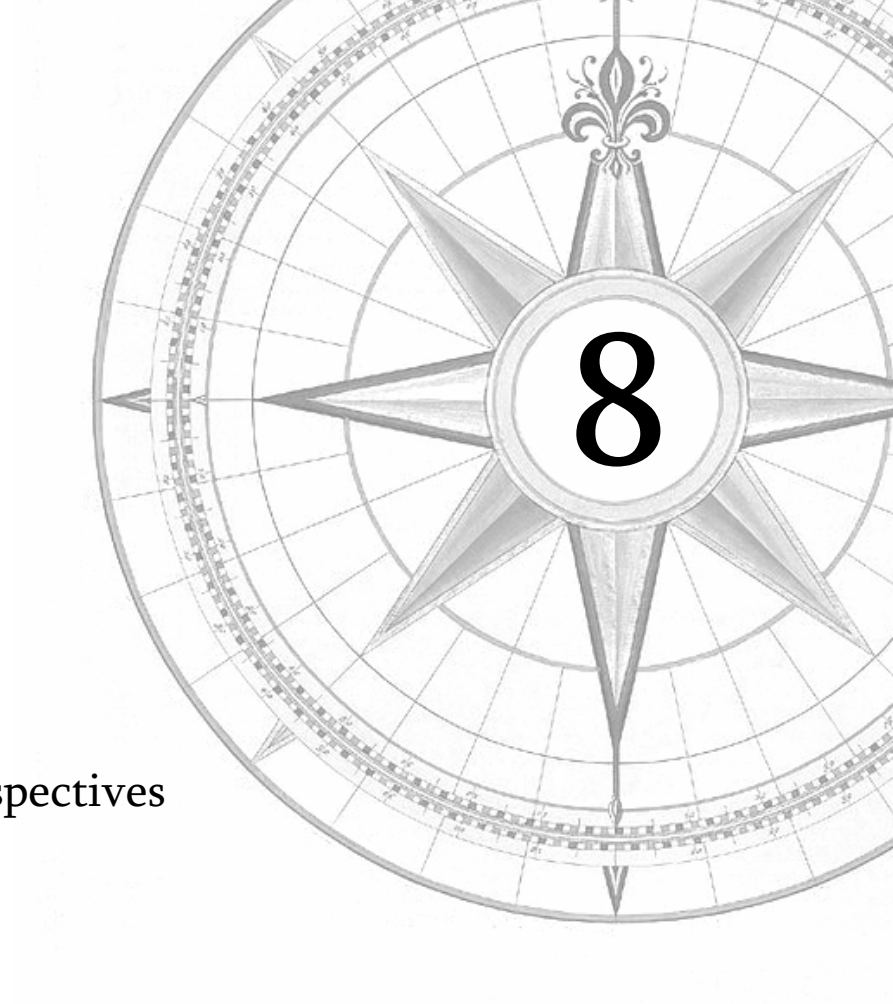

8

Summary and future perspectives

SUMMARY

For over a century, millions of hours of research have been spent on solving the fundamental engineering problems of escaping Earth's gravity well and developing systems for in-space propulsion. Even now, the research and development put into the mechanics of space voyaging is substantial, owing to man's wish to revisit our Moon and make landfall on Mars. The issue of how humans will actually survive and work in space for long periods of time requires input from the whole gamut of physical and biological sciences and has become a great challenge to deep space exploration. A fundamental step in meeting this challenge is: understanding the effects of altered gravity circumstances on cellular organisms. These effects are the subject of this thesis.
Biology (cells), material (microgrooves), and force (gravity) are the interacting aspects of this research, which in the larger playfield of tissue engineering, affect the behaviour and longevity of artificial constructs in living tissue. **Chapter 1** describes broadly our current knowledge of cell biology and cellular responses towards both stationary and dynamic forces. It touches on the fabrication of a structured topography and the various physical phenomena encountered in a changing gravity environment.

FIBROBLAST BEHAVIOUR TOWARDS COMBINED STRETCHING AND MICROGROOVES

The initial research was focused on obtaining a general view of cell responses towards microgrooved surface topography and mechanical loading. **Chapter 2** describes the influence of unilateral cyclic stretch and the resulted tendency of cultured fibroblasts to orient perpendicular to the stress direction. Similar cell alignment can be induced by guiding cells along topographical clues, like microgrooves. The aim of this study was to evaluate cell behaviour on micro-grooved substrates, exposed to cyclic stretching. We hypothesized that cellular shape is mainly determined by topographical clues. On basis of earlier studies, a 10 μm wide square-groove and a 40 μm wide V-shaped groove pattern were used. Smooth substrates served as controls. Onto all substrates, fibroblasts were cultured and 1-Hz cyclic stretching was applied (0, 4, or 8%) for 3-24h. Cells were prepared for scanning electron microscopy, immuno-staining of filamentous actin, alignment measurements, and PCR (collagen-I, fibronectin, α1- and β1-integrins). Results showed that cells aligned on all grooved surfaces, and fluorescence microscopy showed similar orientation of intracellular actin filaments. After 3 hours of stretch, cellular orientation started and after 24 hours the cells had aligned themselves almost entirely. Image analysis showed better orientation with increasing groove depth. Statistical testing proved that the parameters groove type, groove orientation, and time all were significant, but the variation of stretch force was not. Substrates with microgrooves perpendicular to the stretch direction elicit a better cell alignment. The expression of β1-integrin and collagen-I was higher in the stretched samples. In conclusion, we can maintain our hypothesis, as microgrooved topography was most effective in applying strains relative to the long axis of the cell, and only secondary effects of stretch force were present.

FIBROBLAST RESPONSES TO COMBINED HYPERGRAVITY AND MICROGROOVES

Mechanical stress is an important regulator of cell shape and extracellular matrix component production. The notion that changes in cell shape under varying gravity levels contributes to the idea of direct effects of gravity onto cells has led to the study as described in **chapter 3**. This study reports the differences in morphological behaviour between fibroblast cultured on smooth and microgrooved substrata (groove depth: 1 μm, width: 1, 2, 5, 10 μm), which undergo artificial hypergravity by centrifugation (10, 25, and 50 g; or 1g control). The aim of the study was to clarify which of these parameters is more important to determine cell behaviour. Morphological characteristics were investigated using scanning electron microscopy and fluorescence microscopy

in order to obtain qualitative information on cell spreading and alignment. Confocal laser scanning microscopy visualised distribution of actin filaments and vinculin anchoring points through immuno-staining. Finally, expression of collagen type I, fibronectin, and α1- and β1-integrin were investigated by PCR. Microscopy and image analysis showed that the fibroblasts aligned along the groove direction on all textured surfaces. On the smooth substrata (control) cells spread out in a random fashion. The alignment of cells cultured on grooved surfaces increased with higher g-forces until a peak value at 25g. An ANOVA was performed on the data, for all main parameters: topography, gravity force, and time. In this analysis, all parameters proved significant. In addition, most gene levels were reduced by hypergravity. Still, collagen type 1 and fibronectin are seemingly unaffected by time or force. From our data it is concluded 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.

REACTIONS OF FIBROBLASTS TO COMBINED MICROGRAVITY AND MICROGROOVES

Although mechanical stress is important, cells can also experience mechanical unloading by removing the most constant force in nature, namely gravity. **Chapter 4** is about the first of two studies which focused at the effects of simulated microgravity on cell behaviour by describing *in vitro* the differences in morphological behaviour between fibroblast cultured on smooth and microgrooved substrata (groove depth: 0.5 μm, width: 1, 2, 5, 10 μm), that were subjected to simulated microgravity. The aim of the study was to clarify which of these parameters is more dominant to determine cell behaviour. Morphological characteristics were investigated using scanning electron microscopy and fluorescence microscopy in order to obtain qualitative information on cell alignment and area. Confocal laser scanning microscopy visualised distribution of actin filaments and focal adhesion points. Finally, expression of collagen type I, fibronectin, and α1- and β1-integrin were investigated by PCR. Microscopy and image analysis showed that the fibroblasts aligned along the groove direction on all textured surfaces. On the smooth substrata, cells had spread out in a random fashion. The alignment of cells cultured on grooved surfaces decreased under simulated microgravity, especially after 24 hours of culturing. Cell surface areas on grooved substrata were significantly smaller than on smooth substrata but simulated microgravity on the grooved groups resulted in an enlargement of cell area. ANOVA was performed on all main parameters: topography, gravity force, and time. In this analysis, all parameters proved significant. In addition, gene levels were reduced by microgravity particularly those of β1-integrin and fibronectin. From our data it is concluded that the fibroblasts primarily adjust their shape according to morphological environmental cues like substratum surface, whilst a secondary but significant role is played by microgravity conditions.

ACTIVATION OF MAPK PATHWAYS IN FIBROBLAST DURING MICROGRAVITY

Chapter 5 expands the knowledge obtained from the previous study by looking into the intracellular molecular mechanics which mediates the extracellular cell response. Mechanotransduction is the key element behind this study which considers *in vitro* the differences in morphological behaviour between fibroblast cultured on smooth and microgrooved substrata (groove depth: 0.5 μm, width: 1 μm), which were subjected to simulated microgravity. The aim of the study was to clarify which of these parameters was more dominant to determine cell behaviour. Morphological characteristics were investigated using scanning electron microscopy and fluorescence microscopy in order to obtain qualitative information on cell alignment. Confocal laser scanning microscopy visualised distribution of actin filaments and focal adhesion points. Expression of collagen type I, and α1-, β1, β3-integrin were investigated by QPCR. Finally, immunoblotting was applied to visualise MAPK signalling pathways. Microscopy

and image analysis showed that the fibroblasts aligned along the groove direction on all textured surfaces. On the smooth substrata, cells had spread out in a random fashion. The alignment of cells cultured on grooved surfaces under simulated microgravity, after 48 hours of culturing appeared similar to those cultured at 1g, although cell shape was different. ANOVA was performed on all main parameters: topography, gravity force, and time. In this analysis, all parameters showed significant differences. In addition, gene levels were reduced by microgravity particularly those of β3-integrin and collagen, however alpha-1 and beta-1 integrin levels were up-regulated. ERK1/2 was reduced in zero g, however, JNK/SAPK and p38 remained active. The members of the small GTPases family were stimulated under microgravity, particularly RhoA and Cdc42.
The results are in agreement with the hypothesis that the application of microgravity promotes fibroblasts to change their morphological appearance and their expression of cell-substratum proteins through the MAPK intracellular signalling pathways. We conclude that fibroblasts cultured on a grooved pattern adjust their shape accordingly and that this model could be employed as useful model to unravel the effects of gravity perception and cellular response.

MECHANOSENSITIVITY OF FIBROBLASTS TOWARDS INERTIAL SHEAR

To date, inertial shear force, which is inherent in centrifugation set-ups, is described as a theoretical model. From this model it became apparent that inertial shear force results in artefacts which influence the outcome of control samples being centrifuged at 1 x gravity on-board a spacecraft. In **chapter 6** we undertook a study to identify *in vitro* the magnitude of inertial shear forces on fibroblast cells cultured on polystyrene substrata, both smooth and equipped with a surface micro topography (groove depth: 0.25 μm, width: 1 μm). The cells were placed in a centrifuge environment. Samples were subjected to 0, 1.5, and 3.0 Pascal for 0.5, 2, and 4 hours. To ascertain the impact of inertial shear force we looked at both a morphological (scanning electron and fluorescence microscopy) and molecular level (mRNA transcription and Western blotting). The underlying aim was to understand to what extent shear forces can alter cell behaviour and which parameter is more important in determining this cell response. Microscopy and image analysis showed that the fibroblasts aligned along the groove direction on all textured surfaces. On the smooth substrata, cells had spread out in a random fashion. After 4 hours of culturing the alignment of cells cultured on grooved surfaces under inertial shear force appeared dissimilar to those cultured at 0.0 Pascal. Actin filament numbers and thickness were decreased, and talin anchor-points were reduced. ANOVA was performed on the three main parameters: topography, shear force, and time. In this analysis, all parameters proved significant. Furthermore, gene levels were reduced by shear force, particularly those of beta-3-integrin and alpha-1. However, collagen and beta-1 integrin levels were up-regulated. ERK1/2 was present and JNK/SAPK and p38 were increased during inertial shear. The members of the small GTPases family were stimulated under shear force, particularly RhoA and Cdc42.
In addition to the gravity acceleration component of a simulated microgravity study in spaceflight facility, inertial shear force is a force to be taken into account, because of its substantial impact on adherent cell colonies. Inertial shear force artefacts should be considered for interpretation of spaceflight and ground-based data.

NANO-TOPOGRAPHY AND THE ORIENTATION LIMITS OF FIBROBLASTS

One of the main parameters in all of the previous chapters is topography. A structured, groove/ridge surface of known dimensions, of which we know what sort of cell response it can elicit. Since these surfaces mimic the natural surroundings these patterns have been extensively studied. However, collagen fibres, which are the natural surroundings of dermal fibroblast, occur in a smaller range (nano scale) than we currently simulated with our substrates (micro scale). Due

to technical limitations and knowing that collagen fibres usually aggregate into much bigger structures, we resorted to microgrooves up until now. Through cooperation with Philips Research, we have been able to study the differences in morphological behaviour between fibroblasts cultured on smooth and genuine nano-grooved substrata (groove depth: 5 – 350 nm, width: 20 – 1000 nm) *in vitro*. The aim of the study in **chapter 7** was to clarify to what extent cell guidance occurs on increasingly smaller topographies. Pattern templates were made using electron beam lithography, and were subsequently replicated in polystyrene cell culture material using solvent casting. The replicates were investigated with atomic force microscopy (AFM). After seeding with fibroblasts, morphological characteristics were investigated using scanning electron microscopy (SEM) and light microscopy, in order to obtain qualitative and quantitative information on cell alignment. AFM revealed that nanogroove/ridge widths were replicated perfectly, although at deeper levels the grooves became more concave. The smooth substrata had no distinguishable pattern other than a roughness amplitude of 1 nm. Interestingly, microscopy and image analysis showed that after 4 hours fibroblast had adjusted their shape according to nano-topographical features down to cut-off values of 100 nm width and 75 nm depth. After 24 hours culturing time, some fibroblasts even aligned themselves on groove depths as shallow as 35 nm. It appears depth is the most essential parameter in cellular alignment on groove patterns with a pitch ratio of 1:1. On the smooth substrata, cells always spread out in a random fashion. Analysis of variance (ANOVA) demonstrated that both main parameters, topography and culturing time, were significant. We conclude that fibroblast cells cultured on nanotopography experience a threshold feature size of 35 nm; below this value contact guidance does no longer exist.

CLOSING REMARKS AND FUTURE PERSPECTIVES

In this thesis the interaction between static and dynamic factors was investigated from a cell mechanotransduction point of view. By mimicking a fibrillar appearance through application of surface topography onto a biomaterial and subjecting the cells to a stressful environment we were able to obtain insight on the mechano- and gravi-sensing capabilities of primary fibroblast cells.

Ground-based (Earth) gravitational cell biology is a field or research with a bright future: human's curiosity and desire to understand the natural world, from the depths of the oceans to the far reaches of space, joint with the relatively low cost and ease of availability of experimental hardware, will foster further studies. The results described in this thesis and the works of others in this field, have shown that the outcomes of ground-based research are similar to those found in "true" weightlessness conditions.

The micron scale grooved pattern used in the earlier chapters was rather –and perhaps too much– the dominant factor. Therefore future research should expand the knowledge currently obtained by combining cells cultured on a nano-topography and subjecting them to mechanical (un)loading in a variety of ways. This most likely will result in an enhanced interaction between the various parameters. Analysis of cell feedback should be broad: ranging from visualisation of morphological changes of the cell shape by means of microscopy to quantifiable date achieved through molecular analysis methods such as Q-PCR and particularly SDS-PAGE and Western blotting. Monitoring the cell in real-time will not only benefit our understanding of cell behaviour but also will stimulate the development of high-tech experimental hardware. Linking the processes inside the cell to the cells outside appearance, may illuminate the mechanisms by which cells respond to environmental stimuli. This fundamental knowledge could be also beneficial in the construction and application of biomaterials used for repair and regeneration of novel, functional tissues.