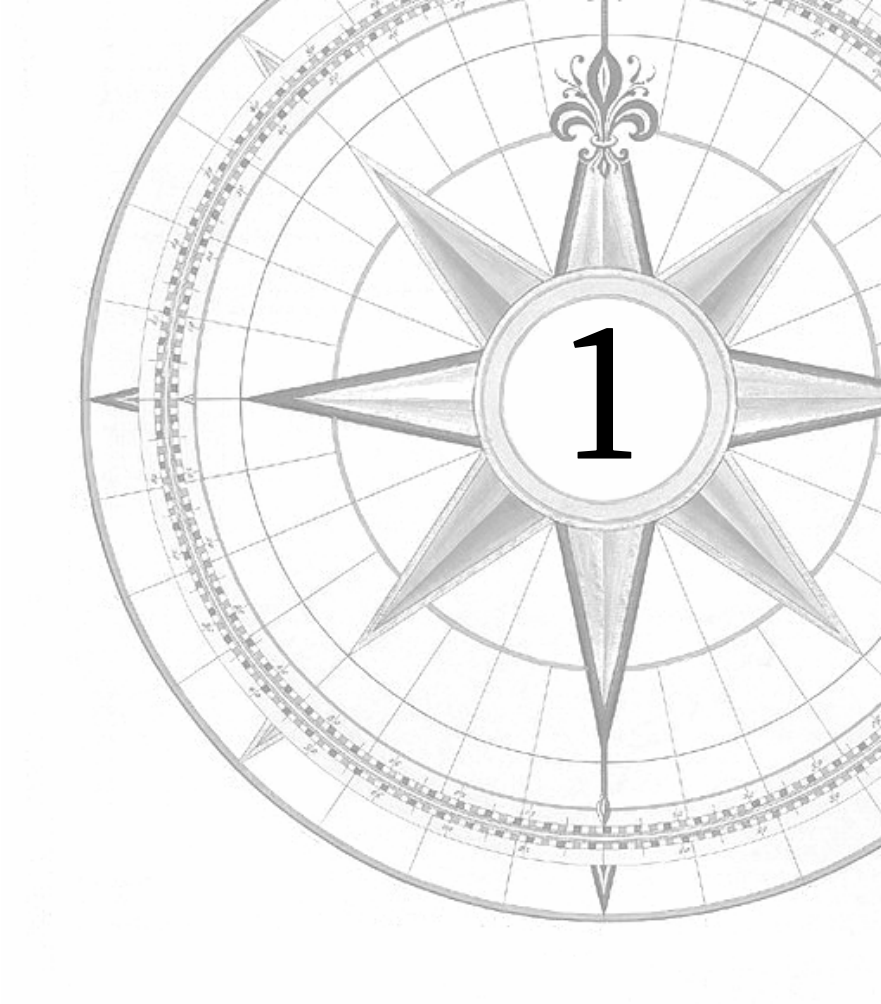

General introduction

INTRODUCTION

The biological challenge for the next decennium will be to obtain more knowledge of systems that allow the culturing of different cell types in three-dimensional structures. This is essential for the controlled regeneration of complex organs, like liver and kidney, which can be used for transplantation purposes. In view of this, we know that the outside environment determines the shape and function of cells. For example, cells cultures on micro-textured culturing materials spread in a very orderly fashion. This condition resembles the situation in living organs, where tissues are characterised by a high degree of organisation. Numerous investigations indicate that there is a direct relationship between the shape and organisation of cells and their differentiated state. The basis of the controlled cell-spreading phenomenon, also known as "contact guidance", is explained by the ability of the cell to sense a specific environment. Apparently, the micro- and nano-morphology of the substrate surface affects the mechanical state of the cell.

In addition to the cellular responses to surface topography, cells also react towards dynamic stresses, such as fluid flow, stretch force, or compression. These forces too affect the mechanical state of the cell. This interaction of static (surface) and dynamic (stress) forces results in the total response of cell towards their (changing) environment. One of such forces is gravity, and is the focus of this thesis. It has become clear form previous studies that cells behave differently under conditions of hypergravity and microgravity (near weightlessness) compared to their appropriate 1g controls. Some more theoretical studies have argued against cells sensing gravity or microgravity altogether. Nevertheless, until now no studies have been performed to examine cell shape changes in situ under various gravity conditions. A change in cell shape in situ under varying g levels contributes to the idea of direct effects of gravity onto cells. Since gravity acts on mass, it might well be expected that initially small changes in cells due to (micro-)gravity result in intracellular mass displacement and/or changes in general cell shape. These small changes might be integrated or amplified thus generating the various changes as seen under micro- or hyper-gravity conditions. Processes as mass displacement and cell shape involve the cytoskeleton system and the intracellular signalling system. Using various microtextured surfaces, combined with settings of hypergravity and simulated microgravity, might well elucidate to what extent cell shape and function are determined by the cell surrounding structures, and in what way they are able to sense (micro-)gravity.

In this introduction we will first discuss the structure of cells and cytoskeleton, and the important role played by the cytoskeleton in cell attachment and locomotion across substrates. We then continue with the design and production of microgrooved substrates, and give a description of how the different cells and tissues respond to these microgrooved surfaces. An introduction in gravity and the physical implications on cell cultures will be discussed, together with a summary of ground-based machines for simulating hyper- and microgravity, as well as the cell response to changes in gravity (gravisensing) in literature. We will end with the research objectives in which a formulation of a study hypothesis and several research questions are posted.

CELL STRUCTURE

Eukaryotic cells have the ability to organise the many components in their interior, to adopt a variety of shapes, to carry out coordinated movements, and provide a communication pathway between cellular protein complexes and organelles. This ability depends on the cytoskeleton – an intricate network of protein filaments that extends throughout the cytoplasm. The cytoskeleton is a highly dynamic structure that is continuously broken down and assembled in order for a cell to change shape, divide, and respond to its environment. The cytoskeleton is not only the "bones" (pre-stressed structures) of a cell but also its "muscles" (tensional forces), and it is directly responsible for large-scale movements such as the crawling of a cell along a surface. The

cytoskeleton of eukaryotic cells consists of three types of protein filaments: intermediate filaments, microtubules, and actin filaments (**Figure 1**). Each type of filament is formed from a different protein subunit. In each case, thousands of subunits assemble into an continuous thread of proteins that sometimes extends across the entire cell [1].

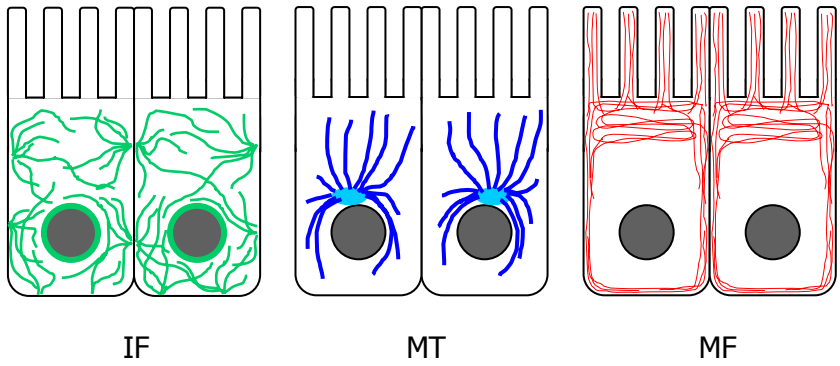

Figure 1. Schematic illustration of the three types of filaments in an epithelial cell lining the gut: intermediate filaments (IF), microtubules (MT), and microfilaments (MF).

Microfilaments

Microfilaments are found in all eukaryotic cells and are essential for many of their movements, especially those involving the cell surface. The microfilaments are a group of proteins consisting of actin, myosin, and several associated proteins. Depending on their association with different proteins, actin filaments can form stiff and relatively permanent structures such as the micro-villi, on the brush-border cells lining the intestine, or small bundles in the cytoplasm that can contract and act like the "muscles" of a cell. Actin filaments can also form temporary structures such as the protrusions formed at the leading edge of a crawling fibroblast, or the contractile ring that pinches the cytoplasm in two when an animal cell divides.

Each actin filament is a twisted chain of identical globular actin molecules, all of which "point" in the same direction along the axis of the chain. Like a microtubule, an actin filament has a structural polarity, with a plus end and a minus end. There is a crucial difference between actin filaments and microtubules; in case of the actin filaments, the organising cluster of proteins is situated at the plus end of the filament.

Actin filaments appear as threads about 7 nm in diameter, this filamentous form is called F-actin. Each filament may be thought of as a two-stranded helix with a twist repeating every 37 nm (**Figure 2**).

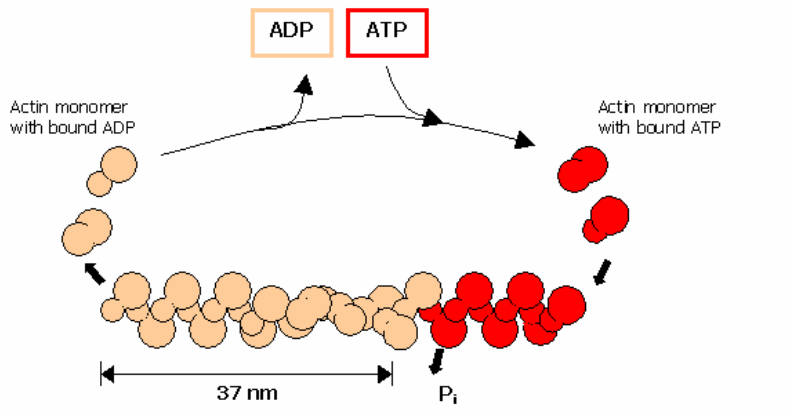

Figure 2. Actin polymerisation requires ATP hydrolysis. The actin monomers carry ATP, which is hydrolysed to ADP after assembly into a growing filament. The ADP stays bound with the actin monomer until the actin monomer dissociates from the filament. Each filament looks like a two-stranded helix with a twist every 37 nm.

In a fibroblast about half of the actin is filamentous, while the other half is free as actin-monomers, that is to say they are bound to actin-binding proteins profilin and thymosin. The binding proteins sequester free actin monomers in the cytoplasm, thereby keeping actin monomers in reserve until they are required. Although actin is found throughout the cytoplasm of a eukaryotic cell, in most cells actin is concentrated in a layer just beneath the plasma membrane.

In this region, called the cell cortex, actin filaments are linked by actin-binding proteins into a meshwork that supports the outer surface of the cell and gives it mechanical strength; the cell cortex also prevents the organelles from touching the inner cellular membrane.
During cell crawling, the cell makes use of internal contractions, to exert a pulling force. These contractions too depend on actin, through the interaction of actin filaments with the motor protein family known as myosin-II. Myosin-II binds and hydrolyzes ATP, which provides the energy for their movement along actin filaments from the minus end of the filament toward the plus end. The myosin filament has a polarity like that of a double-headed arrow, with two sets of heads pointing in opposite directions away from the centre. One set of heads binds to actin filaments in one orientation and moves them one way; the other set binds to other actin filaments in the opposite orientation and moves them in opposite direction. The overall effect is to slide sets of oppositely orientated actin filaments past one another. Therefore, when actin filaments and myosin filaments are organised into a bundle, they can generate a contractile force.
Other actin-associated proteins have various functions. Gelsolin fragments actin filaments into shorter lengths and thus converts an actin gel to a more fluid state. Spectrin forms a meshwork with actin filaments so that it connects to the membrane through intracellular attachment proteins, so called cap-proteins. Myosin-I is a motor protein that moves cell components and vesicles along actin filaments. Tropomyosin binds in the groove of the actin helix, overlapping seven actin monomers and prevents the myosin-II heads from associating with the actin filament. Together with troponin, tropomyosin controls the skeletal muscle contraction [2; 3].

Intermediate filaments

Intermediate filaments have great tensile strength, and their main function is to enable cells to withstand the mechanical stress that occurs when cells are stretched. They are called intermediate because their diameter (8-10 nm) is between that of the thinner actin filaments and the thicker myosin filaments. Intermediate filaments are the toughest and most durable of the three types of cytoskeleton filaments. They typically form a network throughout the cytoplasm, surrounding the nucleus and extending out to the cell periphery. Often they are anchored to the plasma membrane at cell-cell junctions.

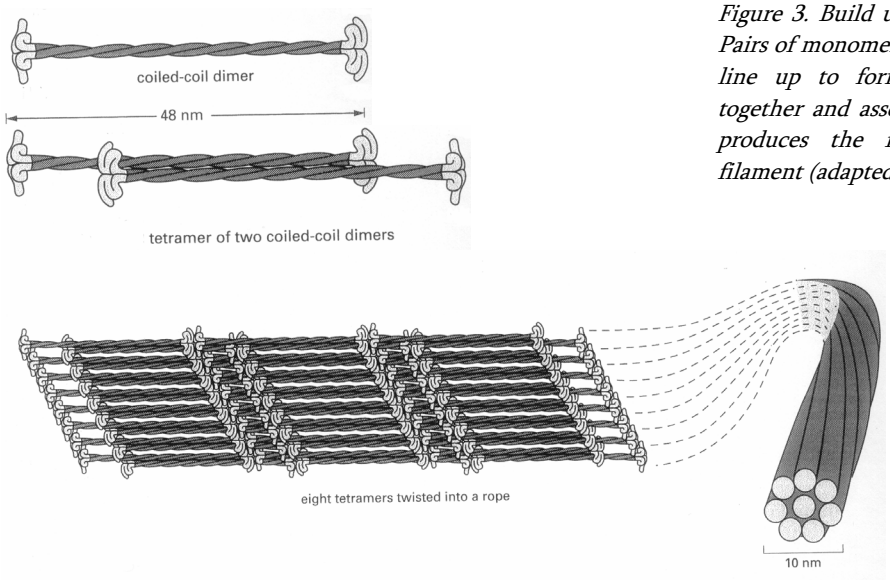

Figure 3. Build up of an intermediate filament. Pairs of monomers form dimers, and two dimers line up to form tetramers. Tetramers pack together and assemble into a helical array that produces the final rope like intermediate filament (adapted from Alberts et al).

Intermediate filaments are like ropes, the strands of this rope – the subunits of intermediate filaments – are dimer-forming filamentous proteins (48 nm in length). These dimers associate to form a tetramer, and the tetramers bind to one another to generate the final rope like intermediate filament (**Figure 3**). A family of fibrous proteins form the intermediate filaments and are grouped into four classes: (1) keratin filaments in epithelial cells; (2) vimentin and vimentin-related filaments in connective-tissue cells, muscle cells (desmin) and glial cells (glial fibrillary acidic protein); (3) neurofilaments in nerve cells; (4) nuclear lamina, present around the cell nucleus. This latter class disassembles and re-forms at each cell-division [2; 3].

Microtubules

Microtubules have a critical organizing role in all eukaryotic cells. They are long and relatively stiff hollow tubes of protein that can rapidly disassemble in one location and reassemble in another. In a typical animal cell, microtubules grow out from a small structure near the centre of the cell, called the centrosome. Extending out toward the cell periphery, they create a system of tracks within the cell, along which vesicles, organelles, and other cell components can be moved. Microtubules can also form permanent structures, called cilia and flagella.

Microtubules are built from molecules of tubulin; each one is itself a dimer of two very similar globular proteins called α-tubulin and β-tubulin. The tubulin subunits stack together to from the wall of the hollow cylindrical microtubule. This appears as a cylinder made of 13 parallel protofilaments, each a linear chain of tubulin subunits with α- and β-tubulin alternating along its length (**Figure 4**). Each protofilament has a structural polarity, and this polarity – the directional arrow embodied in the structure – is the same for all the protofilaments, giving a structural polarity to the microtubule as a whole. The α-tubulin end (at the centrosome) is called the minus end, and the other, the β-tubulin end (at the periphery) it's plus end.

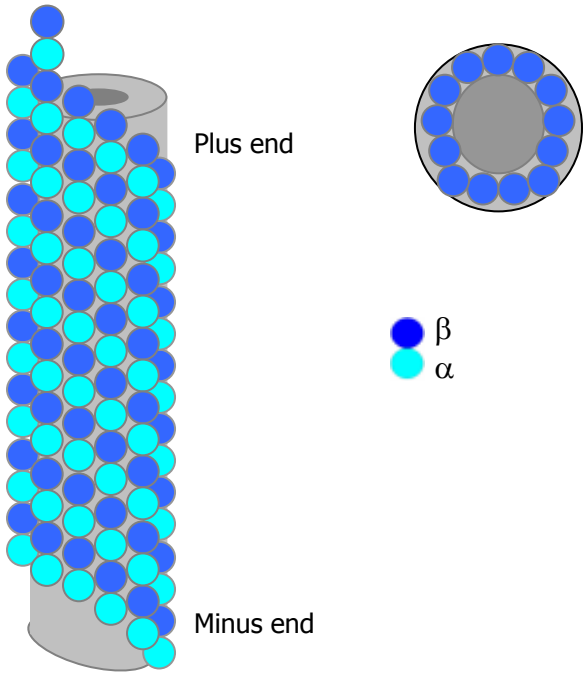

Figure 4. Microtubule structure consists of αβ tubulin dimers which are arranged in protofilaments (one column). The cross section shows 13 subunits, each of which corresponds to a separate tubulin dimer. The orientation of both the tubulin molecules in the protofilaments, and the protofilaments themselves give the microtubule its structural polarity.

Both microtubules and actin filaments are involved in intracellular movements in eukaryotic cells. In both cases the movements are generated by motor proteins, which bind to actin filaments or microtubules and use the energy derived from repeated cycles of adenosine tri-phosphate (ATP) hydrolysis to travel steadily along the microtubule or the actin filament in a single direction. These motor proteins also attach other cell components, and thus transport this cargo along

filaments. The motor protein kinesin moves to the plus end and the protein dynein to the minus end (**Figure 5**) [2 ;3].

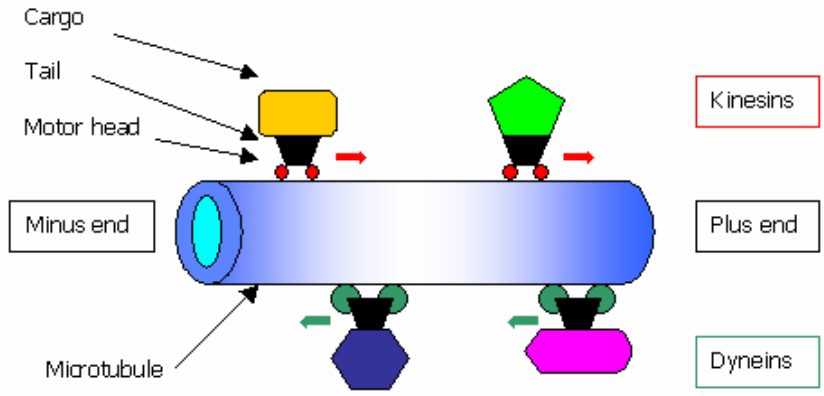

Figure 5. Transport is carried out by motor proteins along microtubules. Kinesins moves cargo toward the plus end and dyneins moves cargo towards the minus end. There are many forms of both types of motor proteins, each of which probably transports a different cargo. The difference in cargo is determined by the tail.

Fibroblast attachment to substrates

If cells are to crawl over a substrate, they must be able to attach to it. Cultured cells adhere to their substratum, as well as to each other. Morphological criteria, to be precise: the gap distances between the cell membrane and the substratum and the presence of submembranous densities, are often used to distinguish between the several types of cell-substratum adhesions. These types are: extracellular matrix (ECM) contacts; close contacts; and focal adhesions. ECM contacts are characterised by large separations (≥100 nm) between the cell and the substratum. ECM sites intracellular appear to be of two kinds, either both α-actinin and vinculin or α-actinin alone. Close contacts have a separation of 30-50 nm. Close contacts are comparable with one half of an adherens cell-cell junction; intracellular they appear to be α-actinin and extracellular fibronectin. Focal adhesions show a 10-20 nm gap between membrane and substratum. They can be compared to one half of a tight cell-cell junction. Intracellular both α-actinin and vinculin are present [1].

The cytoskeleton is bound to ECM proteins at focal adhesions. The formation of a focal adhesion complex (FAC) plays a central role. The FAC is a macromolecular scaffold that mechanically couples the cytoplasmatic portion of integrins to the internal actin cytoskeleton. It contains actin-associated molecules such as vinculin, talin, and α-actinin, as well as many signalling molecules that mediate stimulus-response coupling. The latter include tyrosine and serine protein kinases, inositol lipid kinases, ion channels and even a subset of growth factor receptors [2-8].

The aforementioned integrins form a transmembrane molecular bridge between ECM and the cytoskeleton that distributes mechanical stresses and helps bring forces into balance (**Figure 6**).

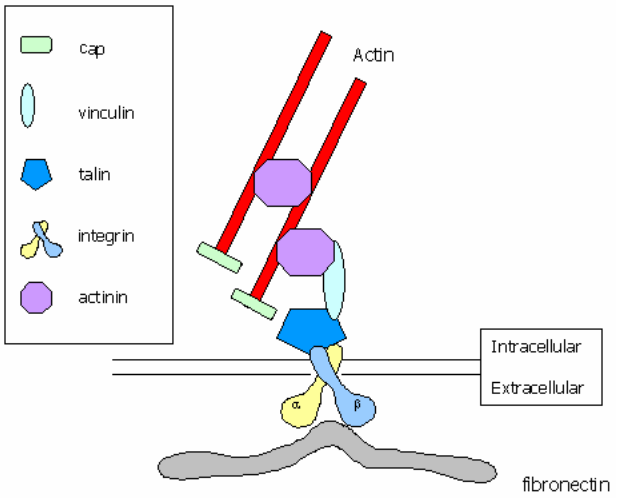

Figure 6. The transmembrane molecular bridge between ECM and the CSK. A number of proteins are involved in focal adhesion.

Integrins bind to ECM components, like fibronectin, vitronectin, or laminin. Integrins are a large family of heterodimeric transmembrane glycoproteins that consist of noncovalently linked alpha (120-170 kDa) and beta (90-100 kDa) subunits. Integrins contain binding sites for divalent cations Mg^{2+} and Ca^{2+}, which are necessary for their adhesive function. The specificity of a given integrin for binding various ligands appears to depend primarily on the extracellular portion of the alpha subunit. However, both subunits are required for an integrin to function properly. The beta subunits have several distinctive features, including tandem repeats of four cysteine-rich regions that are thought to be essential for maintaining the molecular shape. Until now 18 alpha and 8 beta subunits have been identified. From these subunits only some 23 integrins are formed in nature, which implicates that not all possible combinations exist. Matrix proteins often contain a RGD (sequence of the aminoacids arginine, glycine and aspartic acid). This sequence is the part of the matrix protein that is bound by the integrin [3; 4].

The beta subunit of the integrin is important in the connections between actin filaments and integrin (and thereby the outside world). Beta subunit can bind to the major structural proteins in focal adhesions: talin, vinculin, tensin, α-actinin and paxillin. Both tensin and talin strengthen the binding of vinculin and actinin. Vinculin can, besides bind indirectly to integrins via talin and actinin, bind actin and tensin. Paxillin appears important in the organisation and turnover of focal adhesion complexes. The attachment sites are well positioned to act as signal transducing centres to report on changes in the cell's immediate environment. Signalling pathways are mediated through autophosphorylation of the tyrosine kinase Focal Adhesion Kinase (FAK). Besides FAK, which takes up a central position in the signal transduction, other proteins are SrcFK, CAS, Crk and Csk (**Figure 7**) [6; 9-16].

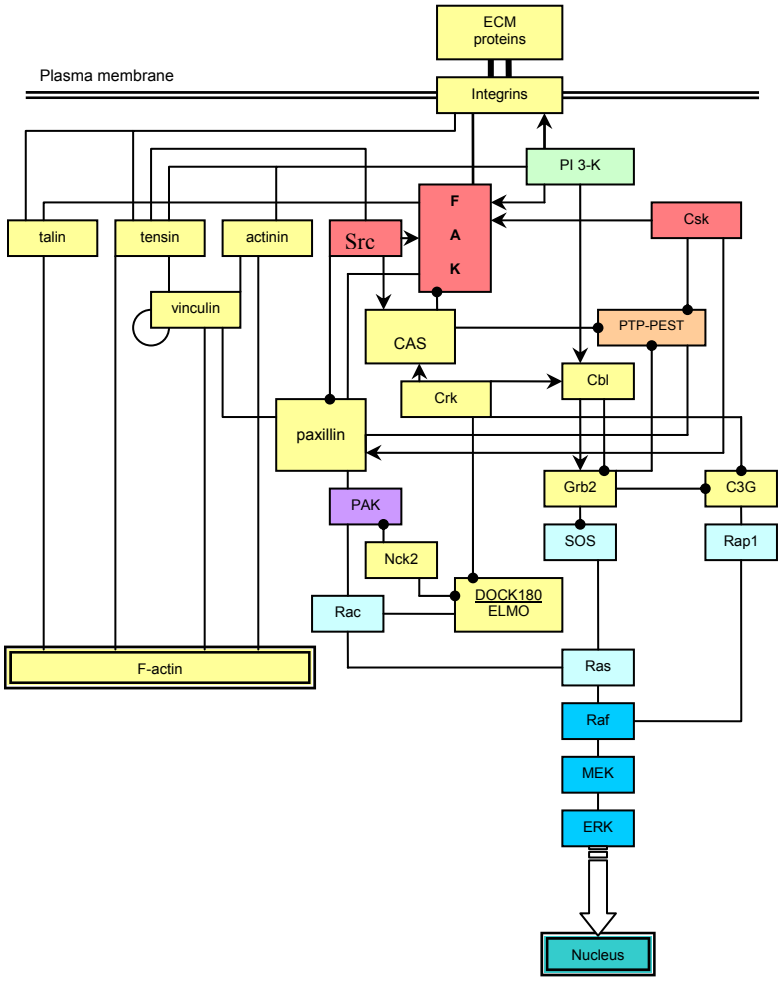

Figure 7. Interaction schematic among the proteins involved in integrin signalling. The integrin-mediated mechanotransduction includes multiple kinases (FAK, SrcFK, and Csk), adaptor molecules (CAS, Crk, Grb2, and paxillin), guanine nucleotide exchange factors (GEFs) (C3G, SOS), and small GTPases (Rap1, Ras) in activating Mitogen-Activating Protein Kinases (MAPKs) (MEK, ERK). Signals through FAK to Rac and PAK play a role in modulating cell adhesion and migration, actin polymerisation and MAP kinase signalling. SH2 interactions are marked by arrows, pointing to phosphotyrosine containing target, SH3 interactions are marked by dots pointing to proline rich target. Black lines indicate either cell-matrix or cell-cell adhesions (adapted from: Zamir et al).

Cell locomotion and the formation of focal adhesions

Cell locomotion is undoubtedly complex, requiring coordinated activity of cytoskeletal, membrane, and adhesion systems. Locomotion involves protrusion and adhesion at the cell front, and contraction and detachment at the rear. In migrating cells, membrane protrusion is driven by actin polymerisation and does not require microtubules; however directional cell movement requires dynamically growing microtubules [17].

A conventional breakdown of crawling along spatial/mechanical lines is shown in **Figure 8**, for a single cell moving over a two-dimensional substrate. Four interrelated processes are essential: (1) forward motility of the membrane at the "front" or leading edge (protrusion); (2) adherence of these protrusions to the surface of the substrate (adhesion); (3) dragging forward of the cell using the anchorage points (traction); the last process (4) is comprised of two mechanistically distinct steps: de-adhesion and retraction of the trailing edge of the cell. Whether this step is actively motile depends on the cell type: strongly adhesive cells such as cultured fibroblasts tend to have a strongly adherent, extended tail and leave behind a trail of cytoplasmatic fragments as they move [18].

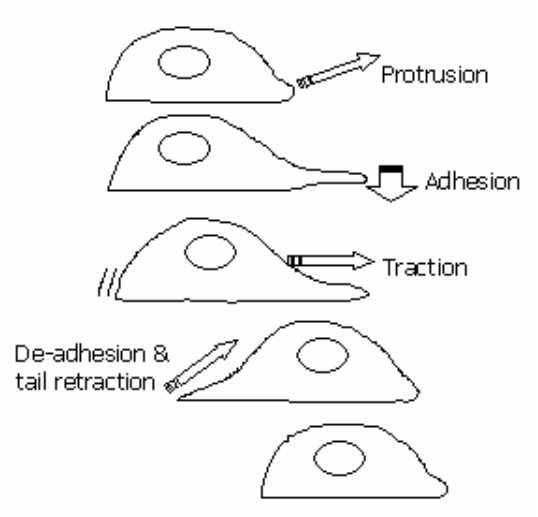

Figure 8. Cell locomotion in cartoon form. Actin polymerisation at the leading edge of the cell pushes the plasma membrane forward. New anchorage points are made between the actin cortex and the substratum surface. Cortical tension then draws the body of the cell forward. The rear of the cell detaches from the substratum and retracts, actin filaments in the retracting tail depolymerise, and the released actin molecules move forward through the cytosol to sites of new polymerisation. This cycle is repeated time and time again, moving the cell forward (adapted from: Mitchison et al 1996).

This dynamic organisation of the actin cytoskeleton, which provides the force for cell motility, is regulated by the members of the Rho family of small GTPases (Guanine Tri-Phosphate). Each of these GTPases act as a molecular switch, cycling between active GTP-bound and inactive GDP-bound state. Guanine-nucleotide-exchange factors (GEFs) facilitate the exchange of GDP for GTP. GTPases-activating proteins (GAPs) increase the rate of GTP hydrolysis of Rho GTPases (**Figure 9**) [3].

The members RhoA, Rac1 and Cdc42 are required for respectively stress fibre formation, lamellipodia and filopodia. Lamellipodia and its accompanying membrane ruffles at the advancing cell front are made up of a laminar meshwork of actin filaments. Lamellipodia are often punctuated by the rib-like filopodia, they can extend as finger-like projections beyond the lamellipodium tip. The adhesion sites in lamellipodia and filopodia are called focal complexes. Focal complexes may be of a transient event and turn over with in a few minutes, or they can differentiate into larger and long-lived focal adhesions. Focal complexes can be induced by Rac1 and Cdc42, and the transition to focal adhesions is put in motion by the upregulation of Rho (**Figure 10**) [19-24]. Rho upregulation comes forth from the activities of Rho effectors formin mDia and Rho kinase (ROCK) [25]. They are responsible for polymerisation of actin bundles and myosin contractility respectively, which in turn is necessary for focal adhesion formation and

maintenance. Both focal complexes and early focal adhesions remain stationary relative to the substrate and serve as traction points for forward translocation [20].

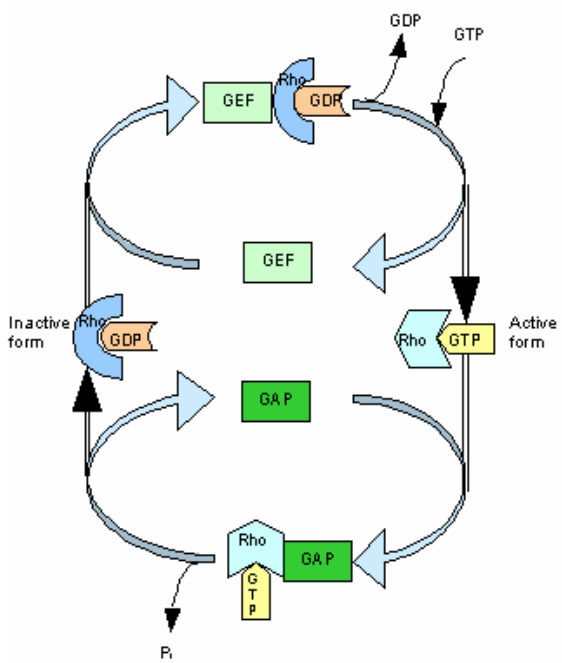

Figure 9. Cycling of Rho protein between its inactive form and active form. Guanine nucleotide-exchange factor (GEF) facilitates dissociation of GDP from Rho. GTP binds spontaneously, and GEF dissociates resulting in the active Rho-GTP form. Hydrolysis of bound GTP to regenerate the inactive Rho-GDP form is accelerated a hundredfold by GTP-activating protein (GAP).

New adhesions sites are formed at the front as the cell moves forward, while the preceding sites are disassembled. At the trailing edge of the cell there are also focal adhesions to be found, they are the remainder of previous protrusion and focal complex assemblies. These focal adhesions can "slide" relative to the substrate and fuse together to form large adhesions.

Microtubule polymerisation dynamics is directly linked to substrate adhesion dynamics. Depolymerisation of microtubules leads to depolarisation of cell shape, increased cytoskeleton contractility and amplified focal adhesion size, changes which are equal to RhoA activation. Activation of Rac1 is associated with repolymerisation of microtubules. Via the Rho GTPases there is extensive cross-talk between microtubule polymerisation with the actin cytoskeleton organisation and substrate adhesion dynamics [19; 20].

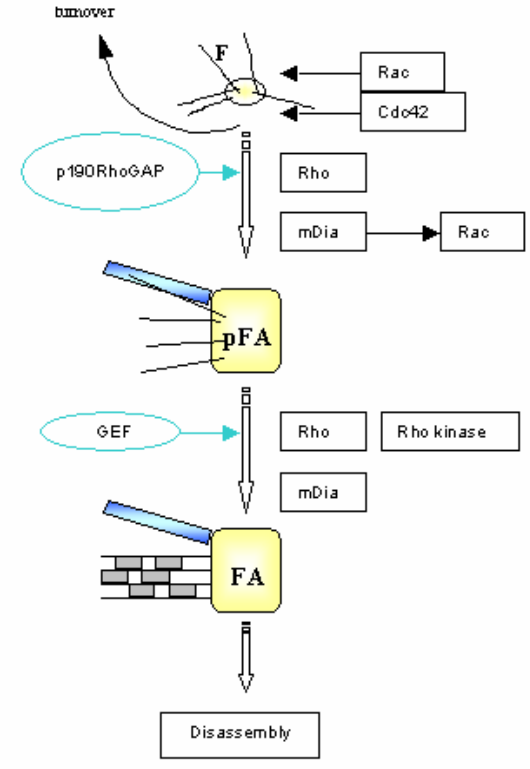

Figure 10. Primary contacts are focal complexes (FC) associated with lamellipodia and filopodia which are formed through Rac and Cdc42 signalling. Lamellipodia and filopodia support protrusion and can either turnover within minutes or differentiate into focal adhesions via Rho upregulation. Inhibitor of this differentiation is probably p190RhoGAP, in order to promote turnover and protrusion. There is reason to believe there is a precursor focal adhesion (pFA) by the finding that Rac can be activated by Rho via mDia, which also stimulates actin polymerisation. The differentiation to focal adhesion requires the recruitment of myosin (grey chequered bars) and the activation of contractility via Rho/Rho kinase, by exchange factors such as GEF. Focal adhesions behind the advancing front stay there and are turned over by disassembly. Microtubules (blue bars) target focal adhesions and could clean up GEF and thereby reduce Rho kinase activity, in this way microtubule-linked signals could decrease the growth of focal complexes or promote disassembly of focal adhesions (adapted from: Small et al 2003).

Microtubules exert their influence on cell polarisation by modulating adhesion site turnover through the point delivery of Rho-family GTPases signals that antagonise myosin contractility at adhesion foci. To allow for molecular crosstalk on the local level, microtubules need to get close to adhesion sites. This targeting of adhesion sites by microtubules is in the range of nanometres. Targeting consists of microtubule growth to the adhesion site and a brief contact between members of the microtubule tip complex and adhesion complex. How this signalling comes about and what microtubule-associated proteins are involved is still under investigation. In order to guide microtubule polymerisation towards adhesion sites; microtubule tip complex proteins interact via an adapter (proposed is an unconventional myosin) with the tensioned actin filaments. This adapter docks onto and moves along actin filaments, thereby steering the microtubule polymerisation into the adhesion site. Obviously, the actin filaments must be linked to the adhesion complex, to allow directed movement of this myosin motor protein (**Figure 11**) [20].

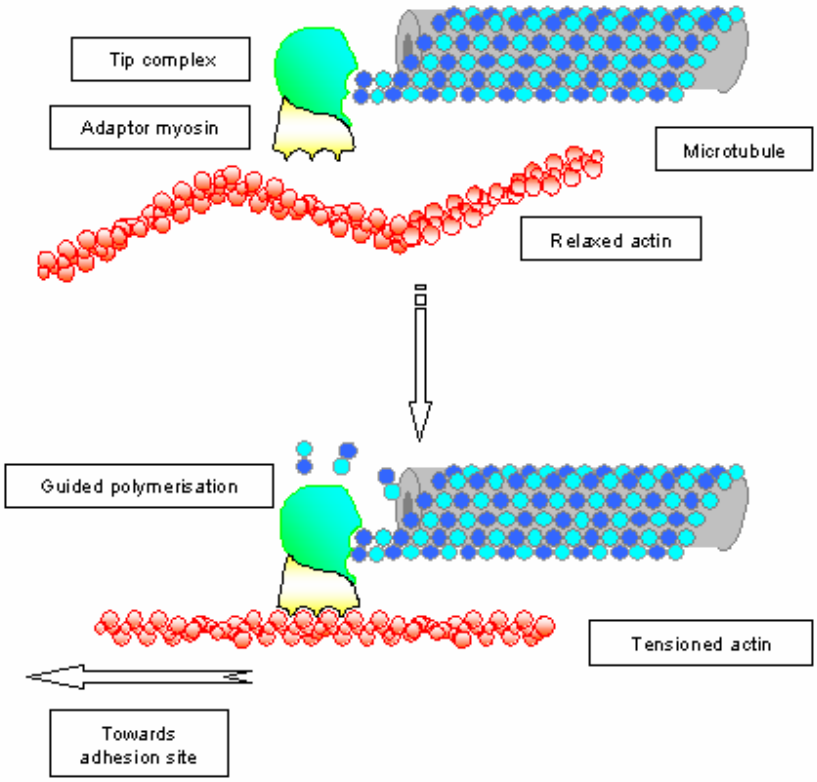

Figure 11. A proposed guidance mechanism of microtubule polymerisation towards adhesion sites. Microtubule polymerisation is stimulated by mechanical stress in the actin filament. Tensioned actin filaments, linked to adhesion foci with their plus end, could be recognised by adaptors associated with the Tip complex proteins which arise in growing microtubules. This adaptor, a possible unconventional type of myosin, attaches to and moves along the tensioned actin, taking the microtubule with it and guiding the microtubule polymerisation in the direction of the adhesion site (adapted from: Small et al 2003).

Cytoskeleton inhibitors

The role of microtubules and microfilaments are also part of continuous investigation, often a specific inhibitor is added and the changes in the cell of interest induced by this substance are observed. Substances like colcemid and nocodazole, which are specific MT inhibitors, or the blocking of kinesin-1 and dynein activity and thereby blocking of the MT motor activity. Also, the use of specific inhibitors for microfilaments like cytochalasin B. Inhibition of both MF and MT by chloropropham and 12-o-tetradecanoyl-phorbol-13-acetate (TPA) have elucidated the importance of the cytoskeleton in growth and cell distribution and of course cell locomotion and shape. Polarisation and locomotion of fibroblasts requires intact MT cytoskeleton. Kaverina *et al.* used contractility inhibitor ML-7 and conclude that regional contractility is modulated by the interfacing of microtubule-linked events with focal adhesions and that microtubules determine cell polarity via this route [26]. Their findings showed that microtubules are dispensable for fibroblast protrusion, but are required for the turnover of substrate adhesions that normally occurs during cell locomotion. Earlier Yokoyama *et al.* applying inhibitors concluded that, although MTs are not essential for elongation of filamentous cell tips, MF are essential for this process [27]. The

interaction of the actin cortex and the MT system has been also been investigated by Dugina *et al.* who induced a cytoskeleton reorganisation with TPA resulting in an actin-rich and MT rich division in the cell. TPA effects were reversible and suppressed by cytochalasin B and colcemid. By targeting MT motor activity through inhibition of kinesin-1 increases the size, but also reduces the number of substrate adhesions thereby mimicking nocodazole effects [28]. The balance of MT minus end capture and release from the centrosome, vital in cell migration, has been uncovered by blocking the dynein activity. Choquet *et al.* used phenylarsine oxide and observed an inhibition of strengthening of CSK linkages, indicating a role for dephosphorylation. They conclude that the strength of integrin-CSK linkages is dependent on matrix rigidity and on its biochemical composition. Matrix rigidity may therefore serve as a guidance cue in a process of mechanotaxis [7]. Another example of a study concerning the connection between cytoskeleton and the outside world through integrins was performed by Uitto *et al.* were the attachment of periodontal ligament epithelial cells to fibronectin, laminin, collagen type I and vitronectin was reduced by arg-gly-asp-ser peptide. The cells treated with this peptide demonstrated prevention of spreading and migration [29]. *et al.*

CELL ADHESION TO MICROGROOVED BIOMATERIALS SURFACES

Tissue Engineering

Tissue engineering, a multidisciplinary scientific field, unravels the fundamentals of structure-function interactions in both normal and diseased tissues. Tissue engineering aims to develop artificial biological substitutes as well as methods to repair damaged or diseased tissues, and ultimately the creation of entire tissue replacements. Driven by the dramatically increasing need for medical implants to replace malfunctioning or lost tissues and organs, tissue engineering has become a billion euro market. The basic principle of tissue engineering is to culture cells and combine them with artificial matrices (scaffolds). These scaffolds are implanted into defects in order to repair damaged tissue or to regenerate new and functional tissue. In the wake of this basic principle is the production of cells, tissues, and matrices. Matrices should be a dynamic microenvironment, not only to manipulate the healing environment, so it can control the structure of regenerated tissue, but also to control the cellular responses of the cells seeded upon the matrices. They should give appropriate signals to the cells, hence mediating their biological activity and function. One way to exert control over cell shape, orientation, metabolic rate, and migration is the use of microgrooved biomaterials [30-40].

Microgrooved surfaces

Cell behaviour can be affected by substrata surface topography. Topography of a substrate can be explained as the morphology of the substrate surface. Surface topography can be divided into roughness and texture [41]. Roughness is characterised by random size and distribution of patterns. Roughness is sometimes applied with a purpose, but it can be the additive result of the material's structure or the fabrication process. Texture is characterised by a deliberate and controlled pattern. Textures are the result of regular surface topography with defined dimensions and surface distribution. A vast array of configurations can be used like: grooves/ridges (v-section, rectangular section, round, multiple grooves, branching, spiral grooves), hills, dots, pits, mesh, and pores [42; 43]. The influence of the topographical morphology of the surface on cell behaviour was first discovered in 1914 by Ross Harrison who used spider webs for cells to migrate along. In 1945, Weiss conducted a range of experiments and described the changes in cell behaviour as "contact guidance". Both Rovensky and Maroudas rediscovered this behaviour in the early 1970s. Ever since, investigators have studied the behaviour of various cell types on a variety of microtextured substrate material [44; 45].

Production of microgrooves

The microtextured pattern can be applied by machining, sintering, or etching the surface. To achieve micron (or even nano) textured dimensions, mostly computer controlled photo-lithographic techniques are used. These techniques, which come forth from the ever expanding electronics microfabrication technology, are relatively cheap and fast, the textured surfaces are of reasonable size, and a wide range of patterns can be applied on a wide range of biocompatible materials [46].

A microtexturing process can be briefly described as follows: a silicon wafer, after cleaning and being dried with filtered air, is coated with a primer and photo-resist. The samples are patterned by exposing to light through a mask that has the desired pattern. The exposed photo-resist is rinsed of. The next step concerns the etching itself, this can be done by either chemicals (wet conditions), UV light for details of down to 1 μm, or electron beams for details of 100 nm or less (dry conditions). An advantage of physical etching over wet technique is that it allows for a higher resolution, and could also be applicable in amorphous materials. Finally, after the etching process the remaining resist is removed (**Figure 12**) [47]. In case of microgrooved substrates the texture dimensions are described as: pitch (or spacing), ridge width, depth and groove width (**Figure 13**) [47]. The microtextured material can be used as a template for the production of polymeric substrates. For this usually solvent casting procedures are used. Here a polymer (e.g. polystyrene) is dissolved in an organic solvent like chloroform. After casting this solution on the silicon template, the chloroform evaporates. This procedure allows the production of vast numbers of identical replicas, and thus is to be preferred over directly using the silicon materials for experimentation.

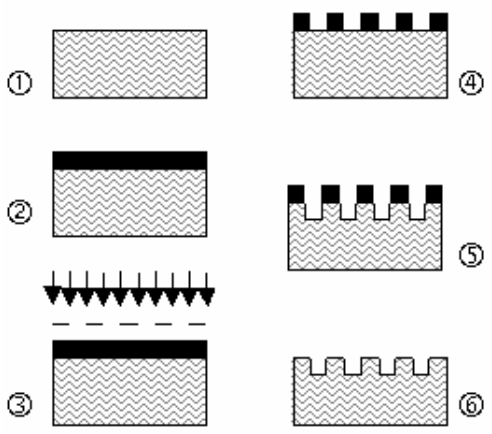

Figure 12. The process of photolithographic etching. The silica material (1) is covered with photo resist (2). The photo resist layer is illuminated through a desired patterned mask (3). The developed resist is washed off (4) and the material is etched by chemicals, UV light or ion beam (5). The remaining resist is removed (adapted from: Walboomers 2000).

Cell response to microgrooved surfaces

Over the years several materials have been used to construct substrates: Polystyrene [46-61], Poly-Lactic Acid [46; 62-69], Poly(glycolic-co-lactic)acid [70-75], Gold [76; 77], Titanium [46; 57; 78-95], Ceramic [72; 83; 96-99], Silicone rubber [46; 67; 68;100-117], Polyethylene Terephthalate [71; 78; 101; 118-123], Perspex [44; 45], nylon [124; 125], glass [126-132], silica [133-136], and gelatine [137-139]. The surfaces of these materials were microtextured or left smooth (control). These substrates were untreated or treated by ultraviolet irradiation, or received a radio frequency glow discharge treatment (RFGD) or both [67; 86; 101; 104; 140-142]. Sometimes the surfaces were coated with ECM proteins like collagen or fibronectin, the aim of those protein coatings is to increase the surface bioactivity [57; 67; 129;143-145]. Transforming growth factor (TGF) has been applied to microtextured implants to reduce scar-tissue formation *in vivo* [52; 64; 96; 98; 146-151].

Numerous cells, seeded upon those materials, have been studied: fibroblasts from different tissues [5; 45-50; 60; 84; 85; 89; 94; 100; 116; 129; 145; 152-162], osteoblast-like cells [51; 62; 63; 71; 80; 81; 83; 87; 91; 93; 163-169], keratinocytes [138; 141; 144; 170; 171], hepatocytes [61; 172], bone marrow cells [62; 63; 71; 99; 173; 174], epithelial cells [44; 52; 82; 175-178], endothelial cells [9; 56; 114; 123; 179-185], various nerve cells [186; 187], smooth muscle cells [74; 75; 107; 110; 147; 188; 189], several tumour cell lines [14; 70; 190-193], polymorphonuclear cells, and macrophages [130; 133; 194-196].
Methods used in these experiments range from proliferation studies, immunocytochemistry, green fluorescent protein techniques to several forms of microscopy: Scanning electron microscopy (SEM), Transmission electron microscopy (TEM), Confocal laser scanning microscopy (CLSM), Interference reflection microscopy (IRM), Dual wavelength time-lapse fluorescent speckle microscopy (FSM), Atomic force microscopy (AFM) and Optical emission spectroscopy (OES); the last two forms are used in microtexture profiling analysis.
Effects of topographical control on cells which have been studied are: cell-orientation, -shape, -migration, -proliferation, -growth, mRNA transcription expressions of integrin, vinculin, fibronectin, and collagen, tyrosine phosphorylation, adhesion strength, and (de)polymerisation of micro-filaments and microtubules.

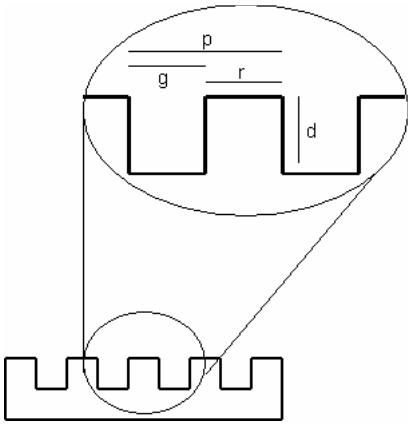

Figure 13. Dimensions of a surface groove pattern as seen in a cross section:
p = pitch, r = ridge- width, g = groove-width, d = groove-depth (adapted from: Walboomers 2000).

What is clear from most studies is that cells elongate and align to the surface grooves. Cells may bridge from one ridge to another ridge, without touching the groove bottom, on patterns up to 2 μm wide. This bridging is independent on the groove depth. Groove/ridge width, ranging from 10 μm to 70 nm, and groove depth, 1 μm to 150 nm, have shown the importance of the ridge in the establishment of topographical control. Microfilaments and vinculin (marker for focal adhesions) appear to be orientated parallel to the surface grooves, this aggregation is most obvious on grooved substrates of 4 μm or less, although, depending on the material used, this alignment has also been observed on larger (> 5 μm) grooved substrates. Vinculin was observed mainly on the ridge of the surface patterns. Depositions of endogenous fibronectin and vitronectin have been studied, and found to be orientated along the surface grooves. As far as migration is concerned; cell protrusions are found extending into the grooves. In case of wider grooves, these cell protrusions extend all the way to the bottom of the grooves. The behaviour of osteoblast-like cells on microtextured surfaces is characterised, besides alignment of cells and cellular extensions, by deposition of collagen fibrils and the formation of calcified nodules. Neither the presence of grooves, nor the dimensions of the grooves have an effect on cell proliferation. Wettability (contact angle between a cell and the substrate surface) and surface free energy (energy required to create a surface of one unit area) influence the cell growth, but play no measurable role in the shape and orientation of cells on microtextured substrates.

Although many studies have shown the cellular alignment along the microgrooved substrates, this field of study continuously pushes the border forward. The grooves now a day are of nanometre scale [56; 133; 134; 196-199]. Once it was thought that cellular effects disappear at about 1 μm level, but Clark proved with 130 nm wide grooves that epithelial cells still responded by alignment of isolated cells and of the cytoskeleton to the grooves [178]. This is interesting in the area of whether the cells react to local patterns of chemical difference or to features such as topography. The shallow grooves loose out to chemical tracks, but when the grooves are deeper than 500 nm topographic effects overwhelm the chemical effects. Besides grooves, pits and pillars, "island" topography on nanometre scale has also found its way in the *in vitro* study of cells [54]. Here, 95 nm high, flat topped discs of different diameters (300 nm to 2.5 μm) have shown to evoke fibroblast cytoskeleton changes and temporal cell morphology. Endothelial cells have also been seeded upon these islands, which resulted in good spreading of the cells, which in turn is claimed to be a good morphology for monolayer cells. The nano-topography provides cues similar to those given by collagen, resulting in a more natural phenotype.

The effects of multigrooved surfaces are also a new area of investigation. Yoshinari *et al.* used a gold alloy as master material and with a trapezoid-shaped diamond cutting tool cut macrogrooved (25 μm high, 150 μm wide) mounds. Subsequently, using a V-shaped tip cut 1 μm deep, 2 μm high microgrooves into the macrogrooves. The multigrooves were able to control the orientation of extra cellular matrix produced by the fibroblasts, and thus production, much better than microgrooves alone or flat surfaces [50].

A recent addition to the different substrates is seeding fibroblasts on a combination of collagen gels and microgrooved or smooth titanium or polystyrene substrates [57]. The gels were first added either to the confluent fibroblast culture on the surface or to the fibroblasts were suspended within the collagen gel and then placed onto the surface. Their results suggest that the order in which fibroblasts encounter substratum and extracellular matrix can influence the eventual matrix-cell interactions, and that substratum topography can influence matrix and cell orientation in zones not immediately in contact with the surface.

A new addition in the use of metal oxide based surfaces was presented by Winkelmann *et al.* which combined titanium, aluminium, vanadium, and niobium in producing patterns (dots and stripes) with one metal as the background and the second metal superimposed on the background metal. The seeded osteoblasts exhibited a pronounced reaction on bimetallic surfaces that contained aluminium. Cells tended to stay away from aluminium. Fibronectin and albumin absorption were significantly greater on non-aluminium regions. The investigators believe that the positive surface charge of aluminium account for the controlling factor in the observed cell behaviour [81].

An area where research is just beginning to focus on is the gene-level responses topography. Dalby *et al.* used microarray to probe for consistent gene changes in response to lithographically produced topography with time. They observed that genes involved in cell signalling, DNA transcription, RNA-protein translation, and ECM formation and remodelling are important in the cell response to the grooves [155].

Tissue response to microgrooved surfaces both in vitro and in vivo

The response of tissues on the use of microgrooved surfaces is described extensively. While some research groups simply observe the effects of soft tissues on the implanted substrates [105], others add cells and growth factors to their elaborate implants and focus "just" on the wound healing for 3 weeks [148]. The migration of an epithelial tissue sheet was found to be enhanced by polystyrene microgrooves, while migration across the microgrooves was inhibited. This pattern found by Dalton *et al.* is similar to that of intact epithelial tissue [176]. By adding Transforming

growth factor-β 1, 2, or 3 (TGF-β) on the substrates the outgrowth of epithelial tissue was inhibited on both smooth and microgrooved surfaces [52]. The relevance of TGF-β in wound healing has been investigated by several researchers [64; 65; 143; 147]. Pandit *et al.* [148] incorporated TGF-β into collagen scaffolds ant observed an enhanced healing process in terms of faster epithelialisation and contraction rate. In an extension to these findings Gehrke *et al.* [200] added TGF-β3 to their microgrooved silicone implants to see whether TGF-β influences the fibrous capsule formation. Although they did not find a significant difference it is still believed that surface topography is important in establishing tissue organisation adjacent to implants, with smooth surfaces generally being associated with fibrous tissue encapsulation. Grooved topographies appear to have promise in reducing encapsulation in the short term, but additional studies employing three-dimensional reconstruction and diverse topographies are needed to understand better the process of connective-tissue organisation adjacent to implants [82; 85; 106]. The formation of fibrous capsule in vivo are quite complicated as mentioned in the studies by Johnson *et al.* [119], Butler *et al.* [118], Shannon *et al.* [201], and Parker *et al.* [67]; implant location, surface charge, collagen types, and coating are just some of the factors involved. Huang *et al.* [146] investigated if a polycaprolactone (PCL) scaffold and TGF-beta1-loaded fibrin glue could be used for tissue engineering applications and found that the scaffold loaded with TGF-beta1 and implanted subperiosteally was found to be richly populated with chondrocytes. Mature bone formation was also identified. They conclude that scaffolds loaded with TGF-beta1 can successfully recruit mesenchymal cells and that chondrogenesis occurred when this construct was implanted subperiosteally.

Researchs groups [82;175] have observed that grooved implants can obstruct epithelial downgrowth on percutaneous devices and improve performance and longevity of percutaneous devices by promoting tissue integration. As wells as, that grooved topographies show reduction of encapsulation and increases in mineralized bone tissue process of connective tissue organisation adjacent to the microtextured implants [85]. This potential of surface grooves to influence matrix and cell orientation and migration in areas immediately adjacent or not immediately in contact with the surface has been described before [57; 176].

Other research groups have seen no orientation along the surface grooves of textured surfaces and no difference between the capsules surrounding smooth or microgrooved implants [105; 106]. Neither have they seen a significant difference in tissue reaction influence around implants, which are either microgrooved, roughened or left smooth [53; 67; 68].

CELLS AND GRAVITATIONAL BIOLOGY

Gravity

Gravity is a force of attraction between all matters in the universe. It is the weakest known force in nature, but it still manages to hold galaxies and the solar system together, because it is always attractive and it can act over very large distances. The other fundamental forces include the strong nuclear force, the weak nuclear force, and electromagnetism, together they are responsible for all of the forces exchanged between matter particles.

Sir Isaac Newton (1642 - 1727) discovered that a force is required to change the speed or direction of movement of an object. He realized that the force called "gravity" must make an apple fall from a tree. Furthermore, he deduced that gravitational forces exist between all objects. Some objects require more force to move than others. The force needed to push an object at a given acceleration rate is proportional to the object's mass. Because of this relationship, gravity is commonly referred to as the resultant acceleration of a mass due to gravitational force (**info box 1**).

Info Box 1

The force of gravity that one body exerts on another can be expressed as:

$$F_{gravity} = (G * m_1 * m_2) / r^2 \quad [1]$$

F = Force of gravity experienced by bodies
G = Gravitational constant: $6.6726 * 10^{-11}\ m^3\ kg^{-1}\ s^{-2}$
m_1 = mass of body one
m_2 = mass of body two
r = radius of body

Newton's second law states that:

$$F = m * a \quad [2]$$

Substitute the 'F' in equation [1] with 'm * a' in equation [2]:

$$m * a = (G * m_1 * m_2) / r^2 \quad [3]$$

Divide both sides by m:

$$a = (G * m) / r^2 \quad [4]$$

At a 90 degree angle:

$$a = g \quad [5]$$

The new formula is:

$$g = (G * m) / r^2 \quad [6]$$

This gives the acceleration due to gravity.

Knowing the gravitational constant, the mass of the Earth, and the distance from the centre of the Earth, you can calculate the gravity of Earth.

Satellites (e.g. the Moon) orbit around the Earth. The satellites move in a circular path around a centre point which is the Earth. The force holding the satellite in its circular path is the centripetal force (a.k.a. centrifugal force). However they also feel the gravitational pull of the Earth. The two forces are equal to each other:

$$F_{centripetal} = F_{gravity}$$

$$(m_1 * v^2) / r = (G * m_1 * m_2) / r^2 \quad [7]$$

When measured at sea level on the surface of Earth, an object experiences what is commonly referred to as "1 g" or "one Earth gravity." If an object was dropped, the resultant acceleration is approximately 9.81 meters per second squared (m/s^2). In Earth's orbit, Earth's gravity force is counteracted by the spacecrafts motion around the earth (Centrifugal Force). The net force on the object is very close to zero. In practice, such accelerations range from about one percent of Earth's gravitational acceleration (onboard aircraft in parabolic flight) to approximately one part in a million (when orbiting the Earth).

Technically, an object is in free fall when gravity and only gravity is allowed to act on it. In other words, it is what most people generally refer to as falling. To really understand free fall one must also understand acceleration. Most people think acceleration is simply when an object increases speed. By its most exact definition however, acceleration is any change in an object's speed or

direction. Satellites orbiting Earth are also considered to be in free fall. To explain this, imagine that one is standing on top of a really tall mountain (**info box 2**). You throw a ball straight forward, the ball will fall to the ground, because Earth's gravitational force directed toward the centre of the Earth will pull it down. If you throw the ball really fast, it not only falls toward the Earth it also travels outward, over the Earth. If you could throw the ball fast enough it would travel so far over the ground that by the time it came down the ground would have curved away from it because the Earth is round. In this case the ball is a satellite orbiting Earth with nothing but gravity acting on it, hence the ball is in free fall.

Info Box 2

An object launched straight forward from a high mountain, will fall to the ground because of Earth's gravity. However, if launched with a high enough speed, it would still fall to the Earth, but at the same rate Earth curves away from it. The object is now in orbit.

Albert Einstein (1879 - 1955) presented in 1915 a revision of Newton's works in his general theory of relativity (a flaw was discovered in Newton's theory, concerning the orbit of Mercury, which was circling the Sun in a way Newton had not predicted). This work completely rewrote the way we look at gravity and even changed our understanding of the very structure of the universe. According to Albert Einstein, there is no difference between the acceleration of gravity and any other acceleration (principle of equivalence). No person or instrument in a closed box could tell the difference between the force of gravity and the force caused by a rocket engine accelerating the box at a uniform 9.81 m/s^2.

Thus, g is the acceleration caused by the gravitational attraction of a body (e.g. a planet). The acceleration so derived has the same dimensions as the acceleration produced by uniform circular motion on a centrifuge. Experiments conducted on ground-based centrifuges indicate that centrifugation provides an excellent simulated gravity environment for gravitational biology research as long as the centrifuge is of sufficient diameter (radius of rotation) and capable of a sufficient rotation rate [202-207].

Physical phenomena

The effects of gravity on a cell can be divided in direct and indirect effects. Direct are the effects of acceleration onto the cells or the intracellular components. Indirect effects are effects on the cell environment, like the ECM or the tissue of which the cell is part of. The researcher should bare in

mind these indirect effects while interpreting the results of cell culture experiments. There are several phenomena which, especially at the cell *in vitro* level, play a considerable role in gravitational biological research, namely sedimentation, hydrostatic pressure, diffusion, convection, and buoyancy [208-210].

Sedimentation

Sedimentation is the downwards movement of a particle relative to its surrounding medium. During this downward movement the medium, such as fluid, there are three kinds of forces on the particle; downward gravity force, upward buoyancy, and upward resistance known as drag force. This drag force is behind the object due to viscosity and also turbulence behind the particle.

Sedimentation is an important difference between an experimental environment on Earth or in space. Statocytes, specialised cells in the root tip of plants, contain amyloplasts. These relatively big and heavy particles play a part in the positive gravitropism of plants. Gravitropism (sometimes called geotropism in older texts) is a key regulatory process, in response to gravity, in plants to insure that roots grow down and shoots grow up in developing seedlings [208; 209] .

Hydrostatic pressure

In situations of hypergravity part of the pressure which is acting on a surface area derives from the weight of the liquid column standing above these surfaces, like culture medium above a monolayer of cells.

Hydrostatic pressure is linearly proportional to g. Hydrostatic pressure due to weight is zero under weightlessness conditions obviously. During ground based hypergravity experiments not only the hydrostatic pressure derived from the liquid column on the surface of the culture plate should be taken into account but also the atmospheric pressure, which is about 100 kPa at sea level [208; 209].

Diffusion

Molecules in a gas or liquid continually move about and collide with each other. This process, diffusion, is better known as the Brownian movement of small particles in solution. Diffusion takes place in response to a concentration gradient. The flow of molecules/particles is opposite to the concentration gradient. Diffusion is not altered under microgravity. Therefore, in the absence of sedimentation, diffusion is under weightlessness conditions the most important remaining means to mix the fluid or gas [208; 209].

Convection

Differences in density also cause movement in a gas or liquid. This known as convection and it appears when a part of a liquid or gas heats up and, thus, expands. The density of the heated liquid or gas decreases. This heated part shall move upwards since it is lighter than its surroundings. This phenomenon is only present under gravity (acceleration) conditions and is absent in weightlessness. The absence of convection during *in vitro* experiments in space results that spent medium culture, metabolic waste products, as well as autocrine growth factors accumulate around the cells and tissues [208; 209; 211].

Diffusion/Convection

The effects of diffusion and convection differ considerably in magnitudes. Local differences in density of the extracellular medium at microgravity can no longer lead to convective currents around cells that are surrounded with free fluid as they would under normal gravity conditions. In the absence of microconvective flows, the mixing of gases, solutes, solvent molecules, and local

temperatures would depend predominantly on diffusion, and thus be reduced dramatically (**info box 3**).
From the info box it can be conceived that convective currents can cover a distance much faster than diffusion. In other words, we can expect that cells may "get stuck in their dirty bathwater" during prolonged exposures to microgravity. Some of the possible consequences of such poor mixing on cells are effects on their cytoskeleton, membrane potential, and cell behaviour [209].

Buoyancy
Buoyancy is the capacity to float in a liquid medium or even a gas. Buoyancy can also result in convection of a part of a liquid or gas to rise. Local changes in temperature or composition can result in density variations and can therefore cause convection. The force of buoyancy works opposite to gravity, and is proportional to the objects volume and the weight of the liquid. Buoyancy driven convection is not present in a weightlessness environment, making it an important difference between ground and space experiments [208; 209].

Info Box 3

A typical diffusion constant of salt in water (e.g. K^+ ions in water) is D = 0.001 mm^2/s.
In order to cover a distance s = 2 mm by diffusion, it would take the K^+ ions approximately:

$t = s^2/D$

$t = 4/0.001 = 4000$ s = 1.1 hrs

s = displacement distance
D = diffusion constant

In contrast, convection currents that results from density differences can cover the same distance much faster. Assume a spherical particle with radius r = 0.1 mm in water. Water density of water $\rho = 1.0$ g/cm^3 and the viscosity $\eta = 0.01$ poise. Assume the specific gravity of the particle is 1% more than water, $\rho = 1.01$ g/cm^3. At normal gravity of g = 9.81 m/s^2 the particle will sink at speed v at which Stokes friction F equals the gravitational pull:

$F = 6\pi r\eta v = 4/3\pi r^3(\rho_{water}-\rho)g$

Consequently,

$v = (2/9\eta)(\rho_{water}-\rho)\ r^2g = 0.2$mm/s

In other words, the K^+ ions in a convective current that resulted from only a 1% difference in density would cover the distance of 2 mm in 10 s, which is 400 times faster as by diffusion alone. In short, convective currents, not diffusion, are responsible for the rapid mixing of gases and solvents.

On the use of ground-based machines or simulating hyper- & microgravity
There are several advantages and reasons to perform ground-based research studies prior to conducting experiments in space. Most ground based research facilities are readily available and the studies may be performed on a day to day basis and result in sound scientific data. Ground based research is interesting to perform basic acceleration studies and test the effects of

accelerations in preparation of real microgravity experiments onboard orbiting spacecraft, sounding rockets, or parabolic flight aircraft. Also, to define the future space-experiment setup, it is interesting to investigate more specifically, the parameters which might be involved in real microgravity conditions. Ground based studies are sometimes necessary to test hardware performance under simulated hypergravity conditions. Particularly, the effects of launch accelerations and vibrations on the test system whether or not in combination with the hardware [209; 212-214].

Ground facilities are differentiated in hypergravity and microgravity. In case of simulating hypergravity, the Medium Sized Centrifuge for Acceleration Research (MidiCAR) is a dedicated centrifuge in which cell or tissue samples may be exposed to accelerations up to 100 times Earth's gravity[215-217].

A more extended range of machines are available to simulate microgravity; The Random Positioning Machine (RPM), sometimes referred to as 3D-clinostat, simulates microgravity on the principle of gravity vector averaging. Since gravity is a vector (it has both a magnitude and a direction), constantly changing the samples position with regard to the Earth's gravity vector direction, the sample may experience this as a zero-gravity environment. The level of simulation within this RPM depends very much on the speed of rotation and the distance of the sample to the centre of rotation [218].

The Free Fall Machine (FFM) can simulate microgravity for long duration experiments. The system is based on the principle of free fall. The free fall periods are at a maximum of about 900 ms and are interrupted by an acceleration of around 20 x g for about 20-80 ms. The hypothesis, developed by Dr. Mesland, is that systems may experience the FFM as a continuous free fall environment if the gravity perception time in the FFM of the system is shorter than the intermediate period of 20-80 ms [219].

The working principle of a parabolic aircraft is exactly the same as that of a FFM, only the time courses are different. To create a microgravity environment the aircraft follows a trajectory: starting from a steady normal horizontal flight, then the aircraft pulls up for about 8 seconds. Engine thrust is then reduced strongly to set-in the parabolic flight. Microgravity is maintained for about 15 sec, after which the aircraft pulls up to come back to steady horizontal flight. The number of parabolas flown per flight hour is between 12 and 15, depending on the interval time between parabolas [209; 213; 214].

Cell response to changes in gravity (gravisensing)

The consequences of basic physics do not mean that it is impossible for cells to sense gravity. After all, they are biological systems of immense complexity that have evolved to expect gravity to be present at all times. Yet, there is no reasonable possibility for cells to sense gravity because all other forces (electrical, polymerisation, surface) in the cellular world are so much greater. Although, cells are indeed too small to experience gravity as a major force, the cellular environment is considerably larger, and it is influenced by gravity through the generation of pressure, and buoyancy-driven convective currents for example. The cells may sense the gravity-mediated changes in their environment, and thus may be able to detect gravity after all, albeit in an indirect way [220-226].

The term gravisensing means, ideally, the detection of the amplitude and direction of inertial acceleration. There a very few direct ways for cells to detect the direction (to tell up from down) because their own weight is too small compared to other forces, but they are able to detect the amplitude of gravity. Some cells are equipped with specialised gravisensing organs or are specialised gravisensors themselves, like hair cells and statocytes in plant roots [227; 228]. Most

cells are not specialised for the task, but respond to microgravity conditions by indirect consequences of the lack of inertial acceleration.
Although it is clear that physical forces, such as those due to gravity, are fundamental regulators of tissue development, little is known about how living cells sense these signals and convert them into biochemical response. This transduction process, which is at the core of gravity sensation, is known as mechanotransduction [229; 230]. Mechanotransduction at work can be observed in the otoliths in the sensory cells of the inner ear. Another well known example is bone-resorption observed in space farers who undergo long term space flight. The change in loading pattern, caused by microgravity, results in immediate bone remodelling [128; 231]. Osteoblasts and osteoclasts, responsible for this remodelling do not possess statoliths, yet the bone cells and other cells within other tissues are sensitive to mechanical forces. A potential gravity sensor could be the nucleus of the eukaryotic cell. The packed DNA inside the nucleus makes the nucleus about 18% denser compared to the rest of the cell. This higher density makes the nucleus sink to the bottom of the cell in 1 g environment; however, the cytoskeleton actively maintains the nucleus in place. This process transfers a nuclear load to the cytoskeletal fibres adjacent to the nucleus and could be another way of transducing information about the direction and magnitude of the gravity field the cell is subjected to [208]. A molecular mechanism for sensing nuclear positioning is until now unknown.
Extensive research has been performed on the lymphocytes in microgravity [195; 232-235]. Depending whether the lymphocytes are free floating or adhered, the mitogenic response of the cells is reduced. It appears that Interleukin-1, -2 activated lymphocytes need to anchor and spread in order for a sound response [236; 237].
In case of fibroblasts, mechanical stress is an important and specific regulator of distinct extracellular matrix (ECM) components. There are different possibilities how genes for an ECM component are regulated by mechanical signals. In a primary response, a cellular mechanotransduction pathway activates available transcription factor, which in turn binds to "mechano-responsive" regulatory element in the ECM gene promoter. A secondary response would be a mechanical signal which induces the transcription and synthesis of nuclear factor, which transactivates an ECM gene (fibronectin is an ECM gene transactivated by transcription factor EGR-1, via the ras/Erk-1/2 pathway). A third possibility is that mechanical stress induces synthesis and/or secretion of growth factor, which indirectly regulates ECM gene expression via auto- or paracrine feedback loop of TGF-β. This latter possibility seems to be responsible for long term adaptive responses of connective tissues to mechanical stress. Gene array studies of the cells grown in microgravity show remarkable effects. There are increases and decreases of message levels for many genes in microgravity. This implies that cells are mounting a massive gene regulatory effort to acclimate to the microgravity environment and maintain homeostasis. Long term focusing on protein expression (proteomics) could supply information on the cells adjustment (acclimation) to the space environment [238].
The effects of altered gravity on the cell membrane and the cytoskeleton have been studied; the more extensive studies try to link the environment factor gravity (usually microgravity) with the functional status of membranes and elements of cytoskeleton. The involvement of membrane structures and cytoskeleton in the processes of reception and realization of gravitational stimulus allows us to evaluate the extent of the direct or indirect influence of gravity on cell functioning in the gravitational field [239; 240]. Gruener *et al.* [241] studied the effects of altered gravity on the aggregation of the nicotinic acetylcholine receptors and the structure of the cytoskeleton in cultured muscle cells. Marked changes were shown in the distribution and organization of actin filaments and a reduced incidence of acetylcholine receptor aggregates. Hence, they concluded that the sensitivity of synaptic receptor aggregation and cytoskeletal morphology suggests that

microgravity alters cell behaviour. Investigations more focussed have revealed changes in both the microfilament and microtubule network. Thinning and redistribution of MF [242] the loss of self-organisation and local disorders of MT as well as a general increase in apoptosis in the early phases of microgravity have all been described [235; 242-244]. Seitzer *et al.* [245] noticed in human fibroblasts a increase in collagen synthesis under microgravity conditions, while increasing g resulted in decreasing collagen production compared to 1 g controls. Croute *et al.* [246] had human dermal fibroblasts undergo hypergravity (2-20 g) for up to 8 days. Changes in cell shape (star shape with fine filopodia) and anchoring point arrangement appeared above 15 g. The centrosome was shown to migrate to the nucleus side compared to the above the nucleus position in 1 g controls. The fibronectin network thickened after 8 day of culture and collagen fibrils appeared linking ordered arrays of fibres. According to their observations hypergravity can induce change in fibroblast cell shape, migration way, and anchorage leading to reorganisation of ECM without a hampering cell proliferation. Recently, Tabony *et a*l. [247] tackled the problem of molecular processes in biological systems affected by gravity. Since (bio)chemical reactions do not depend upon gravity, it is proposed that biological systems might depend on gravity by way of the bifurcation properties of certain non-linear chemical reactions [223; 244; 248]. By way of combining reaction and diffusion the homogenous solution spontaneously self-organises and may determine the morphology that develops. They found that MT shows this behaviour. Experiments carried out under low gravity conditions show that the presence of gravity at the bifurcation time actually triggers the self-organising process. This is an experimental demonstration of how a very simple biochemical system, containing only two molecules, can be gravity sensitive. As microtubule organisation is essential to cellular function, it is quite plausible that the type of processes described in that article provide an underlying explanation for the gravity dependence of living systems at a cellular level.

As already mentioned the cell-matrix adhesion contacts form the physical link from the ECM across the cell membrane to the cytoskeleton. Because of their strategic location, transmembrane proteins within cell-matrix contacts are good candidates for translating mechanical into chemical signals. Two such transmembrane components have been implicated: ion channels and integrins. In mechanosensory nerve and muscle cells, stretch sensitive cation channels are likely to be the actual strain gauges; they might be attached directly to the ECM and/or the cytoskeleton within matrix contacts. In non-excitable cells such as fibroblasts, ion channels could be involved in mechanotransduction as well, but in addition there are integrins which can work as stretch sensors themselves. Immediate consequences of ECM derived forces transmitted to integrins are the Rho-dependent assembly and growth of focal adhesion complexes at these sites, an increase in cytoskeletal tractional force, and a triggering of MAP kinase and NF-κB pathways within the cell. Fibroblasts undergoing tension, within collagen matrices, induce transient activation of MAP kinase (p38 and ERK) indicate that MAP kinases can act as general, but unspecific, transducers of mechanical stress towards the cell interior [249].

Gravity alterations, being a physical environmental signal, causes shift in morphological cell characteristics. Although the direct influences of gravity are more pronounced in vitro and non-direct influences usually expresses themselves in unicellular organism in vivo. Morphogenesis processes like adhesion and locomotion are controlled by the cells' signal transduction systems. And gravity affects such processes. Minor disturbances in this system coming from the environment, due to amplification, may provide significant modulations of the signals. Research of this system at the level of molecular cell reception is of great importance and interest [250].

Like all living biological systems, a cell needs both energy and information in order to function. Gaining insight into the mechanisms responsible for cell gravisensitivity can be acquired by

solving the fundamental problem of molecular physiology. Morphological deformations are the result of spatial disposition changes of cells in the gravitation field. The cell shape is altered at one point [250]. Interaction between cell shape and functions present one of the most intriguing problems in biology. 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, many important details have to be solved. For instance, the biophysical mechanism which converts a mechanical stimulus into a chemical signal. Also the specificity of the cellular response to externally applied mechanical stress. Since forces propagate throughout the cell, any vector stress will be sensed as tension in one part and compression in another part of the cell. One stimulus triggers distinct signals in different locations, resulting in an array of signalling pathways [238]. Wherever a cell response occurs, one should look first of all for an extracellular cause, like a gravity dependent process. Secondly an intracellular, cytoskeletal, cause should be sought in the context of weak zones of the cytoskeleton.

RESEARCH OBJECTIVES

Summary

The components of the cytoskeleton and the focal adhesions of the cell are key players in the cells' adhesion and locomotion across the microtextured surface of substrates. The orientations and morphology of the cells are important, for these cells should resemble correct normal tissue as closely as possible if it wants to be effective in repairing or constructing tissue. Pattern formation or contact guidance is involved in meeting those requirements. Previous studies have expanded the knowledge and understanding how these surface topography principles work and have resulted in the development and improvement of biomaterials used in medical implants. With humans, slowly but steadily, expansion into space, new questions are raised and new problems are faced. Reports of involvement of the cytoskeleton and focal adhesions in relation to microgravity and the changes in cell morphology due to gravity have raised questions how this comes about. Do cells sense changes in gravity directly, what intracellular process and signalling pathways are involved? The field of gravitational cell biology will obtain that knowledge and comprehend the behaviour of cells in changed gravity circumstances. This knowledge could be used in helping the development and manufacturing of tissue engineered constructs suitable for use on this world or beyond.

Research Hypothesis

The response of cells *in vitro* towards micro-structured surfaces will drastically change when exposed to micro- and hyper-gravity conditions, and that this will be a useful tool in elucidating the cell mechanosensing process.

Therefore, the influence of hypergravity and simulated microgravity and substrate surface micro-morphology on cell appearance and differentiation will be investigated.

Research Questions

1. Does the cellular morphological behaviour *in vitro* to standardised, well characterised surfaces relate to the geometrical properties of these surfaces under different gravity conditions?

2. Do the intracellular cytoskeletal components orientation, organisation, and distribution differ between cells cultured on smooth and microtextured surfaces under different gravity conditions?

3. Is the cellular response to microtexture altered if different topography dimensions are used?

4. What intracellular signalling pathways are triggered under simulated microgravity conditions?

5. What genes are up/down-regulated under micro/hyper-gravity conditions?

6. To what topography dimension do cells still respond by adjusting their cell shape?

7. Can the MidiCAR centrifuge be used as a model to study inertial shear force?

8. Evaluation of the suitability of different ground-based machines designed for the simulation of micro/hyper-gravity. Are the results comparable with earlier findings in space?

9. Several scientific publications propose mechanisms for the response of cells to gravity alterations (gravisensing). Can any of these theories be proven true or denied?

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