unexpectedly, most of the selected genes and cytokines were differently expressed or secreted, respectively, depending on which kind of simulation device MCTS were formed. We conclude that during MCTS formation considerable alterations of various cellular molecules occur. Some of them might not be directly related to the process of 3D cell aggregation, but by products generated by device-dependent modifications [29]. A comparative study in space will help to discriminate microgravity-induced alterations from other physical stimuli due to vibration, shearing forces, etc. generated by the simulators. The 2D clinostat rotating constantly at 60 rpm generates residual accelerations below 0.012 g within the distance of ±3 mm around the center of the flask [30]. Cells located at further distance from the rotation axis are exposed to accelerations reaching up to 0.036 g at about ±9 mm. Significant differences in gene expression within these acceleration intervals have been shown by Eiermann et al. [30]. Based on these findings, only cells within the central 6 mm (±0.012 g) of a slide were harvested and analyzed in the present study. An exception had to be made for supernatants which were collected in total, as a distinction...
Figure 3 (See legend on next page.)
between different acceleration levels was not possible. Therefore, supernatants were enriched by cytokines of all cells from the flasks. Whether gravity forces in a range of 0.012 $g$ and 0.036 $g$ have a separated influence on the cell behavior has not yet been clarified [30,31]. Either coating of the flasks in the parts outside the area of interest in order to prevent initial cell attachment or a different geometry of the flasks themselves, with an enlarged area along the rotation axis would be convenient to avoid a mixture in cytokine release.

### Table 2 Cytokines detected in supernatants of FTC-133 cells after 72 h incubation

| Factor       | 2D Clinostat | Random positioning machine |
|--------------|--------------|-----------------------------|
|              | LDD (pg/mL)  | 72 h 1g (pg/mL) | 72 h s-μg (pg/mL) | LDD (pg/mL) | 72 h 1g (pg/mL) | 72 h s-μg (pg/mL) |
| GM-CSF       | 3.2          | 1536 ± 166 | 1172 ± 31* | 9.3          | 468 ± 69* | 1108 ± 120* |
| IFN-γ        | 0.49         | n.d.       | n.d.       | 0.58         | n.d.       | n.d.       |
| IL-2         | 1.0          | 1.9 ± 0.3  | n.d.       | 2.6          | n.d.       | n.d.       |
| IL-3         | 1.1          | n.d.       | n.d.       | 2.0          | n.d.       | n.d.       |
| IL-4         | 4.4          | n.d.       | n.d.       | 3.2          | n.d.       | n.d.       |
| IL-5         | 1.0          | n.d.       | n.d.       | 0.73         | n.d.       | n.d.       |
| IL-6         | 0.84         | 282 ± 37   | 204 ± 17*  | 0.85         | 43.8 ± 84 | 70 ± 15.6* |
| IL-7         | 2.1          | n.d.       | 2.1 ± 0.3  | 5.5          | n.d.       | n.d.       |
| IL-8         | 0.63         | 7604 ± 410 | 5586 ± 267*| 0.49         | 226 ± 31  | 350 ± 36*  |
| IL-10        | 1.1          | n.d.       | n.d.       | 1.5          | n.d.       | n.d.       |
| IL-18        | 3.7          | n.d.       | n.d.       | 3.2          | n.d.       | n.d.       |
| MIP-1α       | 8.2          | 3086 ± 390 | 2198 ± 379*| 6.0          | 113 ± 18  | 137 ± 24  |
| MIP-1β       | 4.0          | 261 ± 46   | 166 ± 28*  | 4.6          | n.d.       | n.d.       |
| MCP-1        | 4.6          | 47 ± 6     | 40 ± 3     | 4.9          | n.d.       | n.d.       |
| TNF-α        | 2.9          | 8.9 ± 1.2  | 7.0 ± 1.3  | 3.1          | n.d.       | n.d.       |
| TNF-β        | 2.3          | 2.3 ± 0.4  | n.d.       | 4.4          | n.d.       | n.d.       |
| BDNF         | 3.0          | 20 ± 1.7   | 16 ± 1.7*  | 6.2          | 41.2 ± 46  | 76.6 ± 4.7* |
| Eotaxin-1    | 3.7          | 118 ± 1.8  | 124 ± 2.3  | 13           | 75.4 ± 9.9 | 99.4 ± 9.8* |
| ICAM1        | 270          | 360 ± 74   | 350 ± 73   | 620          | 1328 ± 274| 1840 ± 150*|
| IL-1α        | 0.24         | n.d.       | n.d.       | 0.19         | 0.85 ± 0.11| 1.60 ± 0.29*|
| IL-1β        | 0.3          | n.d.       | n.d.       | 0.25         | 1.14 ± 0.22| 2.02 ± 0.34*|
| IL-1ra       | 8.7          | n.d.       | n.d.       | 14           | 127 ± 38  | 226 ± 24*  |
| IL-12p40     | 16           | n.d.       | n.d.       | 17           | 128 ± 21  | 206 ± 29*  |
| IL-12p70     | 4.8          | 7.8 ± 0.6  | 7.3 ± 0.9  | 4.5          | 36.0 ± 42  | 46.2 ± 6.1 |
| IL-15        | 39.0         | n.d.       | n.d.       | 41           | 142 ± 27  | 208 ± 28*  |
| IL-17        | 0.51         | 1.14 ± 0.32| 1.0 ± 0.16 | 0.6          | 2.5 ± 0.4  | 4.5 ± 0.6* |
| IL-23        | 51           | n.d.       | n.d.       | 69           | 590 ± 117 | 868 ± 78*  |
| MMP-3        | 5.8          | 260 ± 22   | 256 ± 19   | 7.4          | 512 ± 11.3 | 908 ± 11.1*|
| SCF          | 13           | 24 ± 4     | 19 ± 2     | 9.0          | 590 ± 16.1| 946.6 ± 11.5*|
| VEGF         | 1.4          | 3062 ± 539 | 2814 ± 309 | 4.2          | 6048 ± 791| 5044 ± 677 |

Values are given with mean ± SD; 1g, corresponding ground control; n.d., not detectable; *P <0.05 for device sample vs. corresponding 1g ground control; LDD (Least Detectable Dose)-determined as the mean ± 3 standard deviations of 20 blank readings.
Concerning clinorotation, no relative fluid motion is assumed in a closed completely filled and air bubble-free container exposed to the CN due to its linear and constant acceleration mode [32]. In contrast, chaotic fluid motion is caused on a RPM operating in real random mode due to random changes of speed and direction of the platform movement [33-35]. Although the magnitude of forces of fluid motion, which does not exceed 0.44 dyn/cm² [35], is below that required to trigger cell detachment from surfaces [36,37], it is assumed to be high enough to induce an enhanced convective fluid mixing, which may support nutrient supply and removal of metabolic products and thus optimize the culture conditions for a cell.

Residual accelerations for RPM exposure have to be considered for all 3 g-vector components that means in x, y and z directions. Exemplary measurements of a RPM running at 60–120 °/s showed accelerations due to gravity of −1 to +1 g for each direction [38]. A slower rotation speed produces less residual acceleration but requires a longer time for averaging of the g-vector. The relation of rotation speed, resulting accelerations and the time a systems needs to detect the alterations of the influence of gravity has to be considered. Even though less residual accelerations can be achieved through a decreased rotation speed, a longer exposure stimulus will be inevitably connected which can possibly lead to a permanent stress stimulus, if sensed by the cells [39].

Shear stress has to be considered as a variable differing between the devices. As shown by Goodwin et al. BHK-21 cells exposed to 0.51 dyn/cm² consumed 60-70% less glucose, but achieved higher cell counts and aggregation than cells exposed to 0.92 dyn/cm², both on the integrated rotating wall vessel (RWW) [40]. Furthermore, experiments by Hammond et al. on primary cultures of human renal cortical cells exposed for 6 days to either real microgravity on a space shuttle or cultured on ground-based devices as controls demonstrated 1632 gene changes after the space flight, 5 gene changes after centrifugation and 914 gene changes after cell culturing on the RWW, if a threshold of more than ± three-fold change was set. In addition, as many gene changes in reciprocal as in the same direction were observed, when results from RWW and space cultures were compared [41]. The balancing forces, such as shear, used to offset gravity in RWW systems were suggested to be responsible for changing the character of the culture [41]. Further work by Kayser et al. supports this hypothesis, showing a shear stress dependence of selected de novo gene and protein expression during renal epithelial cell culture in RWW [42]. Therefore, different shear stress situations on both devices used in our study seem to be a cause of differences in the results obtained. The problem of internal shear forces due to residual accelerations has been recognized not only in ground-based facilities, but was also predicted to be responsible for differences between 1 g ground controls and 1 g-in-flight controls using a 1 g-reference centrifuge in space, as described by van Loon et al. [43]. An adjustment of the culture flasks to the direction of the forces is suggested in order to obtain an optimal outcome and comparability of the data. Further experiments are required to clarify stress and gravity-related effects and to determine threshold levels for response time and optimal rotation speed in order to simulate optimal microgravity conditions on ground.

Also vibrations are often suggested as a critical parameter in microgravity simulation. As the corresponding 1 g controls were cultured next to the devices in the same incubator, the vibration effect should be neglected as both samples 1 g and s-μg are exposed to the same external stimulus. The same matter has to be addressed in parabolic flights especially in combination with periods of hypergravity. Experiments with respect to vibration and hypergravity have been performed on two human follicular thyroid cancer cell lines (ML-1 and CGTH W-1) and endothelial cells (EA.hy 926), all suggesting that microgravity effects are stronger than the opposing vibration and hypergravity effects [20-22]. Nevertheless, additional vibration experiments for FTC-133 are of interest as final validation of ground- based and future space data.

A further aspect possibly influencing the results are the different culture containers used due to the geometry of the devices. As the same flasks were used for s-μg and corresponding 1 g-samples and results were analyzed relatively to the in parallel obtained 1g-data, an influence should not be expected for the PCR data. Concerning the MAP data, a possible effect cannot be excluded due to different volume and area proportions.

For the CN, special slide flasks had to be used in order to utilize the harvesting area on the rotation axis to a maximum extent and to adjust a larger amount of samples on the machine. This leads to differences in the proportion of cells/cm² and cells/ml for both devices which might have an impact on the cytokine measurements in the supernatant and might influence interaction and signaling of the cells. For the clonostat, a maximum of 0.056 × 10⁶ cells/cm² were used for 4 h-experiments. Longer experiments of 24–72 hours were seeded with less density in order to prevent overgrowing in the limited culture area of only 9 cm². A culture volume of 20 ml resembles 0.025 × 10⁶ cells/ml. For the RPM equal amounts of cells were used for all time frames, as a bigger culture area of 75 cm² and a larger volume of 320 ml culture media was available. This leads to 0.053 × 10⁶ cells/cm² and 0.0125 × 10⁶ cells/ml. As a consequence, differences in autocrine and paracrine signaling between the cells, due to the differences in cell to area/volume proportion as well as different fluid behavior due to the geometry of the culture flask, clinorotation and random rotation have to be taken into consideration. The hypothesis, that in vitro responses of cells to modified inertial
environments might be a manifestation of modified extracellular convective flow has been suggested by Paul Todd already in 1992 [44]. For future experiments, equal culture conditions should be used in order to obtain an optimal comparability of the data. Still, the unique conditions of real microgravity with the loss of gravity-dependent convection and negligible hydrodynamic shear have to be considered when comparing real and s-μg results [15,44,45].

Conclusion

On both, the CN and RPM, spheroid formation of human thyroid cancer cells was observed, whereas, besides a few exceptions, a considerable number of selected genes or cytokines were expressed or secreted differently, although equal kinds of cells formed MCTS on the two machines. The exceptions were CAV1 and CTGF genes as well as VEGF and eotaxin-1 cytokines. We consider them involved in the process of spheroid formation, because their changes consistently accompanied the MCTS formation in similar manner, when cell sedimentation is prevented by RPM or CN or even in real microgravity in space. The study shows the advantage of searching for gravity-sensitive genes and proteins in comparative approach using different machines for microgravity simulation. Our study clearly shows the necessity to verify results from ground-based simulation approaches to the ones obtained in real microgravity conditions to avoid misinterpretations, to learn and to understand device-specific characteristics and finally choose the appropriate simulation approach.

Methods

Culturing of FTC-133 cells

The human follicular thyroid carcinoma cell line FTC-133 [46] was cultured in RPMI-1640 medium at 37°C and 5% CO2. The medium was supplemented with 100 μg/mL streptomycin, 100 U/mL penicillin and 10% FCS (all Biochrom, Berlin, Germany). One day prior to the CN experiments, cells were seeded in 9 cm² slideflasks (Thermo Scientific, Roskilde, Denmark). A cell count of 5 × 10⁵ cells was disseminated for 4 h experiments, 4 × 10⁵ for 24 h and 2 × 10⁵ for 72 h experiments. For the RPM experiments, cells were grown in T75 cell culture flasks (Sarstedt, Numbrecht, Germany). Cells were seeded at a density of 4 × 10⁶ cells per flask. The cells were randomized to be cultivated as static ground controls (1 g) or under simulated microgravity conditions (s-μg) on either a RPM or a CN. Ground controls were always placed next to the device in the same incubator. Cells and supernatants were harvested after 4 h or 72 h on ice. The supernatants were aspirated and centrifuged at 4°C. Afterwards, the fluid was transferred to another tube and frozen. The pellet was fixed with RNAlater. After removal of the culture supernatant, cells which remained adherent during incubation, were washed once with PBS and then fixed with RNAlater. For this, in case of the CN the slides were remove from their flask and were slowly dipped first in PBS (5 s) and then transferred to RNAlater until harvesting. T75 culture flasks from the RPM were slowly filled with 10 ml PBS while standing vertically, and were very carefully brought into horizontal position to avoid any disturbance of the cells. PBS was aspirated again before the addition of RNAlater. Afterwards, the cells cultured on the RPM were scraped off the whole bottom surface, while cells cultured on the CN samples were harvested from the inner 6 mm of the slide flask only, because cells of this part experience accelerations of ≤0.012 g [30].

Random Positioning Machine (RPM) and 2D clinostat (CN)

For a comparative methodical approach, cells were either cultivated on the Desktop RPM manufactured by Dutch Space (an EADS Astrium company, Leyden, Netherlands) [8] or the Fast Rotating 2D clinostat (DLR, Cologne, Germany) [30]. The Desktop RPM was operated in real random direction mode (60 °/s) and equipped with four T75 flasks per run, fixed to the ground plate, giving a maximum distance of 7.5 cm from the center of rotation. The 2D clinostat constantly rotated with 60 rpm and was loaded with four slideflasks on each of the 6 parallel rotating axes, summing up to a total of 24 flasks per run. Both devices were placed inside an individual incubator with temperature of 37°C and 5% CO2. Prior to the experiment, all flasks were completely filled with media avoiding air bubbles carefully, in order to reassure a minimization of turbulences. For harvesting of CN samples, cells of 16 flasks were pooled. Ten samples (n = 10) were collected for each condition: CN, CN corresponding 1g control, RPM and RPM corresponding 1g control.

Phase contrast microscopy

Phase contrast microscopy was performed for visual observation of the morphology of the cells, using the Axiosvert 25 Microscope (Carl Zeiss Microscopy, LLC, United States).

Cytokine measurements by Multi-Analyte Profiling technology

The release of cytokines was investigated via Multi-Analyte Profiling (MAP) as previously described [16,47,48]. For each condition, five supernatants were collected after 72 h and stored at −80°C until testing. The MAP was carried out by Myriad RBM (Austin, Texas, USA) using the Human Cytokine MAP A and B. Briefly, Micro beads carrying specific antibodies, directed against the target analytes, are used to detect the cytokines. A second biotinylated reporter antibody is added, followed by an excess of streptavidin-phycocerythrin solution to develop the multiplexes and thereby enabling the quantification of the cytokines released, via fluorescence detection [49].
RNA isolation and quantitative real-time PCR

RNA isolation and quantitative real-time PCR were performed according to routine protocols [25,50,51]. For CN samples, only the inner 6 mm were harvested with a scratching device. RPM samples were harvested in total. RNA was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany) following manufacturer instructions. DNase (Qiagen, Hilden, Germany) was added in the process of RNA isolation, in order to diminish residual DNA contaminations. The RNA was quantified via Photometer Ultraspec2010 (Amersham Biosciences, Freiburg, Germany). Reverse transcription was performed using the first strand cDNA synthesis kit (ThermoFisher Scientific, Waltham, US), following manufacturer’s instructions. Quantitative real-time PCR was utilized to determine the expression levels of target genes, shown in Table 1, using the 7500 Real-Time PCR System (Applied Biosystems, Darmstadt, Germany). cDNA-selective Primers were designed to span exon-exon boundaries and to have a Tm of 60°C using Primer Express software (Applied Biosystems, Darmstadt, Germany), and were synthesized by TIB Molbiol (Berlin, Germany). All samples were measured in triplicate and normalized to the housekeeper 18 rRNA. Comparative C_T (ΔΔC_T) methods were used for relative quantification of transcription levels, with 1 g set as 100%.

Statistical Evaluation

Statistical Evaluation was performed using SPSS 15.0 (SPSS, Inc., Chicago, IL, USA). The Mann–Whitney-U-Test was used to compare Ig and s-μg conditions, as well as s-μg adherent cells and s-μg MCTS cells. All data is presented as mean ± standard deviation (SD) with a significance level of p < 0.05. * indicating the comparison of Ig vs. s-μg and ** representing the comparison of s-μg AD vs. s-μg MCTS.

Abbreviations

BDNF: Brain-derived neurotrophic factor; CAV: Caveolin; CN: Clinostat; CTGF: Connective tissue growth factor; EGF: Epidermal growth factor; ERR: Extracellular signal-regulated kinase; FCS: Fetal calf serum; FTC: Follicular thyroid cancer; GFB: Ground-based facilities; GM-CSF: Granulocyte-macrophage colony-stimulating factor; IFN: Interferon; IL: Interleukin; RT-PCR: Reverse transcription–polymerase chain reaction; PDGFR: Platelet-derived growth factor receptor; PKCα: Protein kinase C alpha; RBM: Rules-based medicine; RPM: Random positioning machine; MCP: Monocyte colony-stimulating factor; IFN: Interferon; IL: Interleukin; ITBG1: Integrin beta 1; LDD: Least detectable dose; MAP: Multi-Analyte Profiling; MCP: Monocyte chemotactic protein; MCTS: Multicellular tumor spheroids; MK2: Mitogen-activated protein kinases; MMP: Matrix metalloproteinase; MRNA: Messenger ribonucleic acid; PCR: Polymerase chain reaction; PRINS: Protein kinase C alpha; RT: Reverse transcriptase; RPM: Random positioning machine; RWW: Rotating Wall Vessel; SCF: Stem cell factor; SD: Standard deviation.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

JB, DG and RH designed the study. BW and XM conducted the experiments. BW and JB drafted the manuscript. JP, MW and MI supervised and supported the ground-based experiments in Magdeburg. DG supervised the ground-based experiments in Aarhus and helped with the manuscript. RH and MB supervised and supported the ground-based experiments in Cologne. MG was responsible for the design of the 3D clinostat. All authors read and approved the final manuscript.

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