The effect of combined hypergravity and microgrooved surface topography on the behaviour of fibroblasts

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INTRODUCTION

Cells cultured on textured substrata are very responsive to surface micro- and nanometre features [1-5]. For example, remodelling of the cytoskeleton [4; 6; 7], cell proliferation [6; 8; 9], gene expression and protein synthesis [10] have been shown to be affected by substratum surface geometrical conditions [11]. Also, it is known that surface topography can actively direct cell shape and spreading [10-17]. Evidently, cells are capable of sensing the structural shape of their environment, and determine their form and function appropriately.

How cell behaviour is guided by texture is well known, and it seems that the underlying principle is similar to the processes occurring when mechanical forces are applied to cells. Mechanotransduction of stationary (texture) and dynamic (mechanical strain) forces has led to research in a new topic: interaction between different forces. One of those forces that can be used to study this interaction is gravity. In a laboratory environment, it is very well possible to simulate conditions of altered gravity by using centrifuges for hypergravity and random rotation for microgravity. Although cells seem too small to experience gravity as a major force, cells may sense gravity-mediated changes in their environment [18-20]. Small changes might be integrated or amplified thus generating the various changes as seen under micro- or hyper-gravity conditions [21]. Much research has been conducted on lymphocytes and their behaviour in microgravity [22-26]. Marked differences were found in proliferation, apoptosis, production of cytokines, etc. In case of fibroblasts, mechanical stress is an important regulator of distinct cell shape and extracellular matrix component production [27-29]. Some more theoretical studies have argued against cells sensing gravity or changes therein [30; 31]. Nevertheless, a limited number of studies has been performed to examine cell shape changes under various gravity conditions [23; 32-36]. A change in cell shape measured under varying g levels contributes to the idea of direct effects of gravity onto cells.

Although each force affecting cell behaviour separately is relatively well studied *in vitro*, in the living organism always multiple forces will be present. The interaction between forces however remains poorly understood [37; 38]. Therefore, the two aforementioned parameters: topography and (hyper)gravity will be investigated in this study. Thus, this study evaluates *in vitro* the differences in morphological behaviour between fibroblast cells cultured on polystyrene substrata, both smooth and microgrooved, which are placed in a hypergravity environment. In addition, the up regulation of several proteins involved in cell-surface interaction will be investigated. The underlying aim is to discern which parameter is more important in determining final cell response. Our hypothesis is that cellular shape and orientation is determined by the topographical cues on the substrata and gravity increases the cellular attachment to these substrata. As controls fibroblast cells will be cultured on similar substrata which will remain at normal (Earth) gravity.

MATERIALS AND METHODS

Substrata:

Microtextured patterns were etched in a silicon wafer using lithographic and ion etching techniques as described by Walboomers *et al.* [1]. Ridge- and groove widths were 1, 2, 5, or 10 μm, with a uniform depth of 1.0 μm. Smooth substrata were prepared to serve as the control group. These silicon wafers were used as templates for the production of polystyrene (PS) substrata for cell culturing [39]. Replicas were equipped with 18mm diameter cylinders to obtain culture dishes (**Figure 1**). Before use a radio frequency glow-discharge (RFGD) treatment was applied for 5 min at a pressure of 2.0×10^{-2} mbar (Harrick Scientific Corp., Ossining, NY, USA) to improve wettability of the substrata [40-44].
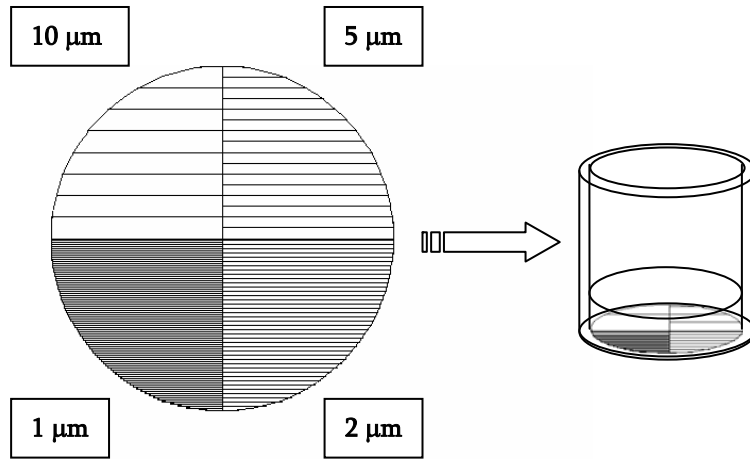
Figure 1. Graphical presentation of a top view of the template surface topography featuring the different groove types and the complete culture dish used in this study (height: 18 mm, width: 18 mm, surface: 269 mm², medium level during experimentation: 4.4 mm). For clarity the drawings are not to scale.

Cell culture:

Rat dermal fibroblasts (RDFs) were obtained from the ventral skin of male Wistar rats [45]. To ensure quick and constant availability, cells were cryo-preserved. Before experimentation, cells were thawed and cultured in α-Modified Eagles Medium (α-MEM) containing Earle's salts, L-glutamine, 10% foetal calf serum (FCS), and gentamicin (50μg/ml). Cells were cultured in a 5% CO_2 incubator set at 37°C in a humidified atmosphere. Experiments were performed with 6 - 8th culture generation cells. Onto all substrata, 1.5×10^4 cells/cm² were seeded. After pre-incubation of 1 h, the culture dishes were inserted into a 12-wells plate for support. The wells plate was placed inside aluminium culture boxes with a humidified atmosphere. These culture boxes in turn were hung into brackets enabling the boxes to tilt/swing so the hypergravity was perpendicular to the surface. Before running, the culture boxes were provided with an appropriate volume of CO_2 to ensure pH stability.

The medium size centrifuge for acceleration research (MidiCAR) was used in this study to simulate hypergravity (**Figure 2**). The centrifuge is housed inside a temperature controlled cabinet and contains a control sample compartment enabling the control samples to undergo the same circumstances, yet at 1 g (Earth) gravity. The culture dishes with a grooved substratum were placed at random inside the 12-wells plate. Previous pilot studies revealed no differences on a cellular level whether the grooves were placed horizontally or vertically with respect to the rotation axis. Centrifuge setting ranges were: 10, 25 or 50 g for two different time periods: 4 or 24 hours. [46-48]. Directly at the end of each time period, the RDF cell layers were washed three times with PBS, fixed, and prepared for further analysis.

Figure 2. The Medium Size Centrifuge for Acceleration Research (MidiCAR) used to simulate hypergravity, in which the samples were subjected up to 10, 25 or 50 times Earth's gravity. The MidiCAR is equipped with a computerised temperature and motor control. The control samples are placed in the upper (non-spinning) compartment of the centrifuge, while the other samples are placed in brackets in the mid and lower section.

Scanning electron microscopy:

To assess cellular morphology of the fibroblasts (n = 4 dishes), scanning electron microscopy (SEM) was used. Cells were washed, fixed for 5 minutes in 2% glutaraldehyde, rinsed for 5 minutes with 0.1 M sodium-cacodylate buffer (pH 7.4), dehydrated in a series of ethanol, and dried in tetramethylsilane. Specimens were sputter coated with gold and examined with a Jeol 6310 SEM.

Immunofluorescence

To observe the cytoskeleton, cells (n = 4 dishes) were 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 Inc., Eugene, OR, USA) diluted in PBS/ 1% Bovine Serum Albumin (BSA) to block non-specific epitopes. Vinculin was stained with mouse monoclonal primary antibodies to vinculin (Sigma, V-9131), followed by labelling with Alexa Fluor 488 goat anti-mouse secondary antibodies IgG. Finally, the specimens were examined with a Biorad MRC 1000 confocal laser scanning microscope (CLSM) system at magnification of 10x.

For quantitative image analysis samples (n = 4 dishes) were stained with Phalloidin-TRITC (Sigma, P-1951), followed by examination with a Leica/Leitz DM RBE Microscope at magnification of 10x.

Image Analysis

The digital immunofluorescence images acquired with the CLSM were loaded into Confocal Assistant (version 4.02) to create overlay images. The Phalloidin-TRITC fluorescence micrographs were analyzed with Scion Image software (Beta Version 4.0.2, Scion Corp., Frederick, MY, USA). The orientation of the cell on the surface was examined. For each sample four fields of view were selected randomly. Within each field two criteria were used for cell selection: (1) the cell is not in contact with other cells and (2) cell is not in contact with the field perimeter. Thereafter, on each cell within the field the following parameters were examined: first, the maximum cell diameter was measured as the longest distance between two edges within the cell borders. Second, the angle between this axis and the grooves (or an arbitrarily selected line for smooth surfaces). This latter measurement will be termed the orientation angle. Cell extensions like filopodia, which could confound the alignment measurement, were not included when assessing the cell orientation. Using Clarks criteria [13], cells oriented at 0–10 degrees from the groove direction were regarded to be aligned.

RT-PCR analysis

Total RNA was isolated from the RDFs with an isolation and stabilisation kit (QIAGEN, Hilden, Germany), and cDNA was synthesised from 1 mg of total RNA (n = 3 x 4 dishes). 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. The PCR products were separated on a 1.5% (w/v) agarose gel, and visualised by ethidium bromide staining. Semi-quantitative analysis of band intensity was performed 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, α1 integrin, β1 integrin gene expressions were normalised to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) values. Forward and reverse primer sequences are shown in **Table 1**.

	5' Primer (Forward)	3'Primer (Reverse)	Size (bp)
Collagen Type I	TGTTCGTGGTTCTCAGGGTAG	TTGTCGTAGCAGGGTTCTTTC	254
Fibronectin	CCTTAAGCCTTCTGCTCTGG	CGGCAAAAGAAAGCAGAACT	301
α1-Integrin	AGCTGGACATAGTCATCGTC	AGTTGTCATGCGATTCTCCG	374
β1-Integrin	AATGTTTCAGTGCAGAGCC	TTGGGATGATGTCGGGAC	262
GAPDH	CGATGCTGGCGCTGAGTAC	CGTTCAGCTCAGGGATGACC	470

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

Statistical analysis:

Acquired quantitative data were analysed using SAS for Windows (Release 9, SAS Institute Inc., Cary, North Carolina, USA). The effects of and the possible interaction between both time or gravity and surface were analysed using two-way analysis of variance (ANOVA) with Scheffé post-test. A probability (p) value less than or equal to 0.05 was considered significant.

RESULTS

Scanning Electron Microscopy

SEM illustrated the accurate reproduction of grooves and ridges in the polystyrene substrata (**Figure 3**). The accuracy of the casting process was apparent in the additional roughness on the ridges of the polystyrene substrata due to the etching process, which was used to fabricate the original silicon wafers. When analysing cell morphology (**Figure 4A & B**), SEM showed that on smooth substrata (control) cells were spread out in a random fashion, while RDF cells aligned along the groove direction on all grooved surfaces. Cells cultured on the substratum quadrants with the smaller grooves (1 and 2 micron, **Figure 4C**) seemed to be more clearly aligned than compared to wider grooved parts of the substrata (5 and 10 micron, **Figure 4D**). The same applied for cells cultured for the shorter time period (4 hours, **Figure 4E**) in comparison to the 24 hours culturing (**Figure 4F**). Although better alignment was observed on most 4 hours samples, some showed a similar alignment with the 24 hours samples. The cells cultured on widely spaced grooved substrata (>5μm) had a flat appearance, and were able to descend into the grooves, whereas the cells cultured on small grooved substratum were nearly always found on top of the ridges (**Figure 4G & H**). In the latter, cellular extensions probing the substratum surface only find the top ridge, resulting in extension of the cellular body along these small ridges.

The cytoskeleton was investigated by staining filamentous actin and vinculin anchor points of the cell focal adhesions. **Figure 5A** shows a 1g smooth substratum sample, the observed cell shape and spreading and random orientation was similar to SEM micrographs. Red staining is that of the actin filaments, which were running parallel and in the long axis of cells. Vinculin staining resulted in a considerable background around the nucleus. Vinculin spots were visible in some photos, positioned at the end of actin bundles, and 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 5B**). The overall view of cells cultured on smooth surfaces at increased g-load did not change notably (**Figure 5C**). This is in contrast to the cells cultured on grooved substrata: with increasing g force the alignment of the cells increased, reaching its optimum at 25g (**Figure 5B, D, E**). At 50 g the cells changed their shape and tended to spread out more, which was not observed for the smooth controls. Figure 5F reveals an abundant staining of vinculin in the micrographs, while actin filament staining in several cells was reduced.
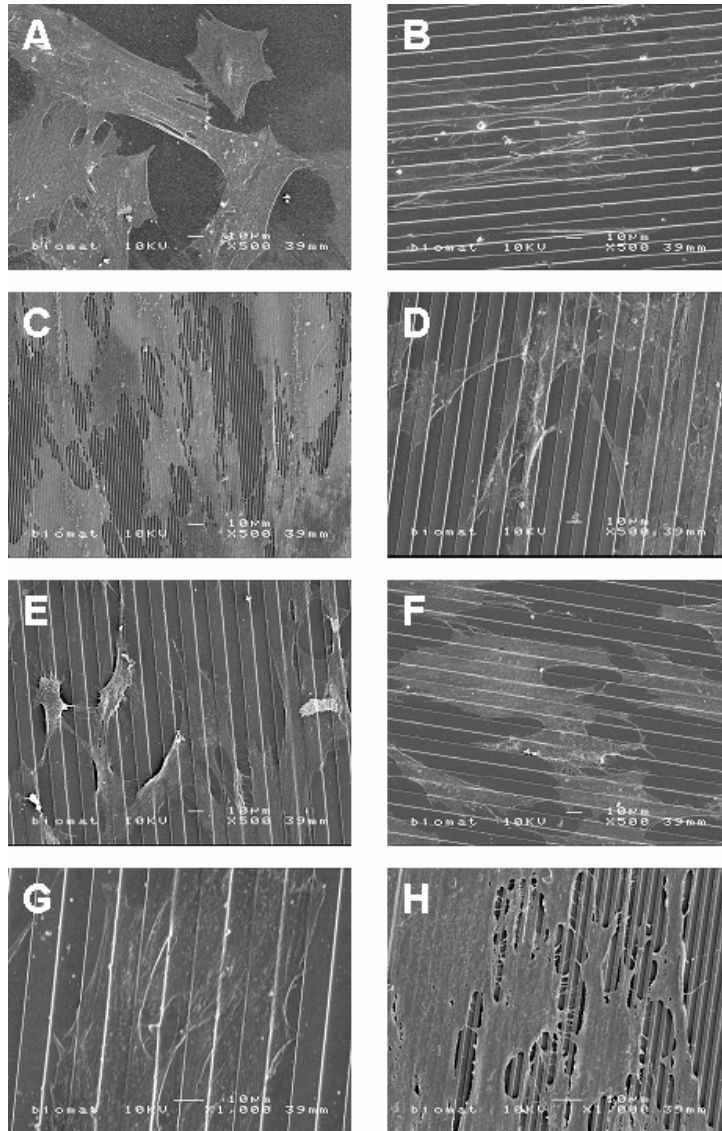
Figure 4. SEM micrographs of RDFs cultured under various conditions. (A) smooth substratum with cells in a random fashion, (B) 10 μm wide grooved substratum displaying aligned cells. Increased alignment of cells cultured on smaller grooves (C, 1 μm wide) in comparison to wide grooves (D, 10 μm wide). After 4 hours of centrifugation, cells display alignment (E, 10 μm wide), after 24 hours however the alignment becomes more apparent as can be seen in cell narrowness (F, 10 μm wide). Cells cultured on wide grooves (10 μm) are spread out in appearance (G) while on smaller grooves (2 μm) the cells can be found on top of the ridges (H). All micrographs were taken from 24 hrs samples, unless otherwise stated (magnification A till F = x500, G,H = x1000)).

Image Analysis

The actin filaments stained with phalloidin-TRITC were clearly visible in fluorescence microscopy. Image analysis confirmed the cellular orientation behaviour, as was seen with the SEM, *i.e.* while the RDFs cultured on grooved substrata in general showed alignment along the grooves; the smooth control samples did not induce any form of alignment. The quantified results for cell alignment are presented as box-whisker plots (**Figure 6**). Such a graph shows the distribution midpoint, the 25th and 75th percentile (boxes), and the largest and smallest observation (whiskers).

Because of the obvious significant differences between smooth and grooved surfaces, it was decided to analyse within both groups instead of between both groups. **Figure 7** depicts the mean angles of RDFs cultured on either smooth or grooved substrata divided over time. On smooth surfaces (**Figure 7A**) no significant change in alignment could be measured with increasing neither gravitational force nor time. The mean angle of cells cultured on grooved surfaces significantly decreased (thus alignment increased) with increasing g-force until it reached a turning point between 25 and 50g where it displayed an increase in mean angle value (**Figure 7B**). This pattern was shown consistently in both subgroups of 4 and 24 hours. The elevated mean angle in the 24 hours subgroups revealed a gravity dependency for this behaviour.
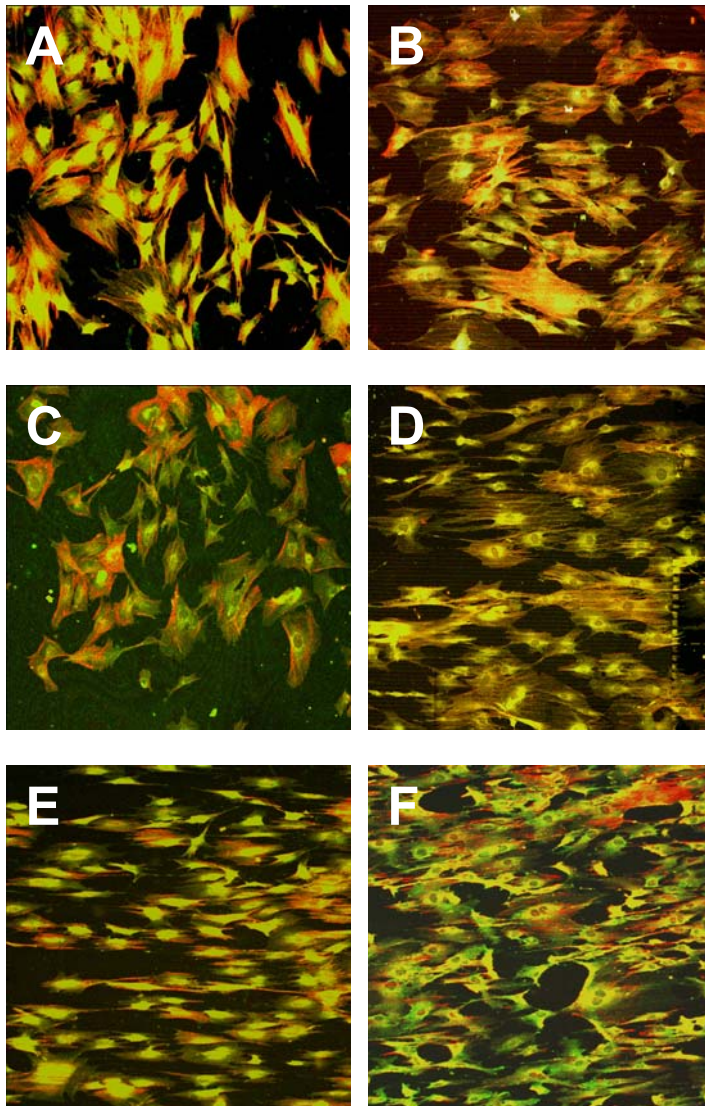
Figure 5. CLSM micrographs of RDFs cultured under several conditions. (A) Smooth substratum showing random orientated cells at normal gravity after 24 hours. (B) Aligned cells on a grooved substratum (10 μm wide) at 1 g. (C) 24 hours at 50 g on a smooth surface results in very little changes to alignment compared to A). Micrograph D is a 24hours/10g sample with 2 μm wide grooves while E is that of a 4hours/25g sample with 1 μm wide grooves, together with B the increased cell alignment with increasing gravitational force is evident, reaching its optimum at 25g. After 24 hours at 50g (F) cells alter their shape and display less alignment and cell surface area is wider (5 μm wide grooves). Colour figure on page 162.

Statistical analysis using an ANOVA was performed on the data, for all main parameters: topography, gravity force, and time. In this analysis, all parameters proved significant. Regarding topography a maximum of 89% of the cells were aligned along the grooved substrata compared to 12% of the smooth substrata.

The amount of gravitational force did influence cell alignment along the grooved topography. Although the effect of gravity from 1 to 10g was not significant, the increase from 10 to 25g and the decrease from 25 to 50g were significant.

As suggested from the graphs shown in **Figure 7**, statistics confirmed that there was no interaction between time and gravity, but over time gravity force became an increasingly important factor affecting cellular alignment. Finally, the effect of time on the alignment percentage was low, but significant. 24 hour samples showed a lower alignment rates compared to their 4 hour counterparts; on grooved substrata this was 63% and 76% respectively.

RT-PCR

The mRNA expression for $\alpha 1$-, and $\beta 1$-integrins, fibronectin, and collagen type I of the RDF cells was examined after each experimental run. An example of the visualised samples, separated on agarose gel, is shown in **Figure 8**, while all RT-PCR sample product ratios are listed in **Table 2**. With respect to all samples, gene expression levels were influenced by both time and gravity.
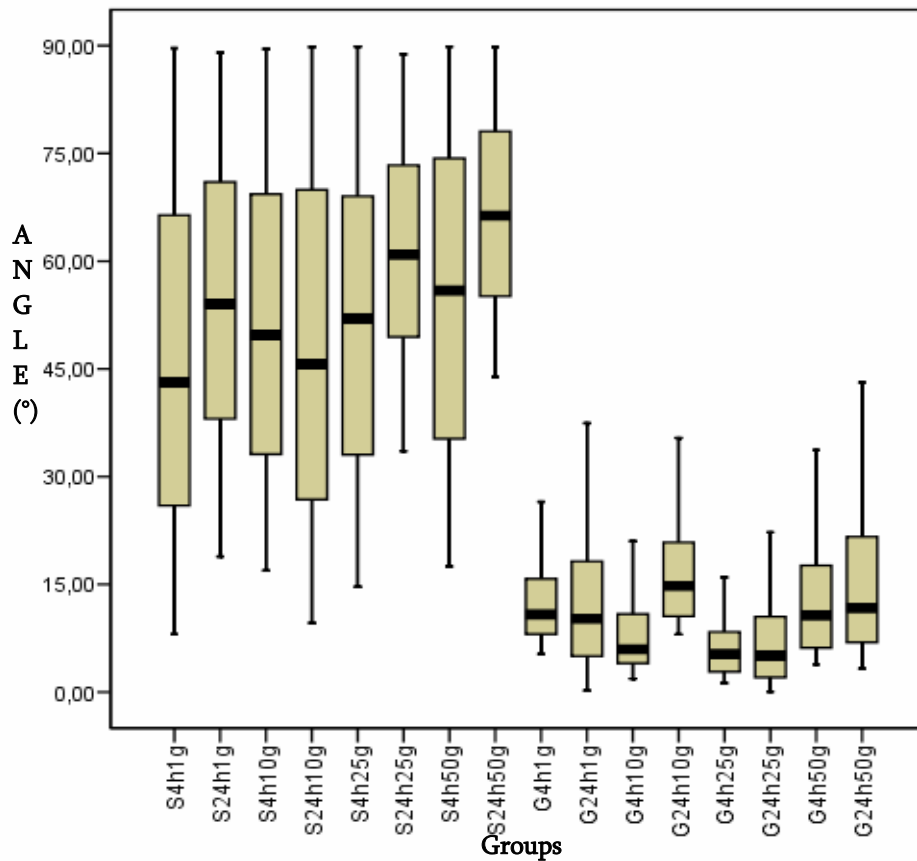
Figure 6. Box-whisker plot showing the distribution of cellular orientation. This graph shows the distribution midpoint, the first and third quartile (boxes), and the largest and smallest observation (whiskers).Of special note are the extreme differences in alignment between smooth and grooved surfaces. This led to investigate within both groups instead of between both groups. For each sample at least 200 individual cells were analysed. S = smooth, G = grooved (all groove widths combined), 4h and 24h stands for the experiment time, and 1, 10, 25 and 50 stands for the applied g-force.
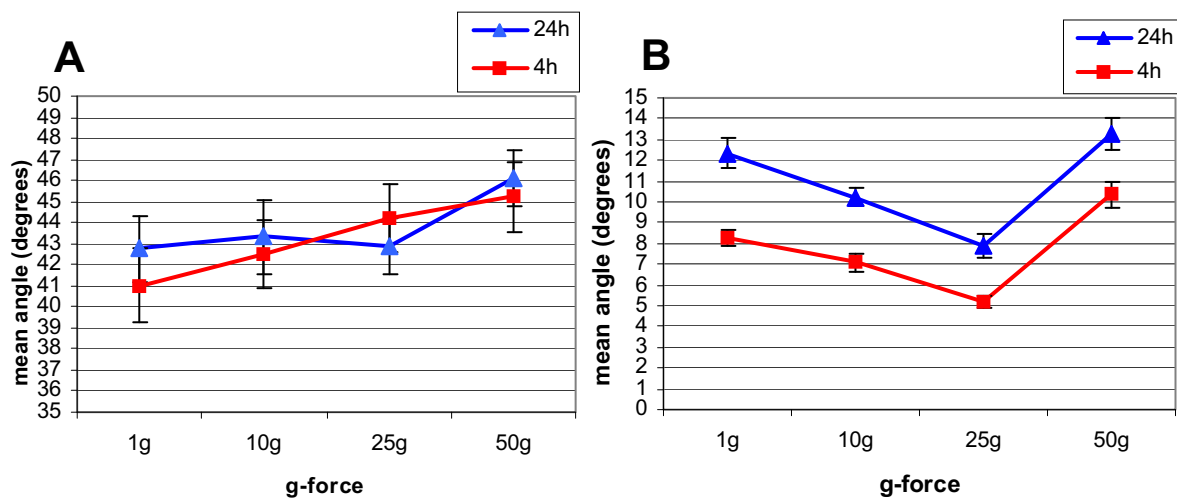

Figure 7. The mean angle and standard error of the mean for both smooth (A) and grooved (B) surfaces divided over time.

Most gene levels were reduced in time as can be seen in the 1g control group; in addition, hypergravity reduced the expression levels even further. The smooth control samples showed that most product values were equal to the housekeeping gene, except the β1-integrin subunit in samples experiencing either 25 or 50 g. The α-1 integrin depression in the 4h/10g group was notable, as it suggested adaptation to changes in environment; however, this was not reflected in its smooth control group. Both collagen type 1 and fibronectin were seemingly unaffected by time or force. Whenever there are reductions of collagen synthesis, there was also substantial decrease of β-1 integrin up-regulation.
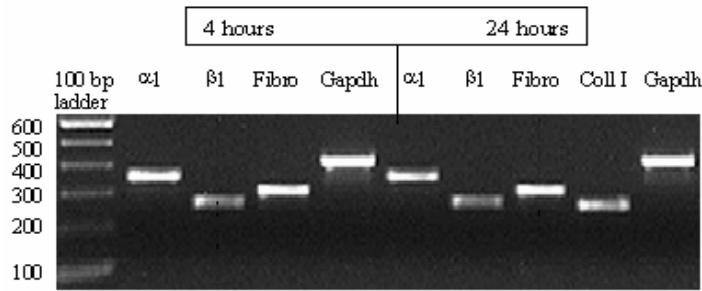

Figure 8. Example of PCR results of cells cultured on a grooved substratum undergoing 25g, visualised on agarose gel.

Group	G4h1g	G24h1g	G4h10g	G24h10g	G4h25g	G24h25g	G4h50g	G24h50g
alpha1	1,00	0,94	0,74	1,00	1,00	0,96	1,00	0,96
beta1	0,96	0,88	0,96	0,89	0,82	0,73	0,84	0,80
fibro	1,00	0,99	1,27	1,00	1,00	0,99	1,00	0,99
coll type 1	n/a	0,96	n/a	0,99	n/a	0,96	n/a	0,99
GAPDH	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00

Group	S4h1g	S24h1g	S4h10g	S24h10g	S4h25g	S24h25g	S4h50g	S24h50g
alpha1	0,97	1,00	0,97	1,01	1,00	1,00	1,00	1,00
beta1	0,76	0,67	0,72	0,50	1,00	0,94	0,99	0,98
fibro	1,01	1,00	0,99	0,98	1,00	1,00	1,00	1,00
coll type 1	n/a	0,84	n/a	0,86	n/a	1,00	n/a	1,00
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 semiquantitative analysis. Shown are the ratios between the gene of interest and the housekeeping gene GAPDH. See Figure 6 for explanation of group abbreviations.

DISCUSSION

This study evaluated *in vitro* the differences in cellular behaviour between fibroblast cells cultured on smooth and microgrooved substrata, which experience artificial hypergravity by centrifugation. From our data it can be concluded that the fibroblasts primarily adjust their shape according to substratum surface (topographical) features. Nevertheless, also apparent effects of the applied hypergravity conditions were measured.

When regarding our study set up, several remarks can be made. Of course, as in any in vitro model, the number of experimental conditions that could be applied is limited. In a real hypergravity environment such as would be present during space flight, cells would experience simultaneous effects of temperature changes, vibration, irradiation, angle between the cell and the direction of acceleration, pH changes, etc. These parameters are all interrelated but too complicated to investigate within one study set-up. Also, in our model possibly enhanced gas solubility in the medium during the centrifugation could arise due to the pressure in the

centrifuge system, and can have led to biochemical effects that are thus not direct, but secondary effects of gravity acting on the cells.

The dimensions of the groove features were based on earlier studies [1; 6; 7; 40]. Scanning electron microscopy, immunofluorescence staining, and subsequent image analysis confirmed that fibroblasts were oriented on the microgrooved surfaces. It was seen that cells on the 10 and 5 micron wide grooved parts were able to reach the bottom of the 1μm deep grooves, whereas on the parts with 2 and 1 micron wide grooves the cells lost contact with the bottom of the grooves. The mechanism behind cell orientation has been postulated in literature [1; 4; 46; 49]. Cell orientation on (microtextured) surfaces starts with the formation of multiple membrane extensions. The cell is probing and exploring the surface in order to adhere and come to a mechanical equilibrium with its environment. Since the immediate surroundings is lacking natural extracellular matrix (ECM) and the relatively low seeding density results in isolated cells, the aforementioned membrane extensions are formed in all directions. Once appropriate attachment sites are found the cell will deposit ECM material (*e.g.* collagen). The cell changes its spherical appearance, spreads on the surface, and active filaments form longitudinal stress fibres [50]. In this study, it was evident that cellular orientation indeed occurred immediately during the cell spreading, and always parallel to the grooves.

Cell movement or orientation as reaction to mechanical stresses, like the hypergravity applied here, might be based on a comparable mechanisms. However, in contrast to the responses to microtextures as described above, the mechanical force (hypergravity) was introduced after the cell has attached and spread out over the surface, and after the cells had most likely established an inter-connective system of ECM, integrins, and cytoskeleton. The subsequent effects of gravity might be primary or secondary: The primary effects of gravity are those which directly affect the inside of the cell and internal components of the cell, while secondary effects are effects of the environment onto cells. These latter effects are the physical phenomena, which appear in an altered gravity situation, where hydrostatic pressure derived from the liquid column on the surface of the culture is linearly proportional to gravity force. In addition the acceleration from a centrifuge increases with increasing distance from the centre of rotation, thus creating a gravity gradient. Regardless of these differences, centrifugation provides an excellent simulated gravity environment for cell biology research as long as the centrifuge (MidiCAR used in this study) is of sufficient diameter in relation to the sample studied.

The SEM and CLSM results, confirmed that on smooth substrata no notable differences were seen on cell shape. The microtextures elicited the main effect on cell behaviour. The possible effects of gravity were less evident but still present, as increased gravity did affect cell shape. The CLSM overview micrographs showed considerable background staining around the nucleus, a phenomenon known to others as well [1; 51]. The magnification was chosen at 10x so that cell alignment measurements were possible over a large number of cells (at least 200 measurements per group). Actin filaments followed cellular alignment and focal adhesions, as shown by vinculin staining, were always located at the end of the actin filament.

The image analysis proved that substrata with microgrooves elicit cell alignment. Because of the obvious differences between smooth and grooved surfaces, study groups were split up and the grooved surfaces were investigated more closely. Concerning alignment there was no significant difference between 1 and 10g. However, gravitational force above 10g significantly enhances cellular orientation and this effect increased when cells are subjected to prolonged exposure of gravity independent of force magnitude. Since cell-cell contact was scarce, which might disrupt cellular orientation along the grooves, the described differences could indeed be assigned to prolonged exposure to gravity. This points to that, although actin cytoskeleton is closely involved in the orientation process, when the orientation is completed the cells are no longer dependent

upon that system to maintain their position. However, hypergravity is still present and exerts a continuous influence making the cytoskeleton respond and provide the cell with the means to maintain equilibrium with its environment. Such an assumption could be further tested in experiments with cytoskeleton inhibitors such as cytochalasin.

For our RT-PCR the production of components vital for cellular attachment was evaluated. Differences in gene expression of fibronectin on grooved versus smooth surfaces have previously been published by Chou et al. [52]. Our results indicated that cells under hypergravitational stimulation showed decreased β1-integrin and collagen-I levels. Generally speaking a lower up-regulation of these components led to a situation of decreased orientation. Combining the statistical analysis of the effects of gravity and the semi-quantitative results of our genes of interest we concluded that over time gravity becomes more apparent resulting in down regulation of certain proteins. Nonetheless, at higher g forces, the 25 g sample groups display the most profound orientation, yet with lowered values of β1-integrin. We could speculate that fibroblasts could rely on another anchor protein member of the β-integrin family in order to help them align, although many other explanations could be given. With increasing gravity (50g) this other pathway also becomes affected. Similar adaptive behaviour of integrin expression has also been described by others [53]. The statistical analysis performed on the fluorescence micrographs revealed two turning points signifying that anchor proteins are up- or down-regulated at different g-forces. The decreased alignment in the 1 g sample groups might be explained by the fact that these cells are only experiencing environmental cues in the shape of microtexture without the additional effect of altered gravity. The increase of β-1 in the 4 hours sample group might be correlated to the enhanced alignment in comparison with 24 hours sample groups.

CONCLUSIONS

Like all living biological systems, a cell needs both energy and information in order to function. Information regarding the mechanisms responsible for cell sensitivity to gravity is gradually being acquired. There is still a large discrepancy between the empirical use of mechanical forces in orthopaedics, dentistry and plastic surgery, and the theoretical understanding of the cellular and molecular mechanisms involved. Progress has been made in discovering the conversion of mechanical into chemical signals, triggering of intracellular signalling pathways, and the mechanisms of mechanically induced gene activation. However, the specificity of cellular responses to externally applied mechanical stress and the interaction between various environmental cues requires more in depth research. From our study, we conclude that the fibroblasts primarily adjust their shape according to morphological environmental cues like substratum surface whilst a secondary, but significant, role is played by hypergravity forces.

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