



The effect of combined simulated
microgravity and microgrooved surface
topography on fibroblasts

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INTRODUCTION

Mechanosensitivity of fibroblast cells towards static forces, e.g. stress provided by a microgrooved surface onto which the cells are cultured or dynamic stress in the shape of for instance substratum deformation by stretch or fluid shear have been well documented. Mechanical stress is an important and specific director of cell shape and stimulus for extracellular matrix component production. [1-9]. On the other hand, cells can also experience mechanical unloading by removing the, at cell level, very weak, but most constant force in nature: gravity. Morphological and functional studies have shown that many cells like osteoblast-like cells and lymphocytes are highly sensitive to altered gravity [10-17].

Although it is generally accepted that the response to environmental stimuli is multi-factorial, research towards the interaction of cells cultured on substratum texture (nano-, micropatterns) and mechanical force is sparse. Such studies are necessary to serve as baseline values for further research aimed at understanding of observed cell response phenomena on a molecular basis [18-22]. Ground based research to simulate microgravity conditions by means of a random positioning machine (RPM), also referred to as 3D clinostat, provides an excellent means to conduct gravitational studies. The RPM is a microweight (microgravity) simulator that is based on the principle of 'gravity-vector-averaging'. Gravity is a vector, i.e. it has a magnitude and direction. During an experimental run in the RPM the sample position with regard to the Earth's gravity vector direction is constantly changing, and as a result the samples will experience a near-weightlessness environment [23-28].

In this study we evaluated in vitro the differences in morphological behaviour between fibroblast cells cultured on polystyrene substrata, both smooth and with a surface microtopography, which were placed in a simulated microgravity environment. In addition to microscopy analysis, the transcription of several proteins involved in cell-surface interaction was investigated, as well as, proteins of the mitogen activated protein kinases (MAPK) pathway which are involved in cell adhesion and motility. The underlying aim was to understand which parameter is more important in determining cell response. Our hypothesis is that cellular shape and orientation is determined by the topographical cues, and a simulated microgravity environment will decrease the cellular orientation to these substrata. As controls fibroblast cells were cultured on similar substrata, which remained at normal (Earth) gravity.

MATERIALS AND METHODS

Substrata:

Microgrooved patterns were made, using a photo lithographic technique and subsequent etching in a silicon wafer as described by Walboomers et al. [1]. This wafer is divided into 4 quadrants with a ridge- and groove width of 1, 2, 5, or 10 μm , with a uniform depth of 0.5 μm . Wafers with a planar surface were used as controls. The silicon wafer was used as template for the production of polystyrene (PS) substrata for cell culturing. PS was solvent cast in manner described by Chesmel and Black [29]. Polystyrene replicas were attached to 18 mm diameter cylinders with polystyrene-chloroform adhesive. Shortly before use a radio frequency glow-discharge (RFGD) treatment was applied for 5 minutes at a pressure of 2.0×10^{-2} mbar (Harrick Scientific Corp., Ossining, NY, USA) to promote cell attachment by improving the wettability of the substrata.

Cell culture:

Rat dermal fibroblasts (RDF) were obtained from the ventral skin of male Wistar rats as described by Freshney [30]. Cells were cultured in CO_2 -independent α -MEM containing Earle's salts (Gibco, Invitrogen Corp., Paisley, Scotland), L-glutamine, 10% FCS, gentamicin (50 $\mu\text{g}/\text{ml}$), in an incubator set at 37 °C with a humidified atmosphere. Experiments were performed with 4 - 8th culture

generation cells. Onto the various substrata, 1.0×10^4 cells/cm² were seeded. Cells were cultured for 4h, after which they were placed in a 12 wells plate for support. Custom made silicone caps closed each well and the tissue culture dish therein (**Figure 1**). Using a type 22G1 needle and applying a little pressure while adding additional medium, air bubbles were removed to prevent unwanted shear force and turbulence during rotation. In this system with only one specific density fluid and absent air bubbles no fluid motion was to be expected, since the media was moving with the same velocity as the cell monolayer. The well plates containing the samples were secured onto the experiment platform. From previous studies it is known that fibroblasts need approximately 4 hours to commence adapting their morphology to a new environment[31]. Therefore experiment times were chosen of 4 and 24 hours.

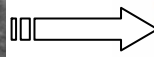
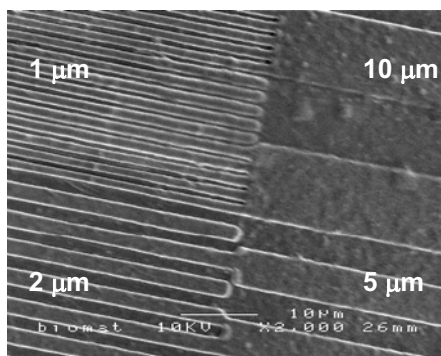


Figure 1. SEM micrograph of a grooved substratum, showing in detail the accurate production of the 4 different groove widths into the polystyrene. Clockwise, starting in upper left: 1, 10, 5, and 2 micron wide and uniform 0.5 micron deep grooves. At right a graphical presentation of a complete polystyrene culture dish used in this study with silicone closing cap securing the insert snug into the well of a 12 wells plate and providing an air bubble free environment.

Simulated microgravity:

The RPM is shown in **Figure 2**. One of the first versions was developed by T. Hoson et al. [32]. We used a similar system, manufactured by Dutch Space (formerly Fokker Space, Leiden, The Netherlands). The outer frame rotated perpendicular to the inner frame, which caused the samples to move randomly in 3 axis. The rotational movement of both frames was powered by two servomotors. A PC user interface with dedicated software controlled the two servomotors onset, rate, and duration of rotation. The rotational velocity of both frames was randomised with a maximum of 60°/sec, direction, and interval was set at random. The samples were fixed in the centre of the inner frame, the largest radius was 57.8 mm to the outermost well. The RPM was accommodated in a temperature-controlled incubator set at 37 °C and capable to be supplied with a 5% CO₂/air gas mixture [33].

For the experiments the cell layers were washed three times with Phosphate Buffered Saline (PBS) and prepared for further analysis immediately after retrieval from the RPM machine.

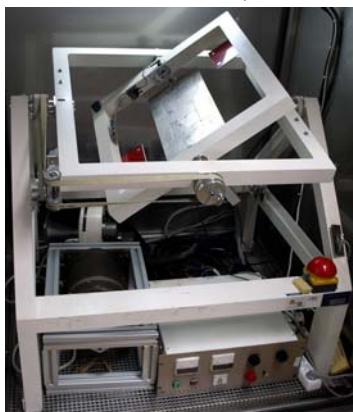


Figure 2. The Random Positioning Machine (RPM) used in this study to simulate weightlessness. The RPM is equipped with a computerised temperature and motor control. The control samples are secured on one of the machine support struts, while experimental samples are placed on the inner frame of the clinostat.

Scanning electron microscopy (SEM):

To assess overall morphology of the fibroblasts, SEM was performed (n = 4). Cells were rinsed, fixed in for 5 minutes in 2% glutaraldehyde, followed by 5 minutes in 0.1 M sodium-cacodylate buffer (pH 7.4), dehydrated in a graded series of ethanol, and dried in tetramethylsilane to air. Specimens were sputter-coated with gold and examined with a Jeol 6310 SEM (Tokyo, Japan).

Immunofluorescence

Components of the cytoskeleton were made visible using fluorescent staining techniques. RDF cells, cultured on microgrooved substrata were rinsed in PBS, pH7.2, fixed for 30 minutes in 2% paraformaldehyde, and permeabilised with 1% Triton X100 for 5 min. Filamentous actin was stained with Alexa Fluor 568 phalloidin (Molecular Probes, A-12380, Leiden, The Netherlands) diluted in 1% Bovine Serum Albumin/PBS to block non-specific epitopes. Vinculin was stained with rabbit polyclonal primary antibodies to vinculin (Santa Cruz, sc-5573), followed by labelling with goat anti-rabbit secondary antibodies IgG with Alexa Fluor 488 (Molecular Probes, A-11034). Finally, the specimens were examined with a Biorad (Hercules, CA, USA) MRC 1024 confocal laser scanning microscope (CLSM) system with a krypton-argon laser at magnification of 40x. The digital immunofluorescence images acquired with the CLSM were loaded into Confocal Assistant (version 4.02, Todd Clark Brelje, USA) to create overlay images. Cytoskeletal components were examined for their overall morphology as well as their orientation with respect to the groove direction. For quantitative image analysis samples were stained with Phalloidin-TRITC (Sigma, P-1951, St. Louis, MO, USA), followed by examination with a Leica/Leitz DM RBE Microscope (Wetzlar, Germany) at magnification of 10x.

Image Analysis

The Phalloidin-TRITC fluorescence micrographs were analyzed with Scion Image software (Beta Version 4.0.2, Scion Corp., Frederick, MY, USA). The orientation of fibroblasts was examined and photographed. For each sample six fields of view were selected randomly. The criteria for cell selection were (1) the cell is not in contact with other cells and (2) the cell is not in contact with the image perimeter. The maximum cell diameter was measured as the longest distance between two edges within the cell borders. The angle between this axis and the grooves (or an arbitrarily selected line for smooth surfaces) was termed the orientation angle. If the average angle was 45 degrees, cells were supposed to lie in an at random orientation. Cell extensions like filopodia, which could confound the alignment measurement, were not included when assessing the cell orientation. Using Clarks criteria [34; 35], cells oriented at 0–10 degrees from the groove direction were regarded to be aligned. The distribution of cytoskeletal patterns with time, gravity force in view of the type of microgrooves and groove direction was described by the percentage of cells in the sample that displayed each pattern.

Cellular surface area was measured with the aforementioned image analyses software. Applying the same criteria for cell selection; cell areas were determined and displayed as μm^2 . Between 850 - 1010 cells were measured per group for both orientation and surface area.

RT-PCR

Total RNA was isolated from fibroblasts with an RNA isolation and stabilisation kit (QIAGEN, Hilden, Germany) and cDNA was synthesised from 1 μg of total RNA with an RT-PCR kit. After initial denaturation for 2 min at 95°C, the samples were amplified for 35 cycles (annealing 55 °C 1 min, elongation 72 °C 2 min, denaturation 95 °C 1 min). The duration of the final elongation reaction was increased to 10 min at 72 °C to permit completion of reaction products. PCR products were separated on a 2% (w/v) agarose gel and visualised by ethidium bromide staining. RT-PCR

products ratios were the result of three replicates and were semi-quantified by band intensity analysis using Quantity One 1-D analysis software for Windows (Version 4.5.0, Bio Rad, Hercules, California, USA). The collagen type I (only 24 hours samples), fibronectin, $\alpha 1$ integrin, $\beta 1$ integrin gene expressions were normalised to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) values. The forward and reverse primer sequences are listed in **Table 1**.

	5' Primer (Forward)	3'Primer (Reverse)	Size (bp)
Collagen Type I	TGTTTCGTGGTTCTCAGGGTAG	TTGTCGTAGCAGGGTTCTTTC	254
Fibronectin	CCTTAAGCCTTCTGCTCTGG	CGGCAAAAGAAAGCAGAACT	301
$\alpha 1$ -Integrin	AGCTGGACATAGTCATCGTC	AGTTGTCATGCGATTCTCCG	374
$\beta 1$ -Integrin	AATGTTTTAGTGCAGAGCC	TTGGGATGATGTCGGGAC	262
GAPDH	CGATGCTGGCGCTGAGTAC	CGTTCAGCTCAGGGATGACC	407

Table 1: *Forward and reverse primer sequences used in this study*

Statistical analysis:

Acquired data from the fluorescence micrographs of cell alignment were analysed using SPSS for Windows (Release 12.0.1, SPSS Inc., Chicago, USA). The effects of and the interaction between both time and/or force and surface were analysed using ANOVA, including a modified least significant difference (Bonferroni) multiple range test to detect significant differences between two distinct groups. Probability (p) value ≤ 0.05 was considered significant.

RESULTS

Scanning Electron Microscopy

The micro topography pattern of grooves and ridges was accurately reproduced in the polystyrene substrata. When observing cell morphology, RDFs cultured on smooth substrata displayed a cell spreading which was considered random (**Figure 3A**), while a orientation along the groove direction was observed when cells were cultured on microgrooved substrata (**Figure 3B**). Upon visual inspection no noticeable difference were seen with regards to cell orientation when comparing the different groove sizes. **Figure 3C** shows a grooved substratum section, which features 1 micron (left side), as well as, 10 micron (right side) wide grooves, both 0.5 micron deep. This micrograph shows clearly that the cells or part of cells on the wider grooves (5 μm and above) were flatter in appearance and were able to descend to the bottom of the grooves, while on the more narrow textures cells remained on top of the ridges, bridging the gaps between the ridges and extending their cell bodies. Although the visual differences were quite small, most 4 hour samples (**Figure 3D**) seemed to showed a better cell alignment towards the grooves when compared to 24 hour samples (**Figure 3E**). In addition, cells cultured on smooth surfaces revealed a larger cell area, especially those which experienced the short experiment time.

Fluorescence microscopy (CLSM)

The cytoskeleton was investigated by staining filamentous actin and vinculin anchor points of the cell focal adhesions. **Figure 4A** shows a 1g smooth substratum sample; the observed cell shape, spreading, and random orientation were similar to SEM micrographs. Red staining is that of the actin filaments, which were always running parallel with the long axis of cells. Vinculin staining for focal adhesions resulted in a considerable background around the nucleus. Vinculin spots were visible in some samples, positioned at the end of actin bundles, and always extended in the direction of the actin bundle. RDFs cultured on grooved surfaces displayed a similar view, as was found on smooth surfaces; however there was clear orientation of the cells and their cytoskeleton (**Figure 4B**). The overall view of cells cultured on smooth surfaces in the RPM did change not notably (**Figure 4C & D**).

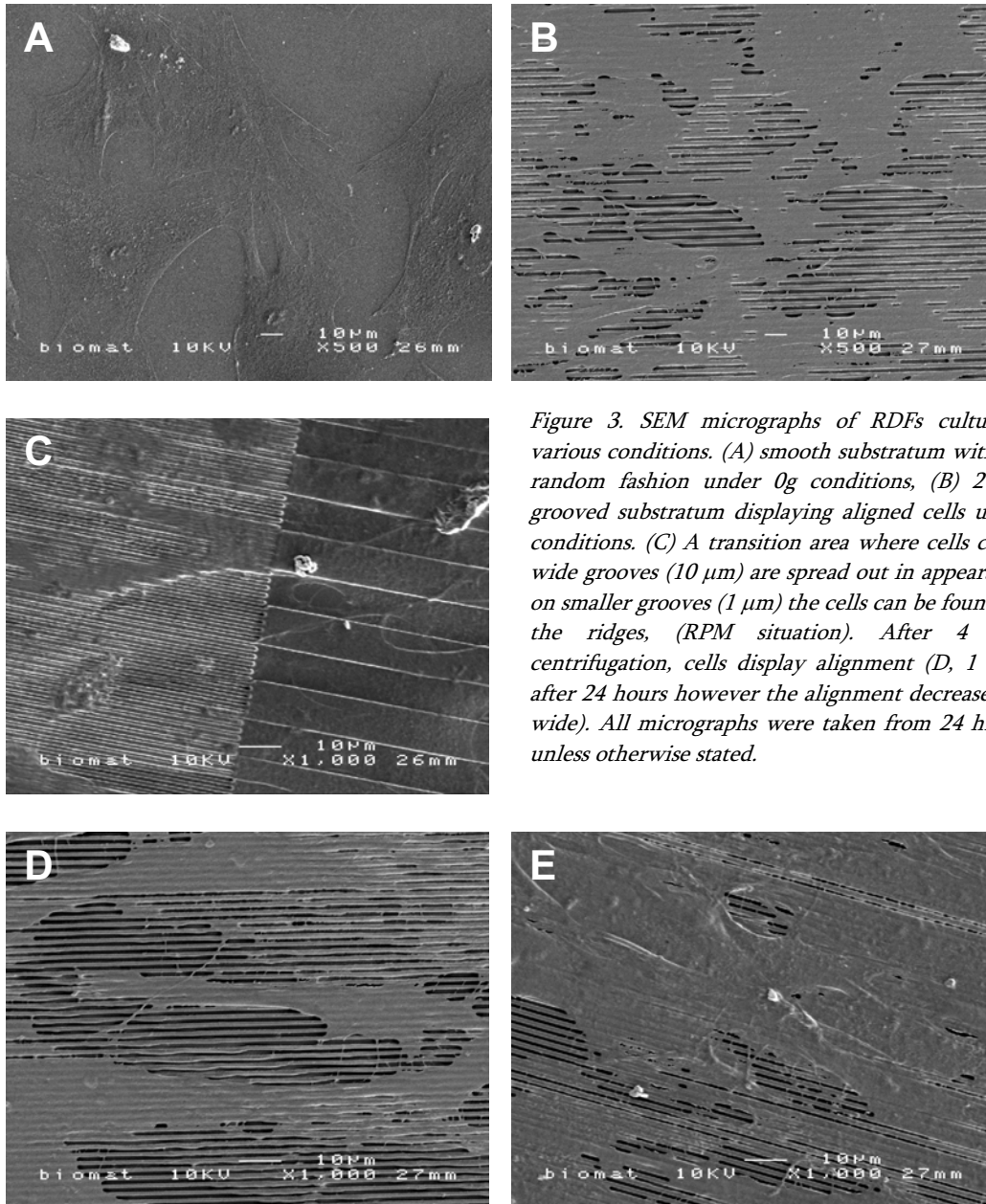


Figure 3. SEM micrographs of RDFs cultured under various conditions. (A) smooth substratum with cells in a random fashion under 0g conditions, (B) 2 μm wide grooved substratum displaying aligned cells under RPM conditions. (C) A transition area where cells cultured on wide grooves (10 μm) are spread out in appearance while on smaller grooves (1 μm) the cells can be found on top of the ridges, (RPM situation). After 4 hours of centrifugation, cells display alignment (D, 1 μm wide), after 24 hours however the alignment decreases (E, 1 μm wide). All micrographs were taken from 24 hrs samples, unless otherwise stated.

This is in contrast to the cells cultured on grooved substrata: in the RPM the alignment of the cells evidently decreased. Under simulated microgravity after 24 hours the cells changed their shape and tended to spread out more, which was not observed for the smooth controls (**Figure 4E & F**).

Image Analysis

Image analysis of the phalloidin-TRITC stained actin filaments showed that cellular orientation of RDFs cultured on all grooved substrata was profound (**Figure 5**). Cells cultured on smooth samples revealed a random orientation in cell spreading. The quantified results for cell orientation are presented in **Figure 6**. No significant differences in alignment were found within the smooth groups neither in time or in gravity. However, besides the obvious significant difference between smooth and grooved substrata, significant differences existed (**Figure 7A**) within the grooved groups as was shown by ANOVA performed on the data. In this analysis all main parameters: topography, gravity, and time proved significant.

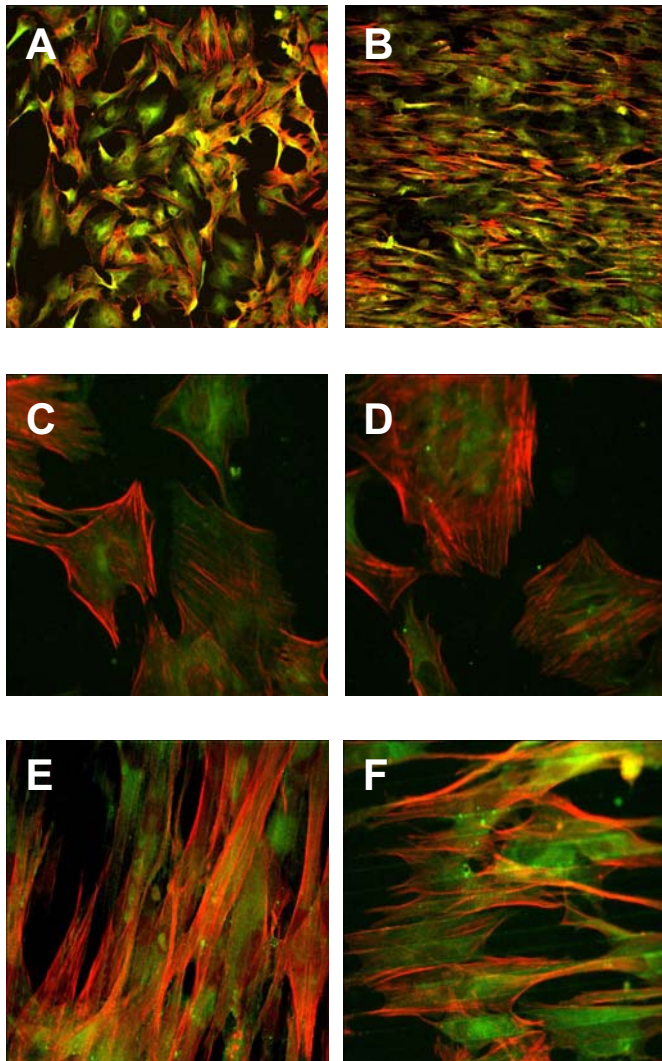


Figure 4. CLSM micrographs of RDFs cultured under several conditions. (A) Smooth substratum showing random orientated cells at normal gravity after 4 hours. (B) Aligned cells on a grooved substratum (5 μm wide) at 1g. (C) Cells cultured on planar substratum after 24 hours in RPM results in little changes in cell shape compared to a 24hours/1g sample (D). In contrast, on topographic substrata cells displayed decreased orientation towards the grooves under near weightlessness conditions; E shows cellular alignment after 4 hours in RPM, while F shows cell orientation after 24 hours. Colour figure on page 163.

Regarding topography, around 75% of the cells cultured on grooved substrata were considered aligned compared to 14% on smooth surfaces. The effect of gravitational loading, although small, is significant. Under simulated microgravity conditions the mean angle of cells cultured on grooved substrata increased, thus overall alignment decreased. This was shown particularly in the 4 hour time groups where alignment decreased from 84 to 78%.

Time played an important role in the cellular orientation: 24 hour groups always showed a significantly decreased alignment towards the grooves. Around 70% of cells were still aligned after 24 hours compared to 81% after 4 hours of culturing.

Comparable to the orientation of the RDFs, there was a significant difference in cell area between smooth and grooved substrata, but also within both groups. Quantified results for cell area are presented in **Figure 7B**. On grooved surfaces overall cell area was about 2480 μm^2 compared to 3700 μm^2 overall on smooth surfaces. In the smooth group the time parameter is of major influence on cell area. Cellular surface decreased over time from 4060 μm^2 to around 3250 μm^2 . An additional effect of simulated microgravity could not be determined in the smooth group. However, within the grooved group, after prolonged culturing, an additional effect was found in cell area: the increase to 2800 μm^2 was significant compared to the other grooved samples which averaged at 2350 μm^2 .

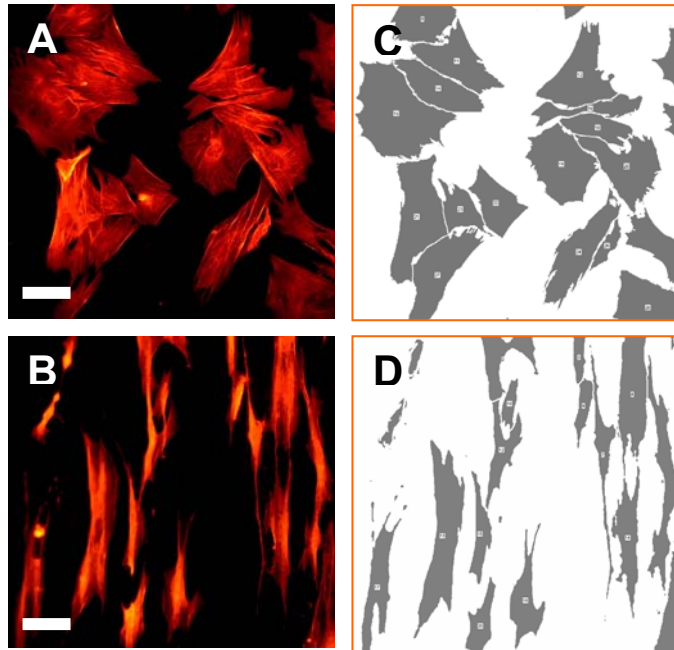


Figure 5. Fluorescence staining with Phalloidin-TRITC of the actin filaments of rat dermal fibroblasts cultured on a smooth (A) and 5 μm wide grooved (B) polystyrene substratum. C and D are examples of digitised threshold images as used for image analysis. Both samples: 24 hours, RPM. Magnification $\times 20$. Bar size = 10 μm .

RT-PCR

After each experimental run, samples were recovered and the mRNA expression for $\alpha 1$ -, and $\beta 1$ -integrins, fibronectin, and collagen type I of the fibroblasts was analysed. An example of the visualised samples, separated on agarose gel, is shown in **Figure 8**. The RT-PCR sample product ratios are listed in **Table 2**. Gene expression was influenced by both time and modelled gravity. All gene levels were reduced in time; 24 hours groups showed a down regulation of the genes of interest when compared to the 4 hours groups. Simulated microgravity additionally reduced the expression levels, particularly those of $\beta 1$ -integrin and fibronectin. While fibroblasts cultured on smooth substrata for 24 hours displayed a reduction of expression, the levels were higher compared with fibroblasts cultured for 24 hours on grooved substrata. However, under simulated microgravity conditions the reverse was seen; cells cultured on grooved substrata showed a higher expression level in comparison to the smooth control group.

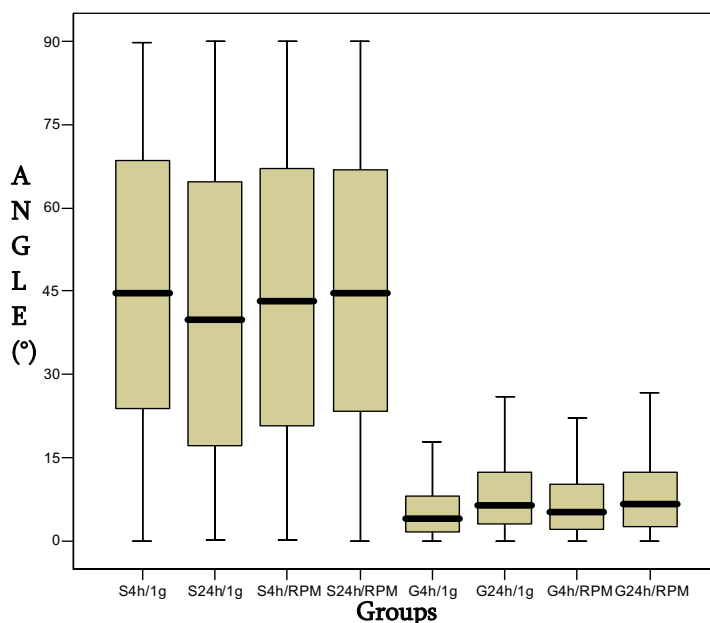


Figure 6. Box-whisker plot showing the distribution of cellular orientation. This graph shows the distribution midpoint, the first and third quartile (boxes), and the largest and smallest observation (whiskers). Of special note are the extreme differences in alignment between smooth and grooves surfaces. This led to investigate within both groups instead of between both groups. For each sample at least 850 individual cells were analysed. S = smooth, G = grooved (all groove widths combined), 4h and 24h stands for the experiment time, and 1g and RPM stands for the applied gravitational force.

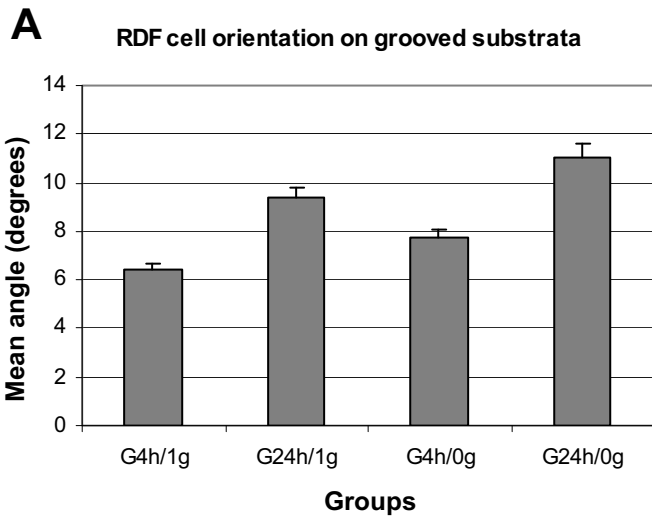


Figure 7. The mean angle and standard error of the mean for both orientation (A) and area (B) of fibroblasts subjected to various parameters. See Figure 6 for explanation of abbreviations.

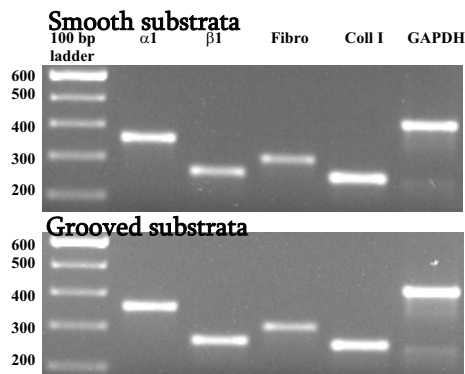
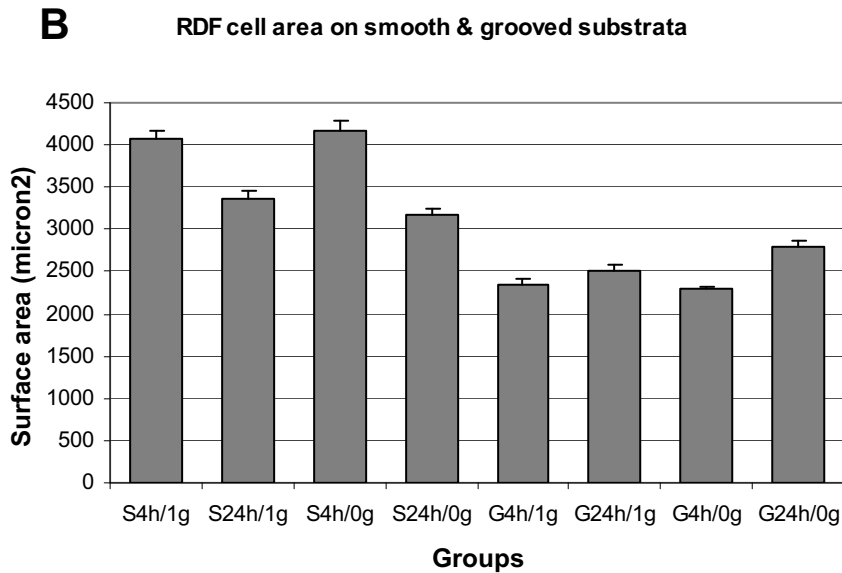


Figure 8. Example of representative PCR results of cells cultured on a smooth (top) and grooved (bottom) substratum subjected to 0g for 24 hours, visualised on agarose gel.

DISCUSSION

The aim of this study was to understand what is essential in determining morphological cell response to substrata and simulated microgravity. Fibroblasts cells were cultured on polystyrene substrata, both planar and topographic, which were mounted inside the RPM which simulated a microgravity environment. Besides studying the morphological cell response like shape and

orientation, the expression of several proteins involved in cell-surface interaction was also investigated.

From our data it can be concluded that RDFs adjust their shape according to micro-topographical features, a secondary, yet significant, role is played by simulated microgravity, particular in combination with prolonged culturing. Under simulated microgravity in the RPM, cells tend to rely more on the stresses encountered from the remaining environmental parameter, i.e. the substratum. It seems that the grooves enhance the cellular-substratum interface.

Regarding the used study design several publications on the use of the RPM or 3-axis clinostat have not only pointed out the usefulness of this research tool to evaluate whether organisms or cells may be sensitive to gravity changes, but also that behaviour of cells to simulated μg conditions on Earth are similar to those found in space flight [10; 24; 36]. Still, it has to be noted that magnitude in simulated microgravity is less pronounced than in true microgravity. The rotation velocity of 60 degrees per second as used in this RPM is recommended for in vitro cell samples. The short radial distance from the cells to both rotational axis and the adequate rotational speed makes it possible to compensate for any perception of gravity stimulus. However, another publication [37] has pointed out that a major characteristic of true microgravity is the absence of buoyancy-driven convective currents. Under microgravity conditions, liquid or gaseous domains which have different temperature or composition cannot rely on convective currents to equalise their density and composition. In contrast, they rely on diffusion, which is a very slow process. The rotation of the samples mounted onto the RPM generates internal motion, which increases the mixing of the sample environment comparable to the levels of normal gravity convection. Another point of concern is the interaction of gravity and thermal fluctuations. Both give rise to changes in cell component behaviour independently of each other. This interaction is difficult to calculate due to the flexibility of the cytoskeleton [27]. Other changes of cell function observed on the RPM might be induced by rotation through the Earth's magnetic field and induction of electric currents. Effects of this theoretically existing influence may make it necessary to shield off the RPM or samples. Stress associated with 3-axis clinorotation needs to be distinguished from effects of simulated microgravity. Still, by using static controls inside the RPM cabinet, we could ensure that the observed alterations were induced by weightlessness. Samples received identical treatment before the start of the experiment and were randomised for 3D clinostat or 1g control. Nevertheless, essential results obtained on the RPM would ideally have to be verified via space experiments, and vice versa, especially to obtain better statistics.

In a rotating set-up such as the RPM, air bubbles present within the closed environment of the cell culture can cause havoc on the cell viability via shear stress [38-40]. The simple, yet effective, solution was the use of a silicone closing cap for each individual well/insert. Combined with CO_2 independent culture medium this resulted in zero air bubbles before and after rotation. Although this design works well in short period experiments, lengthy culturing will require medium changing, and thus other hardware.

<i>Group</i>	<i>G4h1g</i>	<i>G24h1g</i>	<i>G4hRPM</i>	<i>G24hRPM</i>	<i>S4h1g</i>	<i>S24h1g</i>	<i>S4hRPM</i>	<i>S24hRPM</i>
Alpha-1	0,97	0,86	0,99	0,98	1,00	0,88	0,98	0,82
Beta-1	0,96	0,93	0,81	0,76	0,98	0,93	0,95	0,69
Fibro	1,00	0,90	0,98	0,81	0,99	0,80	0,99	0,68
Coll-1	n/a	0,72	n/a	0,83	n/a	0,86	n/a	0,64
GAPDH	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00

n/a: not available

Table 2: *RT-PCR semi-quantitative analysis. Shown are the ratios between the gene of interest and the housekeeping gene GAPDH. See Figure 6 for explanation of group abbreviations.*

In comparison with other studies, several comments can be made, particularly on the expression of certain genes of interest and their importance in cellular function in altered gravitational circumstances; Seitzer et al. [41] reported an increase in collagen synthesis by fibroblasts cultured on thermanox coverslips under real microgravity conditions, although after 20 hours of culturing this increase was reduced to 10% compared to control (1g) samples. Our data show that prolonged culturing will negate such amplifications. This is also seen by Saito et al. [16] who observed an decrease of collagen synthesis (both mRNA and protein) by osteoblast-like cells after 72 hours of simulated microgravity.

Arase et al [17], who compared gene expression levels in human fibroblasts with 3D-clinostat treatment, showed that gene levels of ErbB-2, a proto-oncogene that regulates cell proliferation, was down-regulated. Since several integrins, including β -1, are associated with ErbB-2 [42] and our results show a down-regulation of beta-1 integrin in a microgravity environment, it is therefore possible that altered expression levels of ErbB-2 may play a role in the 3D-clinostat dependent signaling cascade. This finding emphasizes the importance of changed integrin binding in (the absence of) mechanical stress.

As a final remark, we have to emphasize that it makes a considerable difference if the cellular machinery itself would be sensitive to gravity, than if cells simply react to a changed chemical environment due to cessation of gravity-induced convection flows. In the former, gravity would be a fundamental feature at least at some moment of cellular function, and microgravity an important instrument to study its mechanisms of operation. Aside from the immediate consequences for manned space exploration, new insights may be expected in the function and evolution of cell molecular systems. In the latter, novel discoveries could hardly be found and similar experimental results may well be obtained in Earth-based investigations. Therefore, the problem of whether or not effects found in real microgravity experiments are due to direct action on cells is an important question which the RPM may help solve [43].

CONCLUSIONS

Our hypothesis was that topographical cues determine cellular shape and orientation, even if simulated microgravity decreases cellular adherence. Regarding all study results, we conclude that our hypothesis holds. However, the effects of simulated microgravity in the long run will become more apparent on cellular function. RPM resulted in the reduced expression of proteins involved in the cell-surface interface and decreased cellular alignment. Research which includes intracellular signalling will be necessary to further understand the behaviour on the level of cell molecular machinery. We concluded from our data that fibroblasts primarily adjust their shape according to morphological environmental cues, like the substrata surface, thereby underscoring the importance of adhesion in cell behaviour. Simulated microgravity plays a secondary, but significant role.

REFERENCES

1. Walboomers, X. F., Croes, H. J., Ginsel, L. A., and Jansen, J. A. Growth behavior of fibroblasts on microgrooved polystyrene. *Biomaterials* 1998, 1861-1868, 1998.
2. den Braber, E. T., de Ruijter, J. E., Smits, H. T., Ginsel, L. A., von Recum, A. F., and Jansen, J. A. Quantitative analysis of cell proliferation and orientation on substrata with uniform parallel surface micro-grooves. *Biomaterials* 1996, 1093-9, 1996.
3. Clark, P., Connolly, P., Curtis, A. S., Dow, J. A., and Wilkinson, C. D. Cell guidance by ultrafine topography in vitro. *J Cell Sci* 1991, 73-7, 1991.
4. Ingber, D. E. Tensegrity I. Cell structure and hierarchical systems biology. *J Cell Sci* 2003, 1157-73, 2003.

5. Ingber, D. E. Integrins, tensegrity, and mechanotransduction. *Gravit Space Biol Bull* 1997, 49-55, 1997.
6. Curtis, A. and Wilkinson, C. New depths in cell behaviour: reactions of cells to nanotopography. *Biochem Soc Symp* 1999, 15-26, 1999.
7. Brunette, D. M. and Chehroudi, B. The effects of the surface topography of micromachined titanium substrata on cell behavior in vitro and in vivo. *J Biomech Eng* 1999, 49-57, 1999.
8. Oakley, C., Jaeger, N. A., and Brunette, D. M. Sensitivity of fibroblasts and their cytoskeletons to substratum topographies: topographic guidance and topographic compensation by micromachined grooves of different dimensions. *Exp Cell Res* 1997, 413-24, 1997.
9. Chou, L., Firth, J. D., Uitto, V. J., and Brunette, D. M. Substratum surface topography alters cell shape and regulates fibronectin mRNA level, mRNA stability, secretion and assembly in human fibroblasts. *J Cell Sci* 1995, 1563-73, 1995.
10. Uva, B. M., Masini, M. A., Sturla, M., Prato, P., Passalacqua, M., Giuliani, M., Tagliafierro, G., and Strollo, F. Clinorotation-induced weightlessness influences the cytoskeleton of glial cells in culture. *Brain Res* 2002, 132-9, 2002 .
11. Schwarzenberg, M., Pippia, P., Meloni, M. A., Cossu, G., Cogoli-Greuter, M., and Cogoli, A. Signal transduction in T lymphocytes--a comparison of the data from space, the free fall machine and the random positioning machine. *Adv Space Res* 1999, 793-800, 1999.
12. Cogoli, A. and Cogoli-Greuter, M. Activation and proliferation of lymphocytes and other mammalian cells in microgravity. *Adv Space Biol Med* 1997, 33-79, 1997.
13. Rijken, P. J., Boonstra, J., Verkleij, A. J., and de Laat, S. W. Effects of gravity on the cellular response to epidermal growth factor. *Adv Space Biol Med* 1994, 159-88, 1994.
14. Schatten, H., Lewis, M. L., and Chakrabarti, A. Spaceflight and clinorotation cause cytoskeleton and mitochondria changes and increases in apoptosis in cultured cells. *Acta Astronaut* 2001, 399-418, 2001.
15. Grigoryan, E. N., Anton, H. J., and Mitashov, V. I. Microgravity effects on neural retina regeneration in the newt. *Adv Space Res* 1998, 293-301, 1998.
16. Saito, M., Soshi, S., and Fujii, K. Effect of hyper- and microgravity on collagen post-translational controls of MC3T3-E1 osteoblasts. *J Bone Miner Res* 2003, 1695-1705, 2003.
17. Arase, Y., Nomura, J., Sugaya, S., Sugita, K., Kita, K., and Suzuki, N. Effects of 3-D clino-rotation on gene expression in human fibroblast cells. *Cell Biol Int* 2002, 225-33, 2002.
18. Boonstra, J. Growth factor-induced signal transduction in adherent mammalian cells is sensitive to gravity. *FASEB J* 1999, S35-42, 1999.
19. Wang, J. H., Yang, G., Li, Z., and Shen, W. Fibroblasts responses to cyclic mechanical stretching depend on cell orientation to the stretching direction. *J Biomech* 2003, 573-6, 2003.
20. Sciola, L., Cogoli-Greuter, M., Cogoli, A., Spano, A., and Pippia, P. Influence of microgravity on mitogen binding and cytoskeleton in Jurkat cells. *Adv Space Res* 1999, 801-5, 1999.
21. Loesberg, W. A., Walboomers, X. F., van Loon, J. J., and Jansen, J. A. The effect of combined cyclic mechanical stretching and microgrooved surface topography on the behavior of fibroblasts. *J Biomed Mater Res A* 2005, 723-732, 2005.
22. Searby, N. D., Steele, C. R., and Globus, R. K. Influence of increased mechanical loading by hypergravity on the microtubule cytoskeleton and prostaglandin E2 release in primary osteoblasts. *Am J Physiol Cell Physiol* 2005, C148-158, 2005.

23. They, M., Pepin, A., Dressaire, E., Chen, Y., and Bornens, M. Cell distribution of stress fibres in response to the geometry of the adhesive environment. *Cell Motil Cytoskeleton* 2006, 341-55, 2006.
24. Cogoli, M. The fast rotating clinostat: a history of its use in gravitational biology and a comparison of ground-based and flight experiment results. *ASGSB Bull* 1992, 59-67, 1992.
25. Kessler, J. O. The internal dynamics of slowly rotating biological systems. *ASGSB Bull* 1992, 11-21, 1992.
26. Kondrachuk, A. V. and Sirenko, S. P. The theoretical consideration of microgravity effects on a cell. *Adv Space Res* 1996, 165-8, 1996.
27. Briegleb, W. Some qualitative and quantitative aspects of the fast-rotating clinostat as a research tool. *ASGSB Bull* 1992, 23-30, 1992.
28. Sievers, A. and Hejnowicz, Z. How well does the clinostat mimic the effect of microgravity on plant cells and organs? *ASGSB Bull* 1992, 69-75, 1992.
29. Chesmel, K. D. and Black, J. Cellular responses to chemical and morphologic aspects of biomaterial surfaces. I. A novel in vitro model system. *J Biomed Mater Res* 1995, 1089-1099, 1995.
30. Freshney, R. I. Culture of animal cells: a multimedia guide. 99. Chichester, John Wiley & Sons Ltd.
31. Walboomers, X. F., Ginsel, L. A., and Jansen, J. A. Early spreading events of fibroblasts on microgrooved substrates. *J Biomed Mater Res* 2000, 529-534, 2000.
32. Hoson, T., Kamisaka, S., Masuda, Y., and Sievers, A. Changes in plant growth processes under microgravity conditions simulated by a three-dimensional clinostat. *Botanical Mag* 1992, 53-70, 1992.
33. Mesland, D. A. Novel ground-based facilities for research in the effects of weight. *ESA Microgravity News* 1996, 5-10, 1996.
34. Clark, P., Connolly, P., Curtis, A. S., Dow, J. A., and Wilkinson, C. D. Topographical control of cell behaviour: II. Multiple grooved substrata. *Development* 1990, 635-644, 1990.
35. Clark, P., Connolly, P., Curtis, A. S., Dow, J. A., and Wilkinson, C. D. Topographical control of cell behaviour. I. Simple step cues. *Development* 1987, 439-448, 1987.
36. Villa, A., Versari, S., Maier, J. A. M., and Bradamante, S. Cell behaviour in simulated microgravity: a comparison of results obtained with RWV and RPM. *Grav Space Biol* 2005, 89-90, 2005.
37. Albrecht-Buehler, G. The simulation of microgravity conditions on the ground. *ASGSB Bull* 1992, 3-10, 1992.
38. Hammond, T. G. and Hammond, J. M. Optimized suspension culture: the rotating-wall vessel. *Am J Physiol Renal Physiol* 2001, F12-25, 2001.
39. Cherry, R. S. and Hulle, C. T. Cell death in the thin films of bursting bubbles. *Biotechnol Prog* 1992, 11-8, 1992.
40. Kleis, S. J., Schreck, S., and Nerem, R. M. A viscous pump bioreactor. *Biotech Bioeng* 1990, 771-777, 1990.
41. Seitzer, U., Bodo, M., and Muller, P. K. Gravity effects on connective tissue biosynthesis by cultured mesenchymal cells. *Adv Space Res* 1995, 235-8, 1995.
42. Falcioni, R., Antonini, A., Nistico, P., Di Stefano, S., Crescenzi, M., Natali, P. G., and Sacchi, A. Alpha 6 beta 4 and alpha 6 beta 1 integrins associate with ErbB-2 in human carcinoma cell lines. *Exp Cell Res* 1997, 76-85, 1997.
43. Mesland, D. A. Possible actions of gravity on the cellular machinery. *Adv Space Res* 1992, 15-25, 1992.