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MOLECULAR BIOLOGY OF CELL REACTIONS TO SURFACE TOPOGRAPHY

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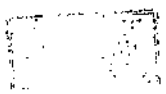

SUMMARY

The contact of cells and surface topography plays a major role in the control of cell growth, differentiation, tissue morphogenesis and pattern formation. The surface topography on which cells grow may alter their cellular interactions and this in turn may affect gene expression. In this study, I identify differentially expressed genes on macrophage-like cells cultured on planar or grooved fused quartz substrata produced by microfabrication. The mRNA differential display method was applied to compare mRNAs from the cells which were cultured on different surface. The differentially expressed genes were detected by reverse transcribed mRNA into cDNA which were used as the template in the polymerase chain reaction (PCR). By using mRNA fingerprinting method, Northern blot confirmation and antibody staining, I found that *c-kit*, Mouse *tctex-1* (t-complex testis expressed 1), *α-N-acetylgalactosaminidase, elastin, NRF1* genes and four unknown DNAs are considerably enhanced by contact with topography. These results indicate that surface topography can regulate and stimulate some specific genes.

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ABBREVIATIONS

ATP	Adenosine triphosphate
BHK	Baby Hamster Kidney cells
cDNA	complementary deoxyribonucleic acid
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
dNTP	2'-deoxyribonucleotides
EtBr	Ethidium bromide
F-actin	Filamentous actin
FAK	Focal adhesion kinase
FITC	Flurescein isothiocyanate
FISH	Fluorescent in situ hybridization
GTP	Guanosine triphosphate
IGF	Insulin-like growth factor
mRNA	Messenger ribonucleic acid
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDS	Polydioxanone suture material
SDS-PAGE	Sodium dodecylsulphate polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy

SDS	Sodium dodecyl sulphate or sodium lauryl sulphate
SH2	Src-homology domain 2
SH3	Src-homology domain 3
UV	Ultra violet light

CHAPTER 1

INTRODUCTION

Many cell types are able to respond to surfaces which bear appreciable topography such as grooves. This includes changes in shape, movement and general morphology (Curtis and Clark, 1990; Curtis and Wilkinson, 1995). Surface topography can affect cell shape in a way which leads cells to become polarised and elongated in the direction of the grooved substrata. Although there is a great deal of information on how the surface topography of an implant might affect the morphology of adjacent cells and tissues both *in vivo* and *in vitro*, there is little information on the effects on cell function at the molecular level.

It has been pointed that cell shape can regulate cell growth (Watt *et al*, 1987), cytoskeleton gene expression (Ben Ze'ev, 1984), collagenase and stromelysin gene expression (Werb *et al.*, 1989) and cell differentiation. Chou *et al* (1995) found that fibroblasts responded to groove topography by increased production of the mRNA for fibronectin and the same group (Brunette *et al.*, 1995) found that bone cell mineralisation was enhanced by growing cells on grooves. An understanding of how cells respond to the surface topography on which they grow could enable us to produce more suitable prosthetic devices, in order to produce the desired cell responses. This could ultimately be used to further enhance wound healing whilst an implant is being applied.

THE HISTORY OF STUDY ON CELL-TOPOGRAPHY REACTION

Topography refers to the shape of the substrate on which cells are growing. Ross Harrison first observed (1910 and 1914) cell reactions to surface topography in cell culture experiments with plasma clots and spider web-fibres and described how the behaviour of the cells was influenced by fine silk threads. Cells were oriented and their movement guided by these fine silk threads. Years later, Weiss (1934) also reported that cell shape changes in correlation with the fibrin network of clotted plasma. Weiss named this phenomenon of cell orientation by underlying topography "contact guidance". Weiss and Garber then carried out a further study using a wide variety of substrates (Weiss and Garber, 1952, Weiss, 1945, 1959).

MATERIALS USED IN VITRO TO STUDY TOPOGRAPHY

For a long time, little research was undertaken *in vitro* on the reactions of cells to surface morphology, mainly because there were few materials with a well defined and constant surface morphology (Singhvi *et al*, 1994). The available techniques for making such topographies lacked a good range of dimensions and the surfaces were complicated due to heterogeneties introduced during fabrication. Photolithographic techniques which were used in the electronics industry solved this problem (Camporese *et al.*, 1981, Meyle *et al*, 1991). Using this technique, features in the micrometer range can be precisely etched onto a variety of substrates, including: quartz (Dunn and Brown, 1986, Wood, 1988), plastic (Clark *et al*, 1987 and 1990), glass (Singhvi *et al*, 1992), and titanium-

coated silicon (Brunette *et al*, 1983, 1986,1988; Chehroudi *et al*, 1990 and 1992).

Early studies on topographically patterned substrata used biological materials (Singhvi, 1994). The employment of this kind of material could lead to misinterpretation of the results. First of all, the chemistry of the material could be affecting the behaviour of the cells (Curtis and Clark, 1990), and thus topography may not be the only factor involved. Secondly, the chemistry of such substrata could be variable. Some biological substrata such as collagen and extracellular matrix fibrils have been shown to align cells *in vitro* (Dunn 1991). Manufacturing substrates has the advantage that the material can be designed to precise dimensions and will be well characterised in terms of chemistry. In this study, I used quartz structures which can be reused after acid cleaning.

Table 1. Materials used for topography study (adapted from Curtis *et al.*, 1997)

Elements
Gold
Titanium (its surface oxidises) (Brunette *et al.*, 1983)
Silicon (its surface oxidises)
Carbon (diamond and diamond-like materials)
Inorganic compounds
Silica (Clark *et al.*, 1990)

Lithium niobate

Silicon nitride (Breckenridge *et al.*, 1995)

Polymers

Polymethylmethacrylate (Clark 1987)

Silicones (den Braber *et al.*, 1995)

Epoxy (unspecified) (Chehroudi *et al.*, 1995)

Polydioxanone

Nylon (Curtis and Seehar 1978)

Cellulose acetate (Curtis and Varde, 1964)

Polyimide (Breckenridge *et al.*, 1995)

Collagen

Fibrin (Weiss, 1964)

Table 1. Materials used for topography study (adapted from Curtis *et al.*, 1997)

SURFACE ENERGY AND CHEMISTRY

Surface energy is an important factor which affects cell adhesion and other cell properties. Boyan *et al* (1996) suggested that the surface charge creates a local environment which decides the surface tension, surface energy and adhesion energy. Hydrophilic and wettable surfaces are high in energy. It is generally accepted that materials with high surface energy are biologically active and promote cell attachment, whereas materials with low surface energy are more likely to produce a capsule of amorphous, scarlike tissue (Baier *et al.,* 1970; 1984; 1988). The surface energy and wettability of a material can be increased temporarily or permanently by radio-frequency glow discharge (Baier *et al.*,

1984; 1988), which can wash away the organic contaminants and, can render implants sterile, highly hydrophilic and very receptive to adhesion (Doundoulakis 1988; Gombotz *et al.*, 1987).

CELL PROPERTIES AFFECTED BY SURFACE TOPOGRAPHY

Cells are exquisitely sensitive to surface topography and, a wide variety of cell properties can be affected. The phenomena are not mutually exclusive, for example, cell adhesion is required for contact guidance, so that the overall response of cells to a surface may be the result of several contributing processes.

-CELL ADHESION

Surface topography can affect cell adhesion, but the particular effect observed can depend on cell type (Curtis and Clark, 1990; Chehroudi and Brunette, 1995; Curtis and Wilkinson 1998), and moreover passage number. For example, primary chondrocytes show no significant alignment to grooves of any width, but 1st and 2nd passage show significant alignment to grooves over 2 microns in width and 1.23 microns in depth (Douglas Hamilton, personal communication). Osteoblast-like cells demonstrated significantly higher levels of cell attachment on rough, sandblasted surfaces with irregular morphologies than on smooth surfaces (Bowers *et al.*, 1992), slightly more oral epithelial cell attached to a microfabricated, grooved surface than to a control smooth surface (Chehroudi *et al*, 1988,1989). Macrophage-like cells prefer roughened surfaces whereas human

gingival fibroblasts prefer smooth surfaces (Kononen *et al.*, 1992). A result of the different responses of cell populations to surface topography is the possibility of cell selection. However, all the cell types that might interact with the surface should be considered.

A possible explanation for greater cell attachment on roughened surfaces is that such surfaces present a greater surface area for cell attachment. However, the regular geometry of micromachined surfaces allows the surface area of smooth and grooved surfaces to be calculated. The increase in epithelial cell attachment on grooved surfaces is not simply the result of the surface area (Chehroudi *et al.*, 1988,1989, and 1990).

-CELL SHAPE

The surface topographies of many implant devices can alter cell shape (Brunette, *et al*, 1988,1995), and there is abundant evidence that cell shape can regulate cell growth (Folkman and Moscona, 1978), cytoskeleton gene expression (Ben-Ze'ev, 1987; Ben-Ze'ev, 1991; Hong and Brunette, 1987; Chou *et al*, 1995) , collagenase and stromelysin gene expression (Werb *et al.*, 1986), extracellular matrix metabolism (McDonald *et al*, 1989) and differentiation (Watt *et al.*, 1988). Indeed, Ben Ze'ev has suggested the concept that the architectural features of eukaryotic cells determine cell shape and contact is becoming a central paradigm of cell biology (Ben Ze'ev, 1987).

There have been some isolated studies done such as, the growth of an oral epithelial cell line on a microgrooved surface altered cell shape by increasing cell height and also the secretion of plasminogen activator (Hong and Brunette, 1987). Similarly, the growth of human gingival fibroblasts on microgrooved surfaces resulted in the alteration of levels and stability of messenger ribonucleic acid (mRNA) for the major cellular-adhesion protein, fibronectin (Chou *et al.*, 1995, 1996). Despite this, it is somewhat surprising that relatively little work has been done about the effects of surface topography on properties associated with cell shape changes, such as gene expression.

-CELL MIGRATION

Cell migration is an important phenomenon in development. It is involved in organ formation, embryogenesis (Nakatsuji *et al.*, 1982), migration of leukocytes in inflammatory response, germ cell migration, tumour cell migration in the circulatory system during metastasis (Parish *et al.*, 1987), and wound healing.

Curtis *et al* (1995) investigated the effects of various grooved substrata on the speed of cell movement. The speed of movement of endothelial and epitenon cells was significantly increased when cells were grown on microgrooved silica, compared with flat surfaces. Neurite extension was also increased about four fold on the grooved substrates. Indeed, Curtis *et al* (1995) suggested that such cell behaviour should have a major effect on the speed of tissue repair. Cell migration is crucial to tissue engineering, playing an important role in the

colonization of biomaterials and the mechanical interlocking of tissue to implant. Efficient wound healing also relies on the migration of fibroblasts and vascular endothelial cells.

SPECIFIC SURFACES

Bowers *et al* (1992) reported that studies involving dental implant surfaces suggested that topography plays a crucial role in implant success. Ratner (1993) noted that our existing biomaterials, although demonstrating acceptable clinical success, are developed on the basis of trial and error optimization, and are not designed in advance to produce the desired biological responses. Overall, the link between surface energy and topography is an undeniable critical factor in cell response to the surface.

-SMOOTHNESS

Fibroblasts and collagen bundles respond to smooth subcutaneous implants by aligning parallel to the implant surface, forming a capsule (Dunn 1976; Thomsen *et al*., 1985; Kasemo *et al*., 1988; Von-Recum *et al*., 1993; Bauman *et al*., 1988). The thickness of the capsule is often correlated to the degree of biocompatibility (Von-Recum *et al*., 1981, 1993; Bauman *et al*., 1988). There is a hypothesis which stresses the role of macrophages and giant cells that accumulate on rough but not on smooth surfaces and which inhibit sarcoma induction. It has been reported that the secretion of immune mediators such as interleukins and tumour necrosis factor and the activation of the complement

system relate directly to the surface area, degree of roughness and surface irregularities of the substratum (Perala *et al.*, 1992; Rahal *et al.*, 1993; Labarre *et al.*, 1993). Thus, smoother surfaces are expected to produce fewer immune-related responses.

-ROUGH/POROUS

It appears that slight roughness and porousity might favour the adhesion of those cells that form focal contacts (Curtis *et al.*, 1964). However, Curtis and Varde (1964) noted that some methods of producing rough surfaces may also alter the surface chemistry. For example, abrasive particles used to roughen the surface may be embedded into the surface; similarly, when reactive ions such as oxygen are used to etch the surface (Clark *et al.*, 1991), they may alter the surface chemistry. Clark *et al* (1991) found that, though an initial exposure of Perspex (polymethyl methacrlate) to oxygen plasma slightly roughens the plastic and increases its adhesiveness for cells. Longer exposures, though increasing the roughness of the plastic, did not further increase its adhesiveness. Murray, Rae, and Rushton (1989), in an *in vitro* experiment showed that rough surfaces on a variety of polymers, glass and stainless steel encouraged macrophages to release mediators that stimulate ion resorption.

-MICROFABRICATED SURFACES

Microfabrication techniques have produced highly ordered and precise topographies on which different aspects of a surface such as depth, repeat

spacing, and shape can be precisely controlled by different steps in the process. These structures include both v-shaped (Chehroudi *et al,* 1990; Orara and Buck, 1979; Dunn, 1982; Brunette, 1986, 1988) and vertical (Clark *et al,* 1990; Wood, 1988; Meyle *et al*, 1991) parallel grooves, with dimensions ranging from; 130 nm- 162 μm wide; 100 nm-92μm deep; 2 μm - 220 μm repeat spacing (Pitch). In general, the findings were that cells aligned to the long axis of the grooves. Figure 1-1 shows that P388D1 macrophage cells align on the groove ridge topography.

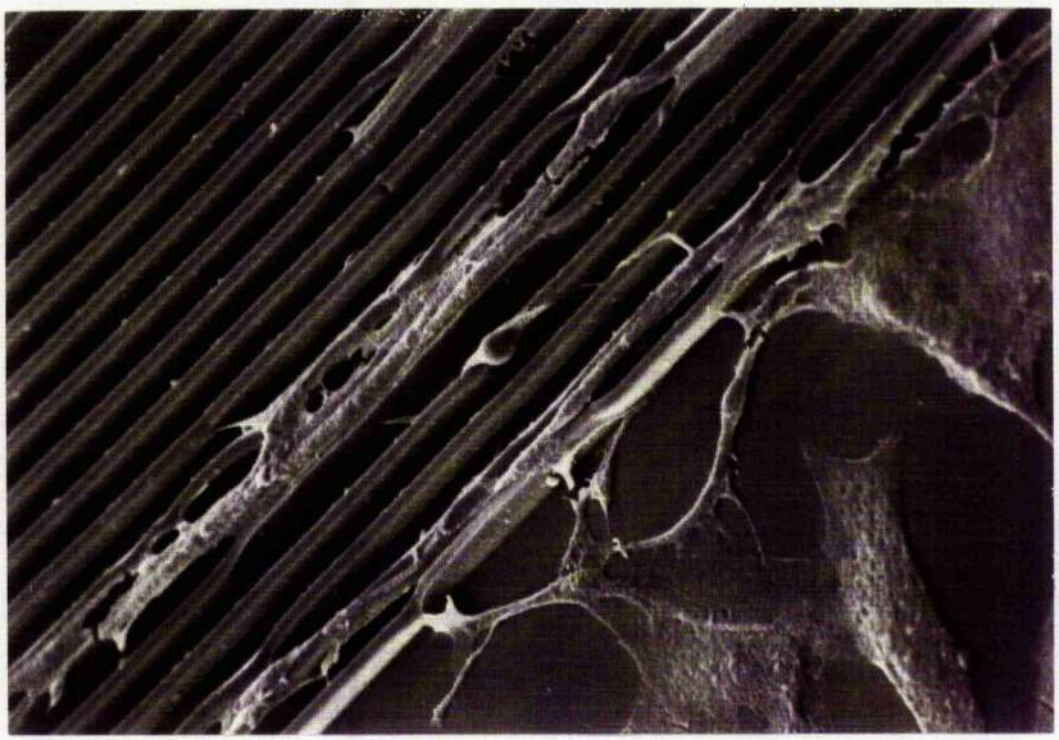


Figure 1-1. P388D1 macrophage cells aligning on grooved surface in comparison with plain surface. (Adapted from Curtis *et al*., 1997)

Brunette *et al* (1986) found that, when using v-shaped grooves, there was no effect on cell orientation when the repeat distance and groove depth was varied. But subsequently the same group showed that cell orientation was dependent on groove depth (Chehroudi *et al.*, 1990; Chehroudi *et al.*, 1992; Hong and Brunette, 1987). These contradictory results may have occurred due to the fact that many of the initial experiments were carried out using groove structures that had dimensions larger than the size of typical cells. Dunn and Brown (1986) showed improved alignment of chick heart fibroblasts on structures of narrower ridge (space between grooves) width, when the cells were grown on grooved substrata with varying groove width, ridge width, and groove depth. They concluded that, over the range 1.6 μm -9.0 μm groove width and 1.4 μm - 23 μm ridge width, the spacing between grooves is more important in alignment than groove width.

Clark *et al* (1990) used Baby Hamster Kidney (BHK) cells and Madin darby canine kidney (MDCK) cells and found that pitch can have a significant effect on cell alignment, but the effect of depth is much greater. Clark *et al* (1991) did further studies and showed that ultrafine grooved substrata with 260 nm width and 100 to 400 nm depth have the same effect on cell guidance. Overall, the grooved guidance on cells of both nano- and micro- scaled structures was cell-type dependent and also related to cell-cell interactions (Clark *et al.*, 1991).

A detailed study showed that depth was of greater importance in determining cell alignment than pitch or width. The effects vary from one cell type to another. Microfabricated surfaces can induce cell contact guidance *in vivo* that results in inhibition of epithelial growth on percutaneous devices (Clark *et al.*, 1991).

-OTHER TOPOGRAPHY

Apart from the surface topography mentioned above, there have also been studies on other topography such as fibers, tubes and spheres. Weiss (1945) first reported the orientation of Schwann cells on glass fibres. Other work has since been carried out. It seems that there are only two studies of cell orientation and spreading on spherical surfaces (Maroudas, 1972; Dunn, 1982). Dunn found that motility and spreading were reduced on beads of less than 50 μm radius. Dunn also found a degree of orientation at right angles to the tube axis in 100 nm diameter tubes in a macrophage migration inhibition test. Recently, Riehle *et al* (1998) tested the behaviour of several cells types in a tube with varied diameters, and found that all cell types can be successfully grown in quartz tubes for up to 21 days. The cells would attach to the concave surfaces even when the radius of curvature was as low as 25 μm. The chondrocytes, osteoblasts and epitenon cells tended to detach from the walls of the tubes, forming long cords of cells attached to the walls at a few points. Endothelial cells did not detach.

GENERAL DISCUSSION

It is well established that the surface topography of an implant is an important factor affecting several cellular functions such as cell shape, migration, adhesion and tissue organisation. Although there is a good deal of information on how the surface topography of an implant can affect the morphology of adjacent cells and tissues, there is little information on the effects on cell function at the molecular level. In the long term, these specific approaches to monitoring the outcomes of altering surface topography, combined with methods such as microfabricating that produce surfaces with precisely calculated surface topographies, have the potential to yield information that will enable future implants to be clothed in "designer topographies" that reproducibly produce desired cell responses.

ROLE OF MACRPHAGES ON TISSUE REPAIR

When implants were first carried out, a lot of macrophages were found in surrounding tissue and on the surface of the implants. Macrophages accumulate after neutrophil influx has ceased through recruitment of peripheral blood monocytes by monocyte-specific chemoattractants generated at the wound site. Monocyte-specific chemotactic factors include ECM protein degradation products such as collagen fragments, elastin peptides, and fibronectin fragments (Clark *et al.*, 1988) as well as enzymatically active thrombin and transforming growth factor (TGF)-β (Wahl *et al.*, 1987).

Once the monocyte arrives in the tissue, the very act of adherence appears to stimulate the monocyte to undergo a phenotypic metamorphosis to a macrophage, including induction of selective messenger RNA expression (Haskill et al., 1988). Macrophages can also release a plethora of biologically active substances such as reactive oxygen intermediates, prostaglandins, leukotrienes, chemotactic factors, growth factors, and enzymes including proteases (Werb and Gordon, 1975).

Although many of the substances released by macrophages at a wound site facilitate the recruitment of additional inflammatory cells and aid the macrophage in tissue decontamination and debridement, growth factors and some chemotactic factors are necessary for the initiation and propagation of granulation tissue (Liebovitch and Ross, 1975). The growth factors that macrophages are known to synthesise and secrete include platelet-derived growth factor (Shimokado *et al.*, 1985), fibroblast growth factor (Baird *et al.*, 1985), TGF-β and the epidermal growth factor-related TGF- α (Madtes *et al.*, 1988). Most of these growth factors stimulate cell migration and proliferation into wounds (Sprugel *et al.*, 1987), but not ECM production (Laato *et al.*, 1987). However, TGF--β specifically induces collagen and fibronectin messenger RNA expression in human dermal fibroblasts and induces fibrosis *in vivo*. Thus, these macrophage factors probably act in a synergistic paracrine fashion to stimulate new tissue formation (Clark, 1989, Review).

THE ROLE OF EPITENON IN TENDON REPAIR

Tendons are highly specialised structures which transmit the forces of muscle contraction to distant bones thereby producing the movement at joints and hold bones together. To fulfil this function, they must be strong, flexible and have the ability to glide. Their strength and flexibility is provided by a core of longitudinally arranged collagen bundles among which are scattered small numbers of tendocytes. Their ability to glide is provided by a coating layer of synovial cells known as epitenon cells.

When a tendon is injured, healing takes place by a proliferation of epitenon cells which migrate into the gap between the divided tendon ends and synthesize collagen which restores the strength and continuity of the whole structure (Linsday *et al.*, 1961). Surgical repair is carried out by holding the tendon ends together with a relatively strong core suture and a fine circumferential suture to approximate the two epitenons. However, two major complications can occur and produce poor results. First, a number of tendon repairs disrupt, usually at an early stage, resulting is a complete loss of function and requiring salvage surgery. Secondly, and much more commonly, fibrous adhesions may form between the tendon and synovial sheath due to uncontrolled migration of epitenon and synovial cells into the synovial space, preventing gliding movements. (Linsday *et al.*, 1961; Rothkopf *et al.*, 1991).

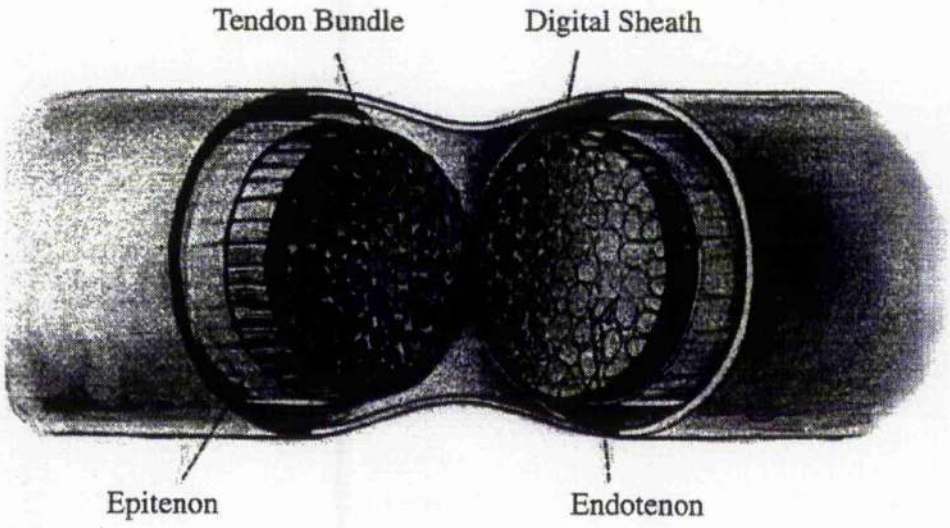


Figure 1-2. Schematic drawing showing the anatomy of a healthy intrasynovial flexor tendon. (Adapted from Gelberman *et al*, 1983).

EXTRACELLULAR MATRIX AND ITS COMPONENTS

Cells are surrounded by extracellular space, which is largely filled by an intricate network of macromolecules constituting the extracellular matrix (ECM) which provides the strength of soft tissues and the rigidity of skeletal tissues (Bernfield, 1989). This matrix is composed of a variety of versatile proteins and polysaccharides that are secreted locally and assembled into an organized meshwork in close association with the surface of the cell that produced them. In connective tissues the matrix is frequently more plentiful than the cells that it surrounds, and it determines the tissue physical properties. The matrix can

become calcified to form the rock-hard structures of bone or teeth, and it also can adopt the ropelike organisation that gives tendons their enormous tensile strength.

Until recently the vertebrate extracellular matrix was thought to serve mainly as a relatively inert scaffolding to stabilize the physical structure of tissues. But it is now clear that the matrix plays a far more active and complex role in regulating the behaviour of the cells that contact it- influencing their development, migration, proliferation, shape and function. The two main classes of extracellular macromolecules that make up the matrix are (1) glycosaminoglycans (GAGs) which are made of polysaccharide chains and are found in the form of proteoglycans. (2) fibrous proteins of two functional types: mainly structural (for example, collagen and elastin) and mainly adhesive (for example, fibronectin and laminin).

-GAGS

Glycosaminoglycans (GAGs) are unbranched polysaccharide chains composed of repeating disaccharide units. One of the two sugar residues in the repeating disaccharide is an amino sugar (N-acetylglucosamine or N-acetylgalactosamine), and the second sugar is usually a uronic acid (glucuronic or iduronec). Hyaluronan (also called hyaluronic acid) is the simplest of the GAGs which serves to create a cell free space into which cells subsequently migrate, when cell migration ends, the excess hyaluronan is generally degraded by the enzyme

hyaluronidase. Hyaluronan is also produced in large quantities during wound repair, and it is an important constituent of joint fluid, where it serves as a lubricant (Prehm, 1984; Toole, 1991).

-COLLAGENS

Collagens are the most abundant components in the extracellular matrix of connective tissues (Bornsein *et al.*, 1980). There are approximately nineteen different types of collagens and other non-structural proteins which contain at least one collagen triple helix as a structural motif. All collagens contain a three stranded helical structure and globular domains which determine the structural role of collagens, for example, type I collagen. Collagen Fibres are used as the reinforcing construction of bone, and various collagen types have been shown to promote the adhesion and migration of a variety of cell types (Dedhar *et al.*, 1987a).

-FIBRONECTIN

Fibronectin is an extracellular adhesive protein that helps cells attach to the matrix. It is composed of two very large subunits joined by a pair of disulphide bonds near their carboxyl termini, each subunit contains a collagen binding region, cell binding region and heparin binding region. A crucial sequence in cell binding domain is Arg-Gly-Asp-Ser (RGDS). The first three amino acids are particularly important, the RGD recognition site, as they are the essential structure recognised by most cells in fibronectin. (Dedhar *et al.*, 1987b; Yamada

et al., 1991). The collagen-binding domain seems to account for the capacity of fibronectin to bind to collagen and gelatin.

Fibronectin is very important in cell adhesion and spreading. It plays a major role in many important physiological processes, such as embryogenesis, wound healing, haemostasis and thrombosis, cell differentiation, maintenance of normal cell morphology, cytoskeletal organisation, and oncogenic transformation (Yamada 1989; Hynes, 1989; Hynes, 1990; Schwarzbauer *et al*, 1991; Potts *et al*, 1994; Watt *et al.*, 1994).

EXTRACELLULAR MATRIX RECEPTORS: THE INTEGRINS

The integrins are a family of heterodimeric membrane glycoproteins expressed on diverse cell types which function as the major receptors for the extracellular matrix and as cell-cell adhesion molecules. As adhesion molecules they play an important role in numerous biological processes such as platelet aggregation, inflammation, immune function, wound healing, tumour metastasis and tissue migration during embryogenesis. In addition, there is now increasing evidence to implicate integrins in signalling pathways, transmitting signals both into and out from cells.

Integrins are the principal receptors used to bind to the extracellular matrix including: collagen, fibronectin and laminin. They are heterodimers that function as transmembrane linkers that mediate bi-directional interactions between the

extracellular matrix and the actin cytoskeleton. They also function as signal transducers, activating various intracellular signalling pathways when activated by matrix binding.

-INTEGRIN STRUCTURE

All integrins consist of two non-covalently associated subunits, α and β. The α subunits's molecular weight range from 150 to 200 kDa while β subunits are 90-110 kDa. (Ruoslahti *et al.*, 1987, Hynes, 1992). Both α and β subunits have a large extracellular domain, a transmembrane segment and a small cytoplasmic domain (Hynes *et al.*, 1989; Reszka *et al.*, 1992; Dipersio *et al.*, 1995). The α subunits which do not contain the I-domain undergo cleavage into heavy and light fragments which are disulphide linked. The extracellular part of the β chain contains a repeating cysteine-rich region, where intrachain disulfide bonding occurs. Integrin structure suggests that by binding to a matrix protein outside the cell and the actin cytoskeleton (via the attachment protein tails and α-actinin) inside the cell, the protein serves as a transmembrane linker.

-INTEGRIN DIVERSITY

The term 'integrins' was originally coined to reflect the role of these receptors in integrating the intracellular cytoskeleton with the extracellular matrix. (Hynes, 1987). Such integration remains a predominant recognized function of integrins,

and extracellular matrix proteins constitute the major category of integrin ligands.

-REGULATION OF INTEGRIN FUNCTION AND SIGNAL TRANSDUCTION

Recent work has shown that regulation of integrin function may involve phosphorylation of the β subunit cytoplasmic domain (Hibbs *et al* 1991). Indeed phosphorylation has been shown to induce homotypic adhesion (Chatila *et al*, 1989). Serine and tyrosine phosphorylation has been detected on the β4 subunit and β1, β3, β5 and β6 subunits all possess potential tyrosine phosphorylation sites. The α subunit cytoplasmic domain does not appear to play a role in the regulation of ligand binding; however, it appears to be important in translating ligand binding into subsequent cellular events. (Hynes *et al*, 1991).

Integrins can activate many growth factors and cytokines of signal transduction pathways (Damsky *et al.*, 1992; Gingell 1993; Richardson *et al.*, 1993; Richardson *et al.*, 1995). Among these pathways, integrins can control both protein tyrosine kinases and members of the rho family of small GTP-binding proteins (Dedhar *et al.*, 1996; Parsons *et al.*, 1996). The existence of variant cytoplasmic domains on several integrin subunits, generated by alternative

splicing, may therefore add even more versatility to intracellular signalling processes.

ROLE OF TYROSINE PHOSPHORYLATION IN CELL ADHESION

Phosphorylation of proteins on tyrosine is a critical element in signaling pathways involved in the regulation of biological processes such as cell growth and differentiation (Hunter, 1987, Ulrich and Schlessinger, 1990), platelet and lymphocyte activation (Ferrell and Martin, 1988, 1989; Gorden and Brugge, 1989; Compbell and Sefton, 1990; June *et al.*, 1990), cell fate determination (Casanova and Struhl, 1989; Kramer *et al.*, 1991) and transformation (Cantley *et al.*, 1991). It has long been known that many of these processes are also modulated by the adhesion of cells to the extracellular matrix (ECM) (Turner *et al.*, 1981; Wittelsberger *et al.*, 1981; Patel and Lodish, 1987; Dike and Farmer, 1988; Adams and Watt, 1990; Nicholson and Watt, 1991). Although the mechanism by which adhesion regulates cell behaviour is still poorly understood, early studies showing elevated levels of phosphotyrosine in focal adhesions (Comoglio *et al.*, 1984; Mather *et al.*, 1985) suggested that protein tyrosine phosphorylation may play an important role in adhesion dependent processes. Several laboratories have now shown that integrin-dependent adhesion of cells to the ECM activates a signalling pathway that leads to phosphorylation of specific proteins on tyrosine (Ferrell and Martin, 1989; Golden and Brugge, 1989; Guan *et al.*, 1991; Kornberg *et al.*, 1991; Shattil and

Brugge, 1991; Burridge *et al.*, 1992; Bockholt and Burridge, 1993; Haimovich *et al.*, 1993; Huang *et al.*, 1993; Lipfert *et al.*, 1992). Furthermore, three of these proteins have been identified as the focal adhesion components paxillin (Burridge *et al.*, 1992), pp125 FAK (Burrige *et al.*, 1992; Guan and Shalloway, 1992; Lipfert *et al.*, 1992) which is a tyrosine kinase which has been shown to correlate with increased tyrosine kinase activity (Guan and Shalloway, 1992; Lipfert *et al.*, 1992) and tensin (Bockholt and Burridge, 1993), suggesting that focal adhesions are important sites of adhesion-induced signal transduction. However, the relationship between these adhesion-induced phosphorylation events and the adhesion dependent regulation of cellular processes remains to be determined. The continued identification and characterization of proteins that are tyrosine phosphorylated in an adhesion-dependent manner, and the relevant kinases, should provide useful information regarding integrin-mediated signaling and ultimately, perhaps, lead to an understanding of the role of adhesion in the regulation of cell behavior.

It has recently been established that the signals received by the transmembrane receptor tyrosine kinases (RTKs) are transmitted further downstream, and eventually into the nucleus, by other signal transduction molecules which possess **S**rc **H**omology 2 (SH2) and SH3 (**S**rc **H**omology 3) domains (Koch *et al.*, 1991). SH2 domains interact strongly and specifically with phosphotyrosine-containing proteins (Pendergast *et al.*, 1991). SH3 domains, on the other hand, recognize proline-rich motifs (Pawson *et al.*, 1993). These

sites, in conjunction with their other binding sites, are likely to play key roles in the formation of adhesive complexes.

Although the function role of these domains in the regulation of tyrosine kinases is less well understood, certain molecules that regulate the generation of adhesive complexes have been identified such as the members of the rho subfamily, ras family, of GTP-binding proteins, namely cdc42, rac, and rho, play a major role in regulating the formation of adhesions. Cdc42 and rac can regulate the formation of filopodia and lamellipodia (Ridley and Hall, 1994). Rho is an essential component of a signal transduction pathway linking growth factor receptor to the assembly of focal adhesions and the polymerisation of actin into stress fibres (Nobes *et al.*, 1995b). Other intermediary messengers involved in this signalling cascade are: tyrosine kinases, including focal adhesion kinase (FAK), which is thought to initiate formation of adhesion; lipid kinases, including phosphatidylinosital phosphate (PIP) 5- and phosphatidylinositoal (PI) 3-kinase are implicated in chemotactic responses and modulation of integrin affinity and mediate platelet-derived growth factor (PDGF) and insulin receptors which regulate the activation of rac (Nobes *et al.*, 1995a; Parker *et al.*, 1995). All these finding imply that tyrosine phosphorylation plays a centre role in focal adhesion assembly and signal transducing pathways.

MECHANISMS OF TOPOGRAPHIC GUIDANCE

Weiss (1945) first investigated how cells are influenced by topographic features. He suggested that a colloidal exudate is preferentially adsorbed along the long axis of a fibre and that this is why cells are oriented in this direction. Curtis and Varde (1964) suggested instead that cells reacted to the topography of fibre and grooves. Their reason for this conclusion was that they found that the cells were sensitive to changes in fibre diameter in the range of 10 to 30 μm. At this dimension orientation of protein adsorption would not be expected. This topographical theory was supported by Rovensky *et al* (1971) who studied cell migration and orientation on grooved substrata and concluded that cell guidance due to topographical features of varying geometrical configuration is a result of differences in the attachment of cells to their contact surfaces. Dunn and Heath (1976) did further studies on fibroblasts on cylindrical substrata of differing diameters. They also suggested that substratum morphology and shape impose restrictions on the formation of linear bundles of microfilaments that are very important in cell adhesion and locomotion.

It is not until 1979 that Ohara and Buck prepared an alternative theory by using grooved substrata with features smaller than cell size. They showed that cells were sufficiently rigid to bridge over grooves, such that cell-substratum contact was confined to the surfaces of the ridges between the grooves. They then suggested that focal contacts can only form on crests on grooved substrata, and that the orientation of the focal contacts themselves is constrained. This restriction influences the direction of the cell cytoskeleton and cell movement.

Dunn (1991) pointed out that there may be more than one response involved in cells and diverse properties of the substratum.

PROJECT AIMS

The initial aim of this project was to investigate if there are any new proteins being synthesised during cells reactions to grooved topography, as cellular alignment and movement on grooved microstructures had been observed previously. The RT-PCR technique was used to find the differential gene expression during the response of cells to topographical stimuli since little previous work has been done and the molecular mechanism remains unclear. The particular changes in mRNA expression which were discovered led me further in investigation of signalling pathways.

CHAPTER 2

MATERIALS AND METHODS

CELL LINES

P388D1 macrophage cell line

P388D1 macrophage like cell line (Koren *et at*, 1975) was supplied frozen from departmental stock, which was a gift from Professor P. Bongrand, Marseille to the department. Cells were recultured continuously (see methods).

Epitenon fibroblasts

Epitenon fibroblasts were isolated from rat flexor tendons of male Sprague Dawley rats by Wojciak, B. (Clin. Exp. Innumol. 93-108; 1993) and maintained in continuous culture in the department and used for up to 30 passages.

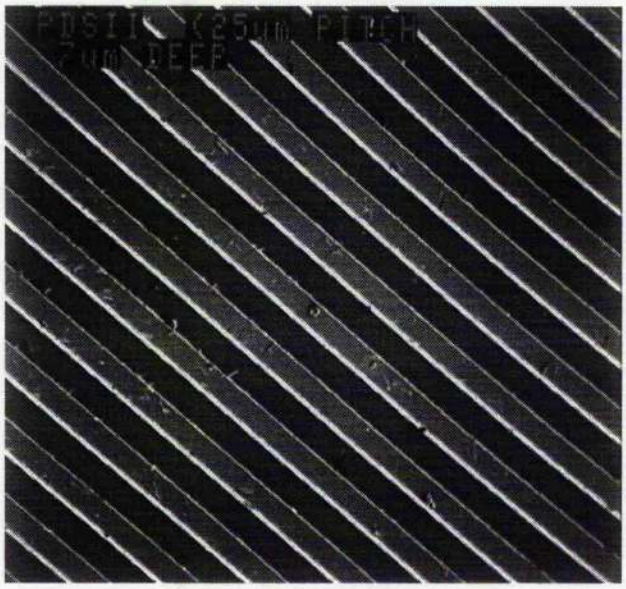
SUBSTRATUM PATTERNING

I used fused silica substrata with micropatterned arrays of parallel grooves and ridges of equal width and a square wave profile. Fused silica samples (Multilab, Newcastle, UK) were cut into 25-mm^2, 1-mm-thick samples. The silica was cleaned by soaking in a solution of 3:1 sulphuric acid (98%): hydrogen peroxide (30%) (otherwise known as permonosuphuric acid or Caro's acid) for 10 minutes at 60 °C followed by rinse in R. O. water and then blown dry with filtered air. The silica was coated with S1818 photoresist (Shipley, Covertry, UK) by spinning on at 4000 rpm for 30 s

followed by a soft bake at 90 °C for 30 minutes. This gave a resist thickness of 1.8 μm. The resist was then patterned by exposing to UV light (HTG mask aligner 200 W mercury lamp, 400 nm) for 10 s through a chrome mask patterned with the required grating pattern using a mask aligner (HTG, San Jose, CA). The exposed resist was removed by immersing the sample in a solution of 1:1 Shipley developer: R. O. water for 70 s followed by a rinse in R. O. water and blown dry. The samples were dry etched in a Reactive Ion Etching Unit (Rie80, Plasma Technology, Bristol, UK) at a rate of approximately 480 watts. After etching, the residual resist was removed in warm acetone, and all samples were etched for an additional minute without protection to ensure uniform surface chemistry.

The studies on cell behaviour were conducted on structures grooves with depths of 1.5, 3 and 6 μm and widths of 5 and 12.5 μm. Behaviour was then compared with plain fused silica substrata as a control.

a.



b.

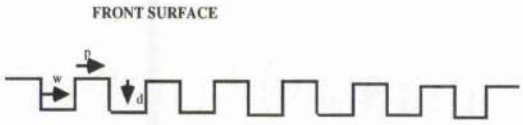


Figure 1a. Scanning electron micrograph (SEM) of a microgrooved substratum (fused silica) with 7μm deep, 25 μm width, SEM by W. Monaghan.

Figure 1b. Diagrammatic cross-section of a microgrooved quartz (fused silica). w=25 μm , p= 25 μm and d= 7 μm .

GENERAL CELL CULTURE

Media Recipes

Water

Water for culture medium and solutions was purified by reverse osmosis and ion exchange, using Mille RO cartridges and MilliQ.

Eagles Water

Water was aliquoted in 250 ml bottles and autoclaved, stored at 4°C.

Bicarbonate

7.5% solution of Sodium Hydrogen Carbonate in high quality water. When fully dissolved, it was sterilised by Millipore ultra-filtration (0.2 mm pore size), dispensed in 20 ml aliquots and stored at room temperature.

Hanks HEPES Buffer

8 g Sodium chloride, 0.4 g Potassium chloride, 0.19 g Calcium chloride ($2H_2O$), 0.2 g Magnesium chloride ($6H_2O$), 1 g D-Glucose, 2.38 g HEPES (N-2 hydroxethyl piperazine-N' 2-ethane sulphonic acid) (Cambridge Research Biochemicals) and 2 ml 0.5% Phenol red in 1 litre water, adjusted to pH 7.5 with 5M NaOH.

HEPES Buffered Water

5.25 g HEPES (N-2 hydroxethyl piperazine-N' 2-ethane sulphonic acid) (Cambridge Research Biochemicals) in 1 litre high quality water, adjusted to pH 7.5, and stored at 4°C.

HEPES Saline buffer (HS)

8 g Sodium chloride, 0.4 g Potassium chloride, 1 g D-Glucose, 2.38 g HEPES and 2 ml 0.5 % Phenol red, adjusted to pH 7.5 with 5M NaOH. Dispensed in 100 ml aliquots autoclaved and stored at 4°C.

Tryptose Phosphate Broth

29.5 g tryptose phosphate broth (Gibco Life Technologies Ltd., Paisley, UK) in 1 litre water, adjusted to pH 7.5, dispensed in 20 ml aliquots and autoclaved, stored at 4°C.

GPSA Anti-biotics

GPSA is a mixture containing Glutamine, the antibiotics Penicillin, Streptomycin, and the antimycotic Fungizone (Amphotericin B). The mixture contains Glutamine (Sigma Chemical Co. , Poole, England) , at a final concentration of 114 mM, Penicillin (Gibco) at a final concentration of 1905 units/ml (5,000 units/ml stock solution), Streptomycin (Gibco) at a final concentration of 1905 µg/ml and Fungizone, at a final concentration of 11.9 µg/ml (Amphotericin B, 250 µg/ml), all prepared in water. GPSA was dispensed in 20 ml aliquots and stored at - 20°C.

BHK Medium

BHK medium was composed of 150 ml of Eagles water, 19 ml of BHK 10X concentrate (BRL, Life Technologies, Paisley, UK), 7 ml of calf serum (Gibco) and 20 ml of tryptose phosphate broth.

RPMI 1640 Medium

RPMI (Roswell Park Memorial Institute) medium was composed of 150 ml Eagles water, 15 ml of RPMI medium (Gibco), 8 ml of Sodium bicarbonate

solution, 5 ml of antibiotics (GPSA solution) and 20 ml of foetal calf serum (Sigma Chemical Co., Poole, England) to give a total volume of 198 ml.

Solutions for Histology

Phosphate Buffered Saline (PBS)

9.86 g Sodium chloride (NaCl), 0.25 g Potassium chloride (KCl), 1.44 g Na_2PO_4, 0.25 g KH_2PO_4, adjusted to pH 7.2 with 5M HCl, stored at room temperature. It was frequently prepared fresh and not stored for more than 3 month because of the risk of fungal or bacterial growth.

Buffered Formaldehyde

100ml of Formaldehyde (40%) (BDH-Merck Ltd., Lutterworth, Leics., England) was added to 900 ml of Phosphate buffered saline.

Kenacid blue (Coomassie blue)

1 g Kenacid blue (Sigma) was dissolved in 430 ml distilled water, 500 ml methanol and 70 ml Acetic acid (Sigma).

Trypan blue solution

2% trypan blue w/v (Sigma) in water. This was freshly prepared shortly before use because of the risk of fungal or bacterial growth.

Solutions for cell dissociation

Versene solution

8 g Sodium chloride (NaCl), 0.4 g Potassium chloride (KCl), 1 g d-Glucose, 2.38 g HEPES, 0.2 g EDTA (ethylenedisminetetra acetic acid) (Sigma) and 2 ml of Phenol Red (0.5%), adjusted to pH 7.5 with 5 M NaOH. Dispensed in 20 ml aliquots and autoclaved, stored at 4°C.

Trypsin solution

Sterile 0.25 % w/v trypsin (Gibco 1:250) dissolved in Hepes buffered saline pH 7.5, was dispensed in 2 ml aliquots, and stored at -20°C. 0.5 ml of this solution was added to 20 ml versene shortly before use.

Medium for freezing cells for storage

9 ml of Foetal calf serum (FCS) was mixed with 1 ml of Dimethyl sulphoxide (DMSO) (BDH-Merck). This was filtered to 0.25µm limits before use.

Cell Culture

P388D1 macrophage-like cells

The **P388D1 cells** were grown in RPMI 1640 medium. Cells were grown in 25 mm TC Falcon flasks and passaged biweekly by removing the cells from the flasks using hand shaking when confluent. Cells were plated at $1x10^5$ cells/ml into polystyrene Petri dishes diameter 9 cm containing the structures and sufficient media was added to ensure that the structures were covered with at least 2mm depth of medium. Other sizes of dish were used for large structures. The volumes of cells added were adjusted to ensure that the plating out density was approximately $5x\ 10^4$ cells per cm^2 of structure. This gives a population of well separated single cells initially. Thus assuming even settling of the cells $5x\ 10^4$ cells/cm^2 area settled on each structure. Structures used had grooves of 2.5, 6 µm depth and 5 µm width. Plain substrata were also used as controls. Cultures were grown for 1hr, 4 hrs and 24 hrs for biochemical and morphometric analysis and for longer periods for video recording for movement analysis.

Epitenon fibroblasts were cultured in BHK medium at 37°C. For experiments they were used between 15 and 25 passages. Cells were plated on test substrata in the same way as P388D1 cells.

Cell suspension

Cell were trypsinised by adding 5 ml trypsin solution and incubated for 3 mins at 37°C until the cells detached. Trypsin activity was stopped by addition of 15 ml medium. Then, cells were aspirated spun at 1500 rpm for 5 min resuspended in the appropriate medium and set at the required concentration.

Cell counting

Cells were counted using a haemocytometer (Fuchs-Rosenthal ruling). The total volume occupied by 8 squares is 10^{-4} ml from which the number of cells/ml can be calculated. The number of cells/ml is equal to the number in 8 squares x 10^4. The number of cells in 16 squares was counted and the mean obtained.

ELECTROPHORESIS

SDS-PAGE Gels for protein

SDS-PAGE (Sodium dodecylsulphate polyacrylamide gel electrophoresis) (Laemmli, 195) is an effective method to separate and size proteins and therefore compare and identify between samples.

Stock solutions

30% acrylamide stock solution (Scotlab Ltd., Coatbridge, Strathclyde, Scotland) 28.5 g Acrylamide, 1.5 g Biscarcrylamie in 100 ml distilled water.

Gel Running buffer 18.5 g Tris, 0.4 g SDS dissolved in 50 ml water, adjusted pH to 8.9 and added water to 100 ml. **Stacking gel buffer** 5.9 g Tris, 0.4 g SDS dissolved in 50 ml water, adjusted to pH 6.7 and added water to 100 ml. **Upper tank buffer (x 5)** 31.6 g Tris, 20 g Glycine, 5 g SDS per litre, diluted x 5 concentration before use. **Lower tank buffer (x 5)** 60.5 g Tris, 5 g SDS, adjusted to pH 8.1 and made up to a final volume of one litre with water.

SDS-PAGE Gel recipes

Stock solution	17.5% separation gel µl	5% stacking gel µl
30% acrylamide	5,250	670
1% Bis-acrylamide	660	520
1 M Tris-HCl pH 8.7	3,030	-
0.25M Tris-HCl pH 6.8	-	2000
20% SDS	45	20
dH_2O	-	780
10% Ammonium persulphate	30	30
TEMED	10	4

Gel preparation

Glass plates were cleaned with Decon and rinsed thoroughly with tap water. Plates, gasket and spacers were wiped with ethanol, then assembled and held in place with clips, the running gel was then added. The running gel was overlaid gently with distilled water and allowed to set in a hot room for 1 hour. When the gel had set, the water was removed and the stacking gel was poured and the comb was inserted. The comb was carefully removed after the gel had set at room temperature for about 15 minutes. The gel was then placed into a tank and held in place using clips. The wells were washed once with distilled water and any bubbles were removed from the base of the gel plates by gently tipping the apparatus to one side.

acrylamide solution was obtained from Scotlab Ltd. (Coatbridge, Strathclyde, Scotland).

Ammonium persulphate was obtained from BIO-RAD (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK).

TEMED (N,N,N',N'-Tetra-methyl-ethylenediamine) was purchased from BIO-RAD.

Reagents for Preparing Protein Samples for Electrophoresis

Isoelectric Focussing (IEF) sample mix

CHES (2-(N-Cyclohexylamino)Ethane-Sulphonic acid)	0.05mM	100mg
Sodium Dodecyl Sulphonate (SDS)	2%	200 mg
Glycerol	10%	1ml

CHES (Sigma)was dissolved in 8.5 ml of water and the pH adjusted to 9.5 with NaOH . The SDS (ICN Biomedicals Ltd., Thame, UK) and the glycerol (BDH)

were then added, the solution was made up to 10 ml with water. The IEF sample mix was aliquoted in 0.5 ml volumes and stored at -20°C.

10µl 2-Mercaptoethanol (BioRad) and 10µl 100mM PMSF (Phenylmethanesulphonyl fluoride; Sigma) (to prevent proteolysis) were added to each aliquot of 0.5 ml IEF sample mix. 1% bromophenol blue (Sigma) solution was boiled and added to sample before loading (e.g. 5µl to a 100µl sample).

SDS Gel staining

Coomassie blue staining

The gels were placed in a dye solution (0.25% w/v Coomassie blue R-250 in 50 % methanol/ 10% acetic acid) for 1 hr, then the gel was destained in 25% isopropanol/ 10 % acetic acid for at least 1 hour, then a further destain for 1 hour in destain solution II (7.5% methanol, 10 % acetic acid) was carried out.

Silver Staining

The gels were fixed for 1 hour in fixer (50% methanol, 12% acetic acid, 0.5 ml 37% formaldehyde per litre). Each gel was then washed twice for 20 min each in 30% ethanol. 1 min in 0.02% sodium thiosulphate ($Na_2S_2O_3 \bullet 5H_2O$). This was followed immediately by three rinses in water. Each gel was then incubated for 20 min in silver nitrate solution (2% $AgNO_3$, 0.75 ml 37% formaldehyde), washed twice in water, and then developed for a maximum of 10 min in developing solution (0.5% NaOH in ethanol). The reaction was stopped with immersion in a mixture of (50 % methanol and 12 % acetic acid. Each gel was dried for 2 hrs at 80°C under vacuum on a 37 cm x 56 cm Whatman chromatography 3 MM paper (Whatman International Ltd., Maidstore, England).

Agarose gel electrophoresis for DNA

This method was performed as described in Sambrook *et at.*, 1989. DNA was electrophoresed in agarose gel containing 0.5 µg/ml Ethidium Bromide in 1x TBE (90 mM Tris, 90 mM Boric acid, pH 8.3, 2 mM EDTA). A range of agarose concentrations (1%-2% w/v) was used depending on the sizes of fragments to be resolved (Sambrook *et at.*, 1989). DNA gel was run for 1 to 2 hours under 90 voltage. 100 bp and 1 kb ladder were used (Gibco).

Denaturing agarose gel electrophoresis for RNA

Prior to electrophoresis, RNA samples (up to 4.5 µl) were denatured by addition of 10 µl of 100% Formamide, 2 µl of 5x MOPS buffer (200 mM MOPS pH 7.0, 50 mM Sodium acetate, 5 mM EDTA), 3.5 µl of Formaldehyde (12.3 M), 1 µl of Ethidium Bromide (1 mg/ml stock), and heated to 70 °C for 5 min. The RNA was electrophoresed on 1% (w/v) agarose gel containing 2.2 M Formaldehyde (Sambrook *et al.*, 1989), using 1x MOPS, in a Phamacia gel running tank (Phamacia Biotech Limited, Herts, UK) with constant circulation from anode to cathode chambers necessary to maintain a constant pH.

Polyacrylamide gels for DNA separation

This Polyacrylamide gel is designed for separation of the products of DNA sequencing reactions. I used it to distinguish PCR products from other DNAs in the mRNA fingerprinting method (see below). The gels were prepared with 6% (w/v) acrylamide (N, N'-methylenebisacrylamide), 7M urea, in 1 x TBE. Polymerisation was catalysed by the addition of 1 ml of 10% (w/v) Ammonium persulphate and 50 µl of TEMED to 150 ml of Acrylamide/urea mix. Each gel was allowed to polymerise overnight before use. Each polymerised gel was pre-

run at 11 W until they reached 50 °C to clean the gels and remove monomer and excess persulphate. Prior to electrophoresis, samples were denatured for 2 mins at 95°C and then loaded onto the gel. The gels were run at 50°C for 2 to 3 hrs depending on the size of DNA to be resolved, and then dried for 2 hrs at 80°C on Whatman chromatography paper under vacuum. Autoradiography was carried out without intensifying screens at room temperature.

Visualisation and photography of gels

DNA and RNA were visualised by UV-induced fluorescence (excitation wavelength 260 nm) of Ethidium Bromide agarose gels. For DNA the Ethidium bromide was added in the gel forming constituents at 0.5 µg/ml, for RNA the ethidium bromide was added at 0.05 µg/µl into the RNA samples before loading. The gels were photographed using Sony UPP-110HA Superior Density Printing Paper (Sony, Japan). Presence of strong 28S and 18S bands in RNA preparations were always seen.

PROTEIN AND NUCLEIC ACID ISOLATION

Protein isolation

The cells were washed once with PBS and the proteins were collected using IEF medium (the recipe for IEF sample mix).

Total RNA isolation

Total RNA was isolated using RNAzol™B (Biogenesis, Poole, UK). Samples were homogenised in 0.8 ml of RNAzol™ B, then transferred to Eppendorf tubes, 80 µl chloroform added. The samples were tightly covered, and shaken vigorously for 15 seconds. The tubes were then kept on ice for 5 minutes. The suspension was centrifuged at 12,000g (4°C) for 15 minutes. This step

removes particulate material. The aqueous phase was collected and RNA precipitated with equal volumes of isopropanol for 45 minutes or overnight at -20°C. The RNA precipitates were centrifuged for 15 minutes and then washed once with 0.8 ml of 75% ethanol.

mRNA isolation

The cell suspensions were washed in PBS prior to preparing a cell pellet by centrifugation. 1 ml Lysis/binding buffer (100 mM Tris-HCl, pH 8.0, 500 mM LiCl, 10 mM EDTA, pH 8.0, 1% LiDS, 5 mM dithiothreitol) (Dynabeads mRNA DIRECT kit, Dynal A/S, Oslo, Norway) was added. Repeated passage of the solution through a pipette tip to obtain complete lysis was performed. The viscosity was reduced by pressing the lysate 3 times through a 21 gauge needle with a 1 ml syringe. The lysate was combined with the Dynabeads Oligo $(dT)_{25}$ (supplied in the kit) and annealed for 3 min at room temperature. The tube was placed in the Dynal Magnetic Particle Concentrator (MPC) to retain the beads for 1 min and the supernatant was removed. The beads were washed with 0.5 ml Wash Buffer with Lithium dodeyl sulphate (10 mM Tris-HCl, pH 8.0, 0.15 M LiCl, 1 mM EDTA and 0.1% LiDS) (supplied in the kit) twice using the MPC device and once with Washing Buffer (10 mM Tris-HCl pH8.0, 0.15 M LiCl and 1 mM EDTA) (supplied in the kit). Finally, a 10 µl aliquot of the 2 mM EDTA pH 8.0 Elution solution (supplied in the kit) was added and kept at 65°C for 2 min. The supernatant containing the mRNA was transfered to a new RNase free tube by using the MPC device.

QUANTITATION OF NUCLEIC ACIDS

For quantitation of DNA and RNA optical density measurements were made in a spectrophometer at a wavelength of 260 nm. An OD_{260}=1 corresponds to 50 µg /ml for double stranded DNA, 40 µg/ml for RNA and 33 µg/ ml for oligonucleotides. When samples had very low concentrations of DNA (<250 ng /ml), the quantity of DNA was estimated by spotting the sample and known standards on the surface of a 1% (w/v) agarose gel containing Ethidium bromide. The gel was photographed using short-wavelength UV illumination (254 nm) and the concentration of the DNA sample was estimated by comparing the intensity of fluorescence in the sample with those of known DNA concentration standards.

cDNA SYNTHESIS

Removal of chromosomal DNA from RNA

Up to 50 µg of total cellular RNA was incubated for 15 min at room temperature with 10 units of DNase I solution (Worthington Biochemical Corp., Freehold, HJ) containing 50% glycerol and 1 mM calcium chloride, pH 5.0. In turn one phenol, one phenol/chloroform (1:1) and one chloroform extraction (200 µl each to up to 50 µl RNA sample) were carried out to remove or inactivate all enzymes, the supernatant was ethanol-precipitated in the presence of 0.3 M Sodium acetate and the RNA was redissolved in diethyl pyrocarbonate (DEPC) treated water, stored at -70°C.

cDNA synthesis

Each RNA sample (2µl) was combined with 1 µl cDNA (1 µM) synthesis poly $(dT)_{12}$ primer and 2 µl sterile H_2O. The tubes were incubated at 70°C for 3 minutes. 5 µl of the cDNA master mix (2µl of 5x first-strand buffer, 2µl of dNTP mix 5mM and 1µl of 200 units/µl MMLV (Moloney murine

leukemia virus) reverse transcriptase (Gibco Life Technologies, Grand Island, NY,USA) was added to each reaction tube and incubated at 42°C for 1 hour in an air incubator. The reactions were terminated by incubating at 75°C for 10 minutes. They were then placed on ice and all cDNA dilutions were stored at -20°C.

PRIMERS

Primers for RNA fingerprinting

The Delta RNA fingerprinting Kit (Clontech Laboratories, Palo Alto, CA, USA) contains 10 arbitrary P primers and 9 oligo $(dT)_n$ T primers. These random primers should prime for 90% of the genes.

P1	5'-ATTAACCCTCACTAAATCCTCCCCA-3'
P2	5'-ATTAACCCTCACTAAATGCTGGAGG-3'
P3	5'-ATTAACCCTCACTAAATGCTGGTGG-3'
P4	5'-ATTAACCCTCACTAAATGCTGGTAG-3'
P5	5'-ATTAACCCTCACTAAAGATCTGACTG-3'
P6	5'-ATTAACCCTCACTAAATGCTGGGTG-3'
P7	5'-ATTAACCCTCACTAAATGCTGTATG-3'
P8	5'-ATTAACCCTCACTAAATGGAGCTGG-3'
P9	5'-ATTAACCCTCACTAAATGTGGCAGG-3'
P1	5'-ATTAACCCTCACTAAAGCACCGTCC-3'
T1	5'-CATTATGCTGAGTGATATCTTTTTTTTTAA-3'
T2	5'-CATTATGCTGAGTGATATCTTTTTTTTTAC-3'
T3	5'-CATTATGCTGAGTGATATCTTTTTTTTTAG-3'
T4	5'-CATTATGCTGAGTGATATCTTTTTTTTTCA-3'
T5	5'-CATTATGCTGAGTGATATCTTTTTTTTTCC-3'
T6	5'-CATTATGCTGAGTGATATCTTTTTTTTTCG-3'
T7	5'-CATTATGCTGAGTGATATCTTTTTTTTTGA-3'
T8	5'-CATTATGCTGAGTGATATCTTTTTTTTTGC-3'
T9	5'-CATTATGCTGAGTGATATCTTTTTTTTTGG-3'

Sequencing Primers

T7 (Amersham International, Little Chalfont, UK): 5'-

TAATACGACTCACTA TAGGG-3'

U19 (Amersham International): 5'-GTTTTCCCAGTCACGACCTT-3'

PCR for mRNA Fingerprinting

PCR reaction

Each cDNA sample was combined with *Taq* DNA polymerase reaction mixture containing 10 x PCR buffer, sterile H_2O, dNTP mix, α-^{32}P dATP and polymerase, and cycled first through 94°C for 5 minutes, 40°C for 5 minutes, and 72°C for 5 minutes. This was followed by 2 cycles of 94°C for 2 minutes, 40°C for 5 minutes, and 68°C for 5 minutes, followed by 22 cycles of 94°C for 1 minute, 60°C for 1 minute and 68°C for 2 minutes.

Autoradiography of PCR products was used to detect different DNAs formed. The products were loaded onto a gel as described above and run as described there. The gels were dried at 80°C under vacuum for 2 hrs, exposed to X-ray film (AGFA Approved., Belgium) at room temperature without intensifying screen to obtain autoradiographies.

X-ray films were developed in (Kodak LX24 (Kodak-Pathe, Sisaone, France) 1:5 dilution in distilled water) for 5 minutes, and then washed in 2 % acetic acid for 1 minute to stop development. The films were then placed in Amfix (Champion Photochemistry, Essex, UK); 1:4 dilution in distilled water, with 2.5% Hardener (Champion Photochemistry) for at least 2 minutes. Finally, the films were rinsed in tap water, and hung up to dry.

Reamplification of DNA fragments from dried polyacrylamide gels

cDNA bands, representing differentially expressed mRNAs, were excised from the dried gels and first boiled in 0.5 ml sterile water for 5 mins, 5 µl of

supernatant was added to 45 µl reaction mix containing 10 x PCR buffer, sterile water, dNTP mix and Taq polymerase together with the original primer sets. cDNAs were reamplified by pcr with a cycling programme of 94°C for 1 minute, 60°C for 1 minute and 68°C for 2 minutes for 20 cycles. Amplified cDNA fragments were purified by using Hybaid recovery DNA Kit II according to the manufacturer's specifications (Hybaid Ltd, Middlesex, UK).

Purification of PCR products

When purified PCR products were required, PCR reactions were separated on 1% (w/v) agarose gel in 1x TAE (40 mM Tris-acetate, pH 7.6, 1 mM EDTA). DNA fragments were visualised by staining with Ethidium bromide (0.5 µg/ ml) and excised in a small slice of agarose gel. The agarose slice was incubated at 70°C until liquid and then purified with the Hybaid recovery DNA Purification Kit II (Hybaid), using the conditions recommended by the manufacturers.

mRNA Fingerprinting Protocol

Figure 2-2 shows an Overview of mRNA fingerprinting protocol

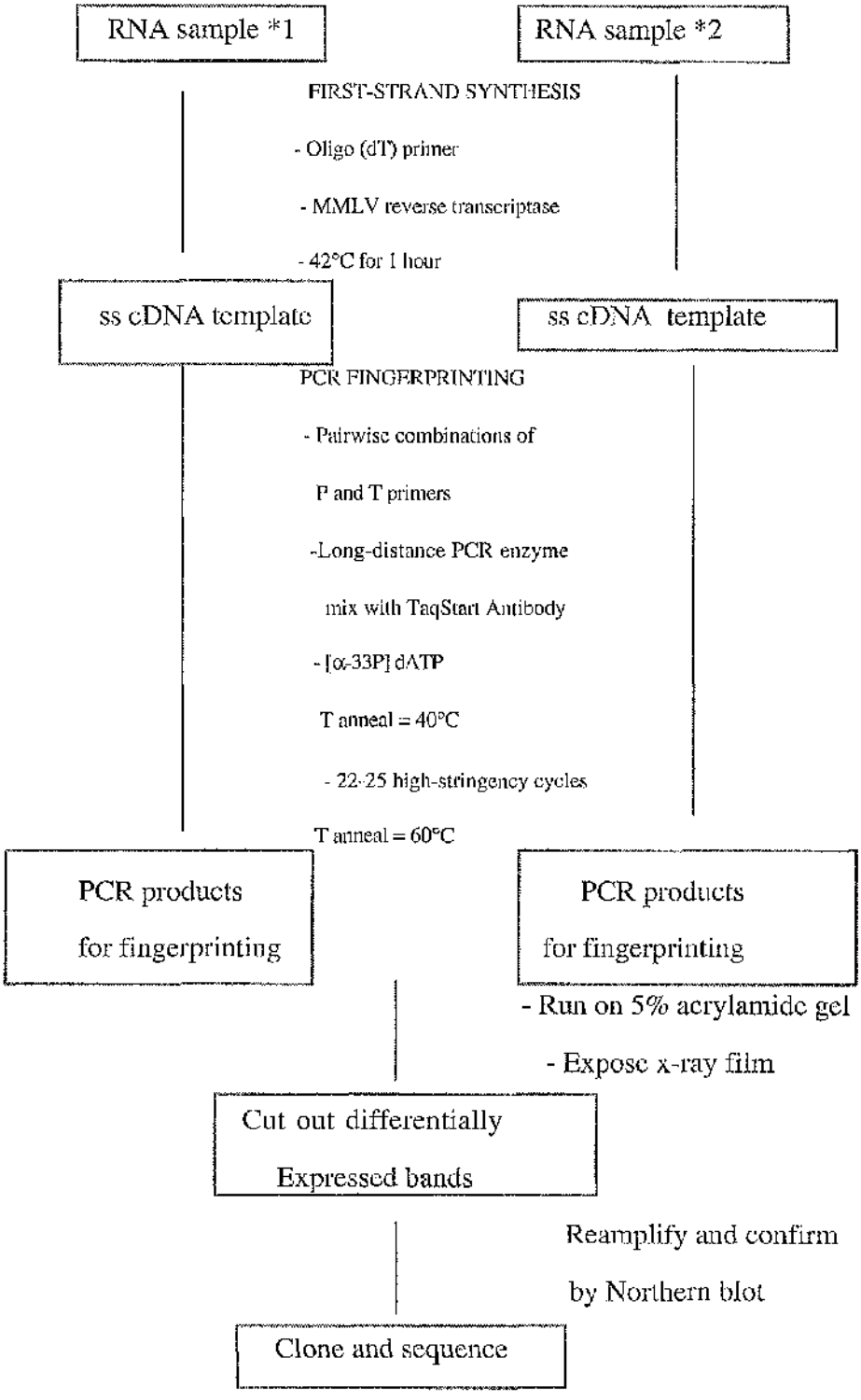


Figure 2-2 Overview of mRNA fingerprinting protocol

CLONING

Bacterial and vector

Vector: pMOSBlue T-vector (Amersham International, Little Chalfont, UK) was used.

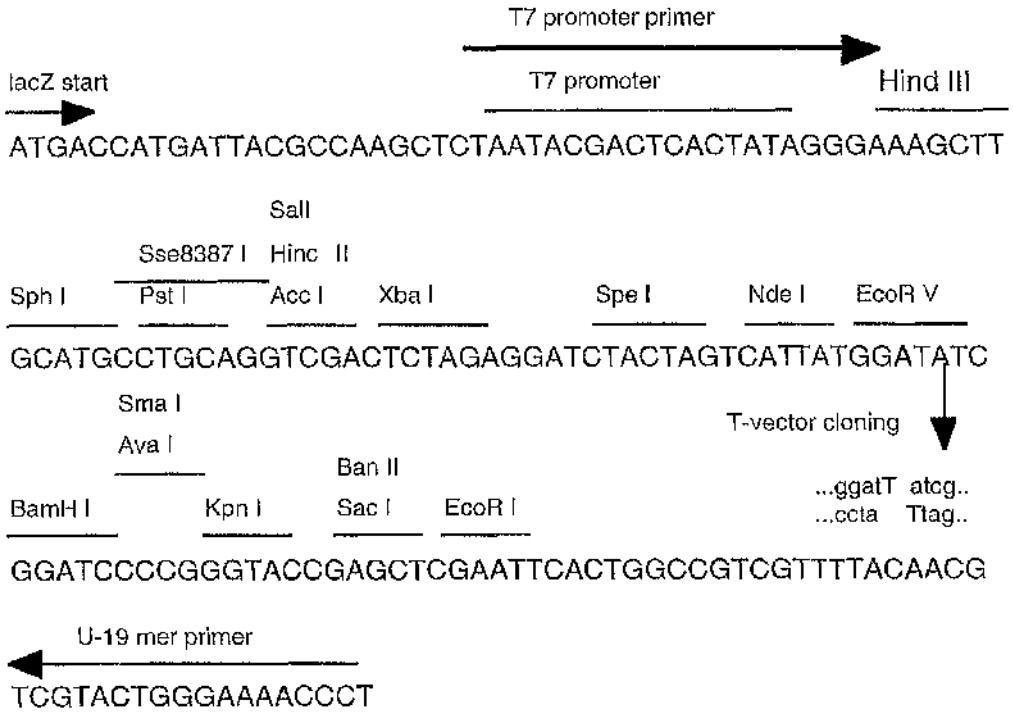


Figure 2-2. pMOSBlue T-vector

E. Coli: pMOSBlue competent cells.

Bacterial growth medium

L-agar

This was made up from 10 g tryptone (Difco Laboratories, Detroit Michigen, USA), 5g yeast extract (Difco), 10 g NaCl, 15 g agar, per litre of water and sterilised by autoclaving.

Luria broth (L broth)

10 g Bacto tryptone (Difco), 5g Bacto yeast extract (Difco) and 10 g NaCl (Sigma, Poole, UK) were dissolved in 900 ml distilled water, then 0.2 ml 5M NaOH was added and the volume made up to 1 litre. The broth was sterilised by autoclaving, allowed to cool below 50°C, then ampicillin (50 µg/ml) and tetracycline (15 µg/ml) (both Sigma) were added.

Antibiotics and indicators

When selection of bacteria in culture was necessary, both ampicillin, at a final concentration of 50 µg/ml (50 µg/ml stock solution in sterile distilled water), and tetracycline, at a final concentration of 15 µg /ml (100mM stock solution in absolute ethanol) were added to the broth. 35 µl of 35 mg/ml 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-gal) and 20 µl 100mM isopropyl-ß-D-thiogalactopyranoside (IPTG) were spread on L agar antibiotic plate in order to detect recombinant clones by the appearance of a white colour. X-gal was dissolved in dimethylformamide and IPTG in distilled water. Both reagents were stored at -20°C.

Ligation in T-vector

For optimal cloning efficiencies, the vector to insert ratio should be in the range of 1 to 7.5. This is because when DNA fragments carry blunt ends, an additional complication arises, because such DNAs are ligated much less efficiently than DNAs carrying protruding complementary termini. They therefore require much higher concentrations of bacteriophage T4 DNA ligase

and foreign DNA. For each ligation reaction, I combined 50 ng DNA with 1 µl 10x ligase buffer, 0.5 µl 100 mM Dithiothreitol 0.5 µl 100mM Adenosine tri-phosphate, 1ml 50µg/ml vector and 0.5 µl T4 DNA ligase. The reaction mix was made up to 10 µl with nuclease-free water, and incubated at 16°C for 2 hours or overnight.

Transformation of E.coli

Transformation was achieved by adding the transforming DNA (1µl of 10 µl ligation reaction) to 20 µl of the competent cells and storing on ice for 30 minutes. The cells were then subjected to a heat shock by incubating them for 40 seconds at 42°C before returning them to the ice for 2 minutes. Transformed cells were then diluted 5 times with pre-warmed SOC medium, The tubes were shaken at 200 to 250 rpm at 37°C for 1 hour. 50 µl of each suspension of transformed bacteria was spread on a 90 mm diameter L agar antibiotic plates containing 35 µl of 50 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-B-galactoside) and 20 µl 100 mM IPTG (isopropyl b-D-thiogalactopyranoside). After the overlay had set the plates were inverted and incubated at 37°C overnight.

Screening of recombinants

Direct colony PCR

Four types of colonies were observed on X-gal and IPTG indicator plates: i) dark blue, ii) white, iii) light blue, and iv) white with a blue centre. Recombinant colonies appeared white. The frequency of these will depend on the length of incubation and the nature of the insert and it is thought to result from the production of small amounts of the lacZ α-peptide, possibly caused by ribosomal frame shifting, second site translation initiation or as α-peptide

fusion protein. Only white colonies were screened. White colonies were picked from the plate containing the transformants using sterile yellow tips.

The bacteria were transferred to a 1 ml tube containing 50 µl of sterile water, and vortexed to disperse the pellet. The tubes were placed in boiling water for 5 minutes to lyse the cells and denature DNase. Then tubes were centrifuged at 12,000 g for 1 minute to remove cell debris, 10 µl of the supernatant was transfered to a fresh 0.5 ml tube and added 40 µl of master amplification mixer (31.8 µl of nuclease-free water, 1 µl of 10mM of each dNTP mix, 1 µl of 5 pmol/µl of each original PCR primers or alternately T7 promoter and U-19 mer primers instead, 5 µl of 10 x Buffer and 1.25 U of AmpliTaq DNA polymerase) with two drops of mineral oil on top. Reactions were first through the program of 35 cycles of 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes , followed by 1 cycle of 72°C for 5 minutes. The PCR products were analysed on a 1.2% agarose gel containing ethidium bromide. Those which contained the insert DNA were cut out and purified using the method described above.

AUTOMATED DNA SEQUENCING

Principle

In this method, the DNA to be sequenced acts as a template for the enzymic synthesis of new DNA starting at a defined primer binding site. A mixture of both deoxy- and dideoxy nucleotides is used in the reaction, at concentrations which create a finite probability that a dideoxynucleotide will be incorporated in place of the usual deoxynucleotide at each nucleotide position in the growing chain.

Incorporating a dedeoxynucleotide blocks further chain elongation, resulting in a population of truncated fragments of varying length. The identity of the chain-terminating nucleotide at each position can be determined by running four separate reactions, each of which contains a single dideoxynucleotide. The result and the sequence is determined by correlating the order of the bands on the gel with the dideoxynucleotide used to generate each band. An alternative approach is to attach a different nucleotide-specific label to each dideoxynucleotide, the reaction can then be run in one tube instead of four, and analysed on a single gel lane. Unlike manual sequencing methods, automated DNA sequences use a scanning laser to detect DNA fragments labelled with fluorescent dyes.

Automated Sequencing Method

In order to obtain DNA of a quality suitable for automatic sequencing it was necesary to obtain relatively large amounts of 3' end primed material. To do this, 90 ng DNA mixed with 8µl of terminator ready reaction mix, 3.2 pmole primer and add sterile water were added to give a total volume of 20µl. The tubes were placed in the thermal cycler with 25 cycles of rapid thermal ramp to 96°C for 30 seconds and rapid thermal ramp to 50°C for 15 seconds followed by rapid thermal ramp to 60°C for 4 minutes then, held at 4°C. The entire 20µl contents of the reaction tubes were transferred to the microcentrifuge tubes containing 50µl 95% ethanol and 2µl 2M Sodium acetate solution (pH, 4.6). The tubes were vortexed and placed on ice for 10 minutes. They were then centrifuged in a microcentrifuge at maximum speed for 30 minutes. The ethanol solution was carefully aspirated off and the pellet was rinsed by adding 250 µl 70% ethanol. All the alcohol solution was removed with a micropipette, and the pellet dried in air. The samples were then ready for use.

NORTHERN BLOT

Preparation of Template Probe

In this method, up to 200 ng of linearised purified DNA was used for radioactive labelling with Ready-To-Go DNA Labelling Kit (dCTP) (Pharmacia Biotech Inc.). 20 µl of distilled water was added to the Reaction Mix tube which contains dATP, dGTP, dTT, FPLC*pure*®Klenow Fragment (4-8 units) and random oligo-deoxyribonucleotides (primarily 9-mers), left on ice for 5 to 60 mins without further disturbance.

The labelling reaction mix was made as follows:

Reconstituted Reaction Mix	20 µl
Denatured DNA	< 25 µl
[α-^{32}P] dCTP(3000 Ci/mmol)	5 µl
Distilled water	to total of 50 µl

The mix was incubated at 37°C for 15 minutes, and then added to a Pre-hybridisation tube (containing Hybond N and Church buffer, (7% SDS, 1%BSA, 1mM EDTA, 0.25 M Na_2HOP_4, pH 7.2). This mix was used as a hybridisation probe in reaction.

Northern Blot

Agarose formaldehyde gels containing RNA were transferred to reinforced nitrocellulose Hybond N (Amersham) by capillary action. RNA was fixed to the membrane by UV treatment as instructed by the manufacturer (Amersham UK). Pre-hybridisation was carried out at 65°C in Church buffer (7%SDS, 1% BSA, 1mM EDTA and 0.25 M $NaHPO_4$). Filters were pre-hybridised for at least 3 hours before addition of denatured radioactive probe (10^5 - 10^6 cpm/ml hybridisation solution) and then hybridised for a minimum

of 16 hrs. The blots were washed at 55°C in washing solution I (2x SSC, 0.1% SDS) for 30 minutes, and then in washing solution II (0.1 x SSC, 0.1% SDS) for 30 mins at 37°C. The washed filters were then covered in Saran Wrap TM clingfilm and exposed to Fuji X-ray film for 1 - 3 days.

ANTISENSE

Antisense was designed against target mRNA as phosphorothioate oligonucleotide with 20 bases in length. The experiments were carried out by applying antisense phosphorothioate oligonucleotide compared with control randomized-sequence phosphorothioate oligonucleotide. Antisense were shipped as lyophilized Na-salts and prepared according to Antisense Start Kit (Biognostike, GmbH, Gottingen, Germany).

Cell Culture Treated with Antisense

Six clean grooved structures with groove 5 μm in depth, and 12.5 μm in width were put in a 24 well plate. Epitenon cells were plated in each well in a fixed volume of 100 μl of BHK medium. Cells were allowed to adhere for at least 1 hour without any further manipulation. 2 μl of antisense oligonucleotide or a control random oligonucleotide molarity were added directly into the respective wells to make a 2 μM solution. After 24 hrs, two wells were treated with antisense to give different time points in the same way as before. 48 hr after first applied antisense. Cells were washed with PBS twice and fixed with 4% paraformaldehyde. And then were stained with Coomassie blue as described above.

Measurement of Cell Length and Width

Cells were fixed and stained with Coomassie blue (as above). Photos were then taken with at 20 x phase contrast objective magnification using a Nikon camera. 100 cells were then counted for both cell length and cell width.

Measurement of cell area, orientation and the Speed of Cell Movement

Cell cultures with and without any treatment were videotaped with at 20 X phase contrast objective magnification using a Cohu 4905 CCD (Brian Reece Scientific Ltd, Newbury, UK) camera or a Panasonic Neuvicon video camera under the lowest light level which would produce a good image. Images were recorded on a Panasonic Model AG-6730 time-lapse video-recorder at 1 frame per minute.

The videos were then analysed by importing images into a Macintosh Power PC 7500/100, and processing with the public domain NIH Image program (developed at the U.S. National Institute of Health and available on the Internet at: http://rsb. omfp.moh.gov/nih-image/). Cell area and cell orientation were also measured with this progress.

Fluorescence microscopy

Antibodies

Antibodies used for immunofluorescent staining were list in table 2-1.

Antibody	Source	Dilution	Incubation time
c-kit	Santa Cruz Biotechnology	1:20	1-4 hours RT
PI-3kinase	Santa Cruz Biotechnology	1:50	1-4 hours RT
NRF-1	Santa Cruz Biotechnology	1:50	1-4 hours RT

Innumofluorescent staining

Cells were plated onto structure at a concentration of $1x10^4$ cells/ml for varying lengths of time. The media and detached cells were removed and structures were washed three times using prewarmed PBS. Cells were fixed in 4% Formalin (Sigma) in PBS for 10 minutes and then permeabilised in 0.5% Triton X-100 in PBS for 15 minutes. After a further three washes in PBS, the structures were blocked in1% BSA in PBS for 30 minutes at room temperature. Structures were then washed three times in PBS before the diluted primary antibody was added. Structures were incubated with first antibody for 2 to 4 hours at room temperature. After incubation with the first antibody, three 5 minutes washes using PBS were carried out before addition of Texas Red labelled secondary antibodies. After three 5 minute washes in PBS, structures were mounted onto slides and examined using a Nikon fluorescence microscopy.

IN SITU HYBRIDISATION

Specimen Preparation

Rat tendon sections (Wojciak *et al.*, 1995) were de-waxed and rehydrated in DEPC treated water before incubated at 60°C in 2xSSC for 10 mins. Sections were placed in fresh DEPC treated water before the proteinase K (PK) digestion was carried out. The sections were dried by the tissue and placed on humid chamber. 300 µl of PK dilutions at a concentration of 15 mg/ml in PBS was added and incubated at 37°C for 1 hour. The sections were washed in PBS and

then post fixed in pre-cooled pHCHO for 20 mins at 4°C. The sections were washed again and placed back to humid chamber and 300 µl of prehybridisation solution were added and incubated at 37°C for 1 hour. 100 µl of 10 µM biotin labelled probe solution were applied to each slide and covered with clean coverslip and incubated at 37°C overnight. The slides were washed at 37°C for 5 mins alternatively in 4xSSC/30% Formamide, 2xSSC/30% Formamide and 0.2 xSSC/30% Formamide. The sections were washed and detected by using Texas Red labelled avidin (Oncor, Galthesurg, MD 20884, USA).

CHAPTER 3

SDS PAGE GELS AND mRNA FINGERPRINTING TO DETECT DIFFERENTIALLY EXPRESSED GENES

INTRODUCTION

Surface topography plays a very important role in controlling cell shape, orientation and adhesion of mammalian cells (Curtis and Varde, 1964; Brunette, 1986a; Curtis and Clark, 1990; Dunn and Brown, 1986). Although there is a great deal of information on how surface topography can affect the morphology of cells and tissues, there is little information on the effects on cell function at the molecular level. Several studies have focused on the role of cytoskeletal elements such as microfilament bundles (Dunn and Heath, 1976; Ben-Ze'ev, 1986), focal contacts (Ohara and Buck, 1979) and microtubules (Oakley and Brunette, 1993), all these structures are observed to align to topographic features such as grooves.

It is known that cell shape can regulate cell growth (Watt, 1988; Folkman and Moscona, 1978), cytoskeletal gene expression (Ben Ze'ev, 1984), extracellular matrix metabolism (Watt, 1986; McDonald, 1989) and cell differentiation. The question has been raised that if surface topography can affect cell shape which in turn regulates several cellular functions, then surface topography should stimulate different gene expression which could lead to new protein synthesis. As described above, there is only one study on how topography influences cells

at the molecular level, in which Chou *et al* (1996) found that fibronectin mRNA level is increased by growing cells on grooved surfaces.

SDS-PAGE Methods to Detect Part of the Differentially Synthesised Proteins

The focus of this investigation was to study if there are any new proteins synthesised when cells are grown on grooved surfaces in comparison with cells on a flat surface. Total protein was extracted after growing cells on the different surfaces and total protein samples were compared by electrophoresis in SDS-polyacrylamide gel.

There are many detection methods available for the visualisation of proteins, some of which were used in this investigation. Direct detection of bands on the gel after electrophoresis is accomplished by immediately treating the gel with a dye, such as Coomassie blue. Coomassie blue is a triphenylmethane anionic dye which preferentially forms dye complexes with proteins in the gel matrix. This method is rapid and will detect protein as low as 2 ng per band. For increased sensitivity, silver stain is also used which can detect proteins between 0.5 and 2 ng per band. ^{35}S labelling with methionine of cell proteins can give high sensitivity of detection of newly synthesised proteins, and the radioactive label is then observed by exposing a photographic film to the dried gel, the bands are then visualised upon a development of the film. It is very likely that new proteins synthesised during the reaction of the cells to their topography might

locate on the cell-surface contact area. I attempted to extract the surface proteins by washing away most cell residues with 0.5% Triton-100 in PBS, and extracting the proteins which were left attached on the surface, to examine the differences.

In this part of the study, I applied all the three methods described above to P388D1 macrophage-like cells to examine if there were any differentially synthesised proteins between cells which were grown on flat and grooved surfaces.

RESULTS

P388D1 macrophage-like cells were cultured on grooves of 12.5 μm width and 88 nm depth and also on planar fused silica (quartz) substrata as controls. The crude cell proteins were harvested after 4 hours. The proteins were then checked on a SDS PAGE gel, before the gel was stained with Coomassie blue. Figure 3-1 shows approximately 20 bands in each lane which shows there are no obvious differences between the control and those cells growing on grooved structure. This result failed to demonstrate that cells respond by changing their patterns of protein synthesis on different surface topography such as grooved and planar surfaces.

The same type of experiment was repeated but with ^{35}S-methionine incorporation. In this experiment, ^{35}S-methionine solution was added to the

medium straight after the cells were placed on the structures. The cells were then incubated on the structure overnight before carrying out the protein extraction. The distribution of protein bands in SDS gel were visualised upon development of the film after exposure to X-ray film. Figure 3-2 shows up to 50 bands in each lane. Although some slightly darker bands were observed, it is hard to define the quantity and how many of the proteins in one band with the similar molecular weight.

In order to work with a more defined group of proteins and specifically focus on those proteins associated with focal adhesion areas. I used 0.5% Triton-100 to treat cells after 4 hours culture. The intention is to wash away most parts of the cell body and leave the proteins which attach to the surfaces. The protein were extracted and checked in a SDS gel using silver staining (see figure 3-3). Rather surprisingly, it was observed that there were two extra bands which were present in the sample of cells which were grown on the grooved surface and missing from those in control lane. The experiments were repeated and showed exactly the same results.

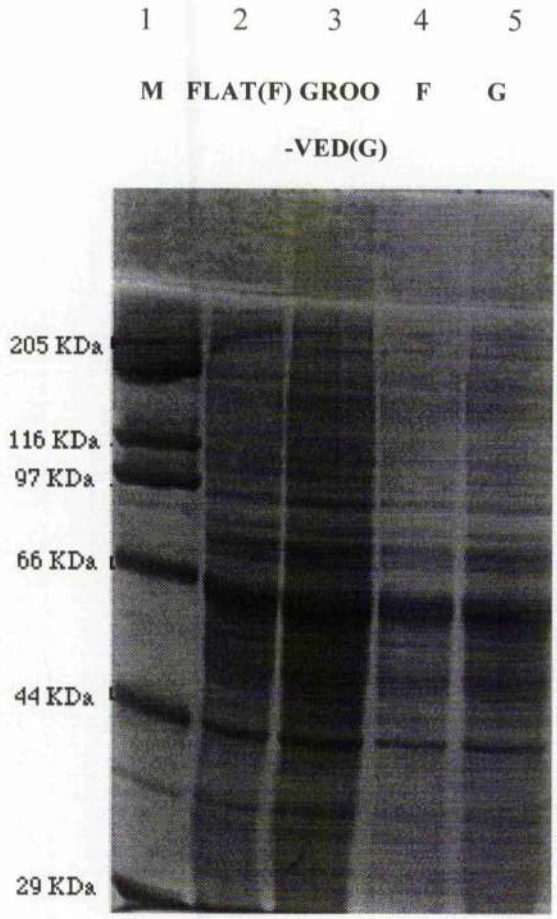


Figure 3-1. SDS PAGE of P388D1 macrophages like cells after culturing for 4 hours on grooved fused silica compared with those cultured on a flat surface. The gel is stained with Coomassie blue. Lane 1, 10 µl standard molecular weight marker; Lane 2, 25 µl of sample from growing on flat surface; Lane 3, 25 µl of sample from cells were growing on microgrooved surface, whereas Lane 4 and 5 were run in the same way except loading 50 µl of the sample instead.

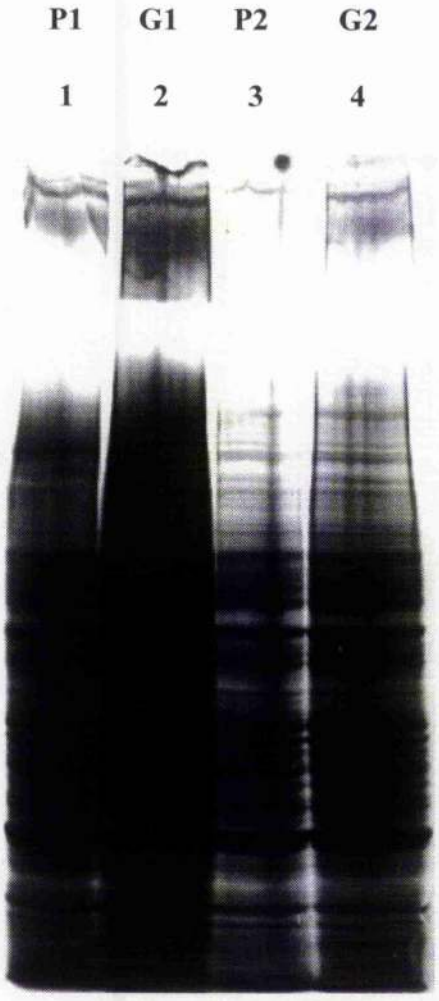


Figure 3-2. ^{35}S-methionine incorporation. ^{35}S-methionine was added to the medium straight after cells were seeded. Protein was extracted 16 hours after culturing and examined in SDS PAGE gel. Lane 1, 50 µl of sample from cells growing on flat surface; Lane 2, 50 µl of sample from cells growing on a microgrooved surface, whereas Lane 3 and 4 were run in the same way except 25 µl of the sample was applied.

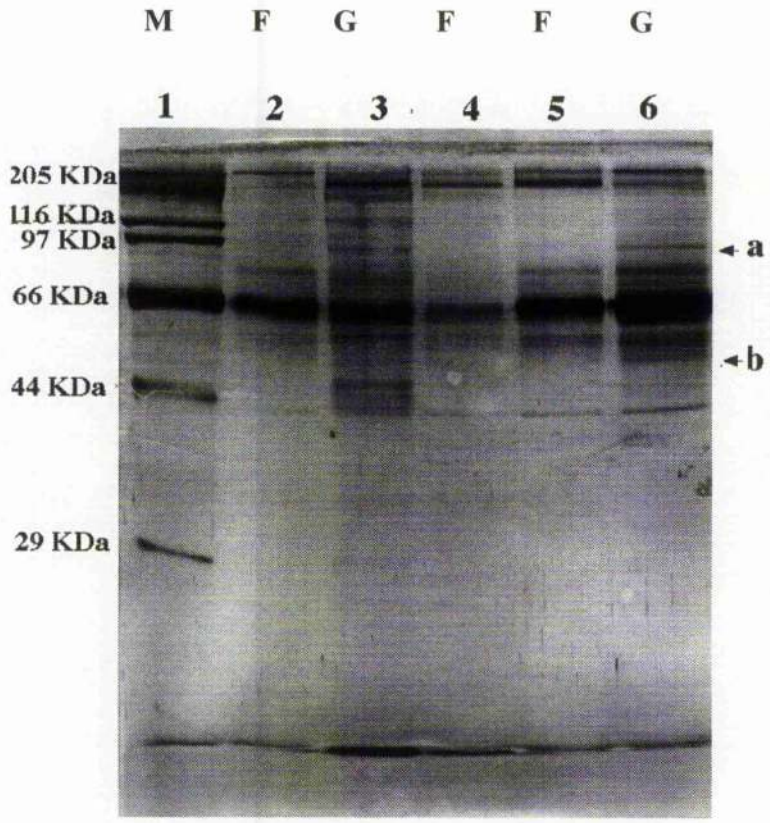


Figure 3-2. Silver stained SDS PAGE gel of P388D1 macrophages after 4 hours culturing, cells were first washed with 0.5% Triton-100 in PBS. Lane 1, 10 µl standard molecular weight marker; Lane 2, 25 µl of sample from cells growing on a flat surface; Lane 3, 25 µl of sample from cells growing on microgrooved surface; lane 4, 10 µl of sample from cells growing on flat surface, whereas Lane 5 and 6 in the same way as lane 2 and lane 3 except 50 µl of the sample were applied. Band a and b only appear in cells which were cultured on grooved surface and missing from flat surface.

mRNA Fingerprinting

My interest in differential gene expression is to identify those genes which were regulated by the surface topography, possibly leading to new protein synthesis. An mRNA fingerprinting method was used in this study. mRNA fingerprinting is a powerful tool which enables researchers to identify mRNAs that are expressed in one RNA population but missing in another (Diachenko *et al.*, 1996). It is also a highly reproducible method for detecting differentially expressed RNAs.

The Delta RNA fingerprinting kit used here is based on improvements to the methods described by McClelland *et al.* (1993). The protocol consists of two stages: cDNA synthesis and PCR fingerprinting. Total RNA was extracted from the samples to be compared, and first-strand cDNA is reverse transcripted from the RNAs with oligo $(dT)_{11}NM$ as primers, where N and M = A, G, or C.

The cDNA was then used in PCR fingerprinting reactions, sequences are amplified in the presence of [α-^{32}P] Dalta-based on chance homology to arbitrary P and anchored T primers. There are 10 P primers and 9 T primers as listed in Chapter 2. Each P primer is 25 nt long, while each T primer is 29 nt long. The longer primer length means that, following three initial low-stringency cycles at an annealling temperature of 40°C, it will allow the primers to anneal and intiate DNA synthesis. Because of the low stringency, each P primer will

bind sites on many cDNAs with imperfect and/or incomplete matches. This temperature can be increased to 60°C for the final 22-25 cycles. The products of these early cycles are then amplified during 22-25 high-stringency PCR cycles. These PCR reactions produce characteristic "fingerprints" of the starting RNA. The P primers are designed to be incapable of forming significant, stable secondary structures, and the 3'-end of each primer does not contain significant complementarity to any other sequence in the primer. Furthermore, the nine bases at the 3'- end favour common sequence motifs found in the coding region of eukaryotic messenger RNAs. Whereas, the T primers used here have the general structure 5'-anchor-$(dT)_9N\text{-}_1N\text{-}_1$, where $N\text{-}_1$=A,G, or C. The primers with the terminal position (i.e., 5'-anchor-$(dT)_9N\text{-}_1T$) are not included due to the high backgrounds which could be produced.

In this study, I compared the mRNA from cell populations grown on flat surface with that from cell populations grown on micro-grooved fused silica (quartz) structures to discover, if topographical cues can stimulate differential gene expression.

RESULTS

MESSENGER RNA FINGERPRINTING

P388D1 Macrophage-like cells

Theoretical consideration suggests that with 10 arbitrary P primers and 9 T primers, there are 90 possible combinations of upstream and downstream

primers. Furthermore, each P primer can be used alone or in pair-wise combinations with other P primers. Similarly, the T primers can be used alone or in combination with other T primers. Thus, one could display 10,000 of the mRNA series that are present in a mammalian cell. In this study, I tried 90 combinations of primer sets between P and T primers to obtain differential display.

P388D1 macrophage-like cells were cultured on the grooved surface and compared with those cultured on planar surface. This experiment was a comparison of the messenger RNA fingerprintings of P388D1 macrophage-like cells which had been cultured for 1 hour and 3 hours on either type of surfaces of flat quartz or grooved quartz structures (1.5 μm deep; 12.5 μm wide). The total RNAs were extracted from the cells which were cultured on different surfaces. The total RNAs were then reverse transcribed into cDNAs. cDNA were amplifed in PCR reaction and PCR products were examined in a denaturing 5% polyacrylymide/8M urea gel. The gel was exposed to a X-ray film and the fingerprinting could then be compared.

Figure 3-4 shows the messenger RNA fingerprinting of P388D1 macrophage-like cells which were cultured on either non-grooved and grooved structure (1.5 μm deep; 12.5 μm wide) for one hour. The odd-numbered lanes correspond to mRNA from the cells cultured on flat surface (control) whereas even-numbered lanes represent mRNA from the grooved surface. Lane 1 and 2 shows the

fingerprints in the presence of primers P1 and T10 and second two lanes are in the presence of primers P2 and T7. The third two lanes are in the presence of primers P1 and T9. The last two lanes in the presence of P1 and T8. This fingerprinting shows that with the use of the combination of the primers, differentially expressed bands can be observed with each of the four sets of primer pair tested.

Figure 3-5 represents a mRNA fingerprinting in the exactly the same way as described above except that P388D1 macrophages were cultured for four hours instead of one hour. Reamplified cDNA samples were loaded in the same way and compared between every two lanes (odd lane and even lane as marked). The primer pairs were used for any two lanes in the order are : P2 and T7; P3 and T8; P2 and T6; P2 amd T9; P1 and T3. Differentially expressed bands can also be observed in this fingerprinting as indicated by the arrows.

Epitenon Cells

Epitenon Cells were cultured on flat (control) or grooved (1.5 μm deep; 12.5 μm wide) fused silica quartz for 1 hour , 4 hours and 24 hours. cDNAs were then reamplified as described above and were run in a denaturing 5% polyacrylamide/8M urea gel, in 0.5x TBE buffer. Differential displayed mRNA fingerprints were shown in Figure 3-6 and figure 3-7.

Figure 3-6 shows messenger RNA fingerprinting from cells cultured for one hour on either non-grooved or grooved quartz substrata. The experiments were carried out as described above. The primers used for every two lanes in order are: P1 and T9; P3 and T4; P4 and T5; P4 and T7; P5 and T6; P5 and T7; P6 and T8. The mRNA fingerprinting presented here is one of three fingerprinting done and shows the most differential display.

Figure 3-7 is a messenger RNA fingerprinting obtained from growing epitenon cells for 4 hours in the same way as described above. The primers used here are: P1 and T9; P2 and T8; P2 and T7; P6 and T7; P7 and T8; P7 and T7. The differentially expressed bands can be observed with each of the six sets of primers used here.

In this study, I made 90 combinations of primer sets between T and P primers to obtain a differential display. Different fingerprints were obtained from applying the different combinations of primers sets. Figure 3-8 shows the fingerprints from Epitenon cells cultured for 4 hours with primer sets of P1 and T1. When compared with the figure 3-7 shown above, this fingerprinting indicates that no different bands can be observed. This confirms that the results above are true differences and not just an artefact of the method used to observe the samples.

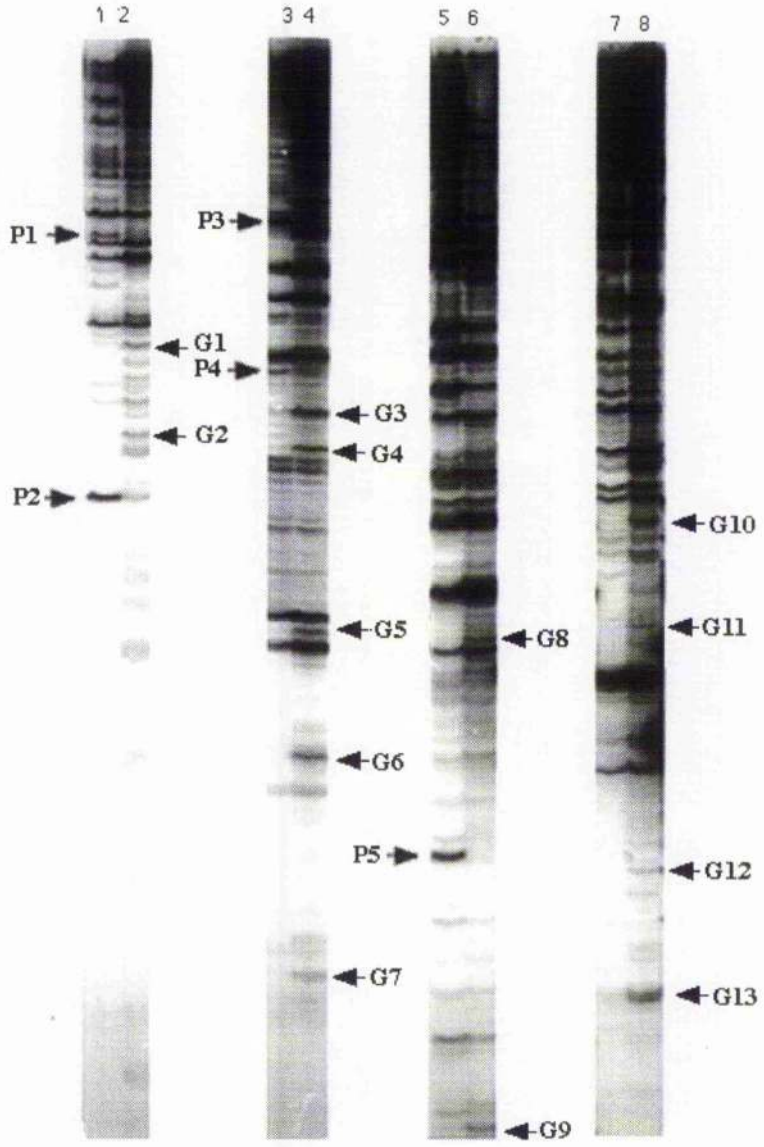


Figure 3-4. Autoradiograph showing messenger RNA fingerprinting of P388D1 macrophage-like cells cultured on non-grooved and grooved surface for one hour. The odd-numbered lanes correspond to mRNA from the cells cultured on non-grooved surface whereas even-numbered lanes represent mRNA from the cells cultured on grooved surface. Several candidate cDNA tags that appear to be differently expressed are marked by arrows. P indicates the bands from control where as G stands for grooved surface.

A B

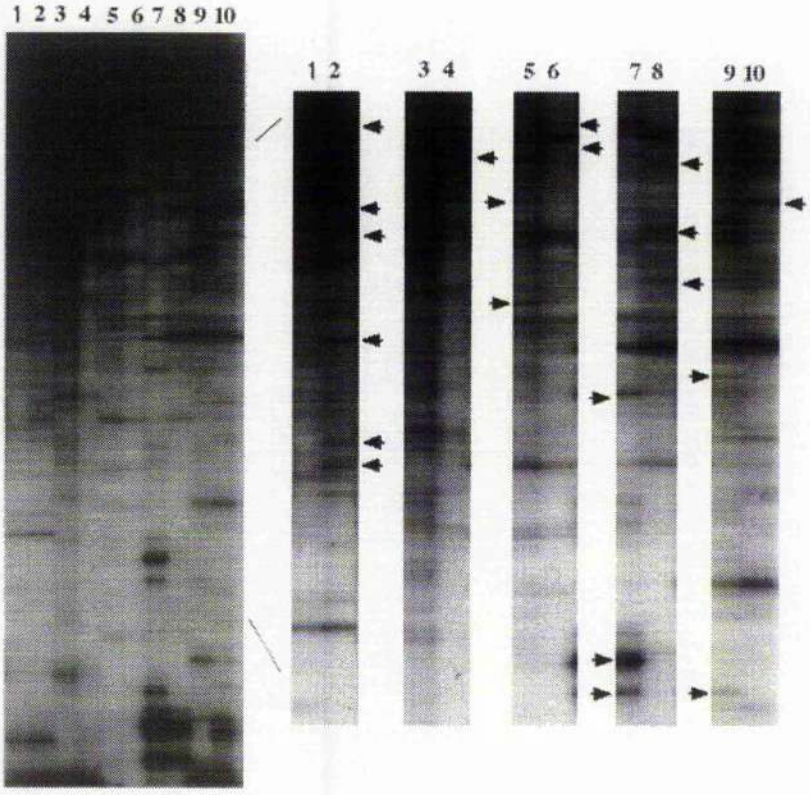


Figure 3-5. Autoradiograph showing messenger RNA fingerprints (amplified DNAs by RT-PCR) of P388D1 macrophage-like cells cultured on non-grooved and grooved surface for 4 hours. The odd-numbered lanes correspond to mRNA from the cells cultured on non-grooved surfaces whereas even-numbered lanes represent mRNA from the cells cultured on grooved surface. Several candidate cDNA tags that appear to be differently expressed are marked by arrows

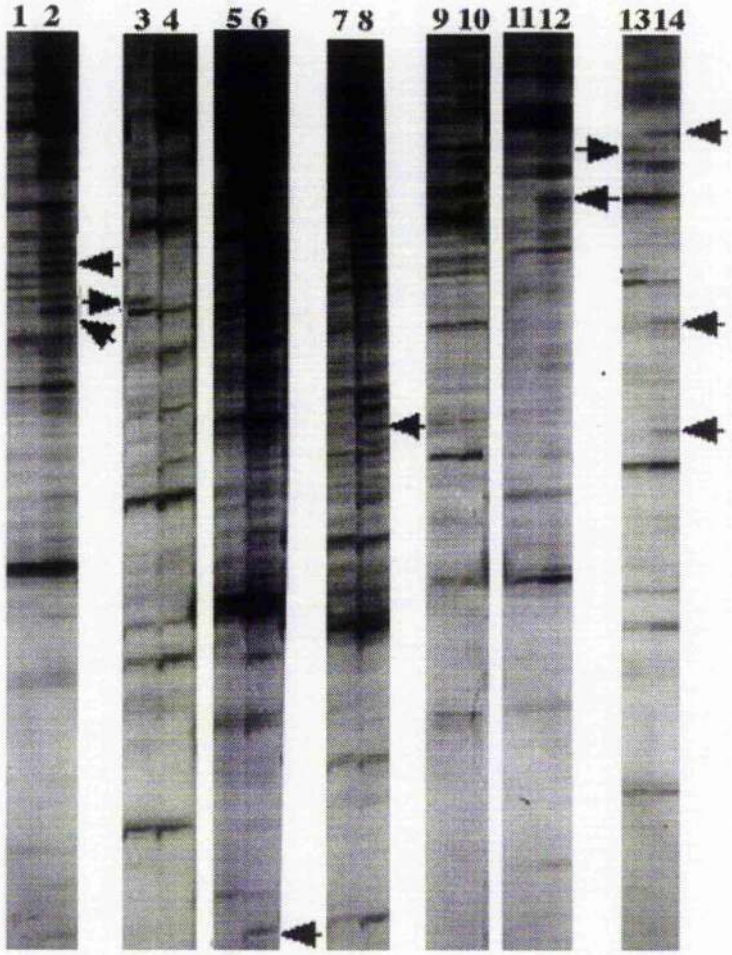


Figure 3-6. Autoradiograph showing messenger RNA fingerprints (amplified DNAs by RT-PCR) of Epitenon cells cultured on non-grooved and grooved surface for one hour. The odd-numbered lanes correspond to mRNA from the cells cultured on non-grooved surface whereas even-numbered lanes represent mRNA from the cells cultured on grooved surface. Several candidate cDNA tags that appear to be differently expressed are marked by arrows.

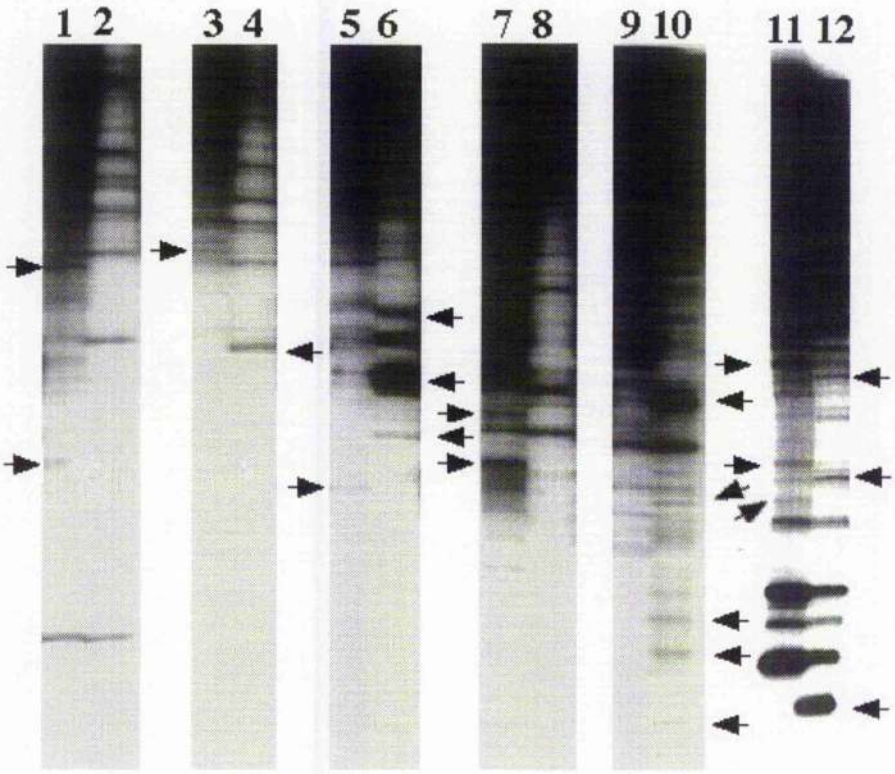


Figure 3-7. Autoradiograph showing messenger RNA fingerprints of Epitenon cells cultured on non-grooved and grooved surface for 4 hours. The odd-numbered lanes correspond to mRNA from the cells cultured on non-grooved surface whereas even-numbered lanes represent mRNA from the cells cultured on grooved surface. Several candidate cDNA tags that appear to be differently expressed are marked by arrows

Figure 3-8 Autoradiograph reversed grey scale negative scan showing messenger RNA fingerprints (amplified cDNAs by RT-PCR) of Epitenon cells which had been cultured for four hours on non-grooved or grooved (6μm deep; 12.5 μm wide) quartz structures using the same cDNA source as figure 3-7. Lane 1: non-grooved (control); lane 2: grooved; the samples were amplified in the presence of primers P1 and T1;. There is no different band. This figure indicates that with different combination of primer pair, the same fingerprints could be obtained between the cells cultured on grooved and non-grooved surfaces (This is a negative scan).

DISCUSSION

Initially it was decided to look at the protein populations between the cells grown on different topography surfaces, since it has been reported that fibronectin mRNA level has been increased when growing cells on a grooved surface compared with that on a flat surface. Using the SDS-PAGE method , two differences in protein experiments could be seen between cells grown on planar and cells grown on grooved surfaces. These might be proteins which are involved in contact guidance and molecules of extracellular matrix. It would be interesting to observe important proteins such as fibronectin, collegen and FAK by applying western blot. Alternatively, invesgations employing mRNA fingerprinting could be used to study the differences at mRNA level.

Messenger RNA fingerprinting is a powerful way to compare genes that are differentially expressed from two different circumstances. In most cases, even a single change in culture condition will result in the changes in gene expression over time and will usually affect tens or even hundreds of genes.

This study has proved that topography can affect cells where they attach and plays an important role not only in affecting cell shape but also stimulating different gene expression as early as one hour after the cells were seeded. Although only two types of cells are examined here, both macrophages and epitenon cells are strongly guided by surface topography. It was also noticed

that sometimes the samples from different sources (for example, cells with different passages) show different fingerprints. Possibly the reason for this is that, this method is very sensitive and can detect even rarely expressed mRNAs, in this case, very small changes in a mRNAs component can affect the fingerprinting results. But it is believed those mRNAs which were expressed differentially in this condition should always be detectable by using random primers which can cover up to 95% of genes. Apart from this, the gap between in vivo and in vitro still exists. It would be interesting to find out how other cell types varied in response to topography. This part of the work would be looked at in our laboratory in the future. Since this technique is also improving, it could be predicted that much progress will be made (see "Discussion", chapter 7).

Finally, to identify the differential expressed genes, it is necessary to carry out cloning and sequencing experiments (see Chapter 4).

CHAPTER 4

CLONING AND SEQUENCING

INTRODUCTION

In order to discover the identities of the differentially expressed DNA bands, they were recovered from the denatured 5% polyacrylamide/8M urea gel, and reamplified using the original primers. Reamplified DNAs often contain multiple cDNA species. Therefore, cloning is important to obtain the right cDNA fragment in order to carry out DNA sequencing, followed by confirmation with Northern blotting.

The pMOS*Blue* blunt ended cloning kit (Amersham) used has been designed to allow researchers the versatility to clone into pMOS*Blue* vector a choice of DNA fragments. The fragments can possess any termini, whether blunt ends, 5'-overhangs or 3'-overhangs. Consequently, DNA fragments from molecular biology techniques such as PCR, using proof-reading or non-proof-reading DNA polymerases, restriction digests and cDNA synthesis can be simply cloned into pMOS*Blue*.

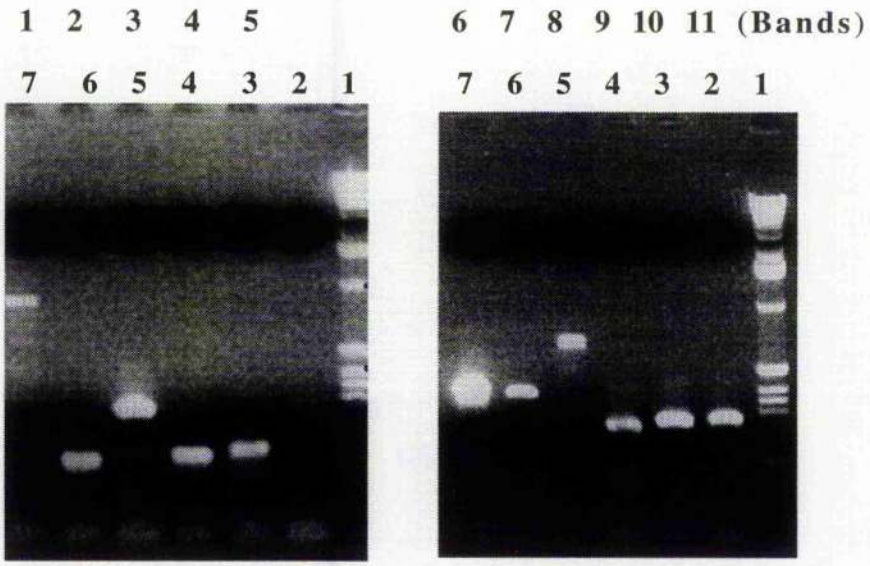
The cloned and purified DNAs were then sequenced with the use of a fluorescent automated DNA sequencer (Material and Methods). Unlike manual sequencing methods, which generally use a radioactive label and visualise the banding pattern by autoradiography, automated DNA sequencing use a scanning laser to detect DNA fragments labelled with fluorescent dyes. All the sequences

were entered into the Basic Local Alignment Search Tool (BLAST) "BLASTN" program. This sequence comparison tool compares a nucleotide query sequence against a nucleotide sequence database (Altschul *et al*, 1990). It is absolutely essential to confirm the differential expression of the cloned fragments by Northern analysis, with actual protein expression being confirmed by antibody methods.

RESULTS

REAMPLIFICATION OF DIFFERENTIALLY EXPRESSED mRNA FINGERPRINTING BANDS

In this study, the bands were selected from the up-regulated samples from P388D1 macrophage-like cells which had been cultured for four hours on grooved quartz (see Discussion). Only the most intense bands were excised and purified (see Chapter 2). A 1 Kb ladder is used to show the size of the bands. Seventeen differentially expressed bands which were present in grooved surface and absent from the control were excised from the denatured 5% polyacrylamide/8M urea gel. These DNAs were recovered from the dried denatured polyacrylamide gel and reamplified using the corresponding primer sets. Figure 4-1, figure 4-2 are reamplified DNAs were checked in 1.5% agarose gels. These results have proved that some of the reamplified DNA contain more than one species on the same band and the sizes vary from 100 bp to 700 bp.



A B

Figure 4-1. Both figure 4-1A and Figure 4-1B show differential displayed bands were cut out from the 5% polyacrylamide/8M urea gel and reamplified by PCR. PCR reaction was as suggested by the manufacturer's PCR parameters (94°C for 1 minute, 42°C for 1 minute and 68°C for 2 minutes with 20 cycles) using the original primers which listed above, and run in 1.5% agarose gel. Reading from right to left, lane 1 is 1 Kb DNA molecular marker, Lane 2 to lane 7 are reamplified bands. Named as band 1 band 2, band 3, band 4, band 5, band 6 and so on till band 11.

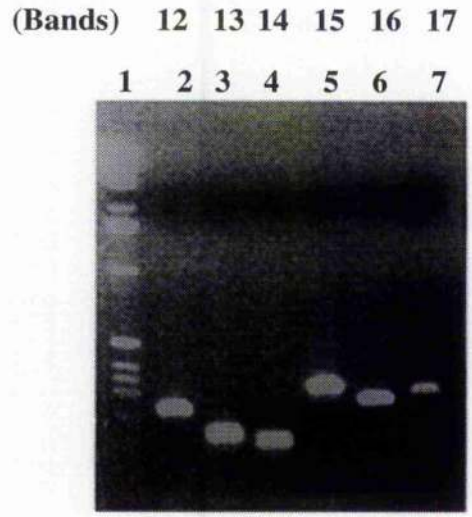


Figure 4-2. Differential displayed bands were cut out from the 5% polyacrylamide/8M urea gel and reamplified by PCR. The PCR reaction was as suggested by the manufacturer's PCR parameters (94°C for 1 minute, 42°C for 1 minute and 68°C for 2 minutes with 20 cycles) using the original primers and run in 1.5% agarose gel. Reading from left to right, Lane 1 is 1 Kb DNA molecular marker, Lane 2 to lane 7 are reamplified bands called as band 12, band 13, band 14, band 15, band 16 and band 17.

cDNA CLONING

Fifteen reamplified DNA fragments were cloned into the TA cloning vector. As described above, each reamplified DNA fragment may contain more than one cDNA species, though minor, these other cDNAs will also be cloned into the vector. Therefore, three colonies were picked up from each clone in order to get the correct clone. Figure 4-3 shows the cDNAs which were screened by PCR using either the vector primers (T7 and U19) or the original primers. The use of T7 promoter and U-19mer primers also enables the orientation of the insert to be determined by combining one vector-directed primer with one insert specific primer. The PCR reaction was performed as suggested by the manufacturer's PCR parameters of 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes and followed by a cycle of 72°C for 5 minutes. The PCR products were then examined in a 2% agarose gel containing ethidium bromide to check if this is a real or false positive. PCR products were then excised from agarose gel and purified in the same way as described above (see Chapter 2). Prior to carrying out the sequencing, DNA samples were checked in a 2 % agarose gel for purity and concentration. Figure 4-3 shows clean DNA bands which are ready for the sequencing.

Every clone was checked in the same fashion as described above. There are 45 DNA colonies in total from which 15 clones were prepared for sequencing analysis.

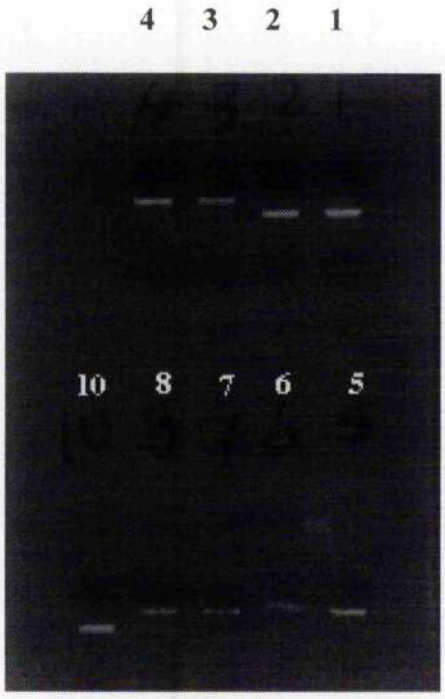


Figure 4-3. 2% agarose gel of cDNAs which were screened by PCR using either the vector primers (T7 and U19) or original primers. The PCR reaction was performed as suggested by the manufacturer's PCR parameters of 94°C for I minute, 55°C for 1 minute and 72°C for 2 minutes and followed by a cycle of 72°C for 5 minutes. PCR products were run in a gel and DNA bands were cut off and cleaned as described above. The concentrations of the cleaned DNAs were checked in a 2 % agarose gel. Clean, single DNA bands were observed in this gel.

SEQUENCING

The cloned and purified DNAs were then sequenced with an automated DNA sequencer (See Chapter 2). All sequencing were entered into the basic local alignment search tool (BLAST) "blastn" program on Netscape. This sequence comparison tool compares a nucleotide query sequence against a nucleotide sequence database (Altschul *et al.*, 1990). Only if three sequencing results, which are from the same source (same clone) and showed the same match were the samples were then used for Northern blot.

The sequencing results listed below include four sequences which have no homology, thus, they are novel DNAs.

Band 1)

TGATGCTGTAGGTCGATCTAGAGGACTACTAGTCATATGGATTAT

TAACCCTCACTAAATGCTGGGGAGACCATTTAAATACATTGTGAC

CTGTGTGATCATGCAGAAGAACGGTGCTGGGTTACACTCCGCAAG

TTCCTGCTTCTGGGACAGCTCCACAGACGGAAGCTGCACAGTCCG

ATGGGAGAACAAGACCATGTACTGCATCGTCAGTACCTTCGGACT

GTCCATCTGACCACCTTGCCAGCCTCAGCCTCGTGGCCCCAGCATT

TAGTGAGGGTTAATAATCGGATCCCCGGGTACCGAGCTCGAATTC

ACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAAAAAA

The closest match found for this sequence is close to the mouse *tctex-1*

mRNA complete cDNAs which the size is 680 bp. Regions of homology are shown below, and the probability of random relatedness is 2.2×10^{-79}.

```
Query: 19  CTGGGGAGACCATTTAAATACATTGTGACCTGTGTGATCAT 59
           ||||||||||||||||||||||||||||||||||||||||
Sbjct:181  CTGGGGAGACCATTTAAATACATTGTGACCTGTGTGATCAT 221

Query: 60   GCAGAAGAACGGTGCTGGGTTACACTCCGCAAGTTCCTGC 99
           ||||||||||||||||||||||||||||||||||||||||
Sbjct: 222 GCAGAAGAACGGTGCTGGGTTACACTCCGCAAGTTCCTGC 261

Query: 100 TTCTGGGACAGCTCCACAGACGGAAGCTGCACAGTCCGAT 139
           ||||||||||||||||||||||||||||||||||||||||
Sbjct: 262 TTCTGGGACAGCTCCACAGACGGAAGCTGCACAGTCCGAT 301

Query: 140 GGGAGAACAAGACCATGTACTGCATCGTCAGTACCTTCGG 179
           ||||||||||||||||||||||||||||||||||||||||
Sbjct: 302 GGGAGAACAAGACCATGTACTGCATCGTCAGTACCTTCGG 341

Query: 180 ACTGTCCATCTGACCACCTTGCCAGCCTCAGCCTCGTGGC 219
           ||||||||||||||||||||||||||||||||||||||||
Sbjct: 342 ACTGTCCATCTGACCACCTTGCCAGCCTCAGCCTCGTGGT 381

Query: 220 CCCAGCATTT 229
           ||||||||||
Sbjct: 382 CCCAGCATTT 391
```

Band 2)

TGNTGCTGCAGGTCGACTCTAGNGGTCTACTAGTCATATGGATTA

TTAACCCTCACTAAATGCTGGGGTTGGCAGTGTCCTCCTCATTCCT

CGCACGATGGCTGACCAGCAGGCTCGGCGACAGGAGAGAAAGCC

AAAGGACCGGAGGAAAGTGAGCGTCCACCCCCAGCATTTAGTGAG

GGTTAATAATCGGATCCCCGGGTACCGAGCTCGAATTCACTGGCC

GTCGTTTTACAACGTCGTGACTGGGAAAA

The closest match found for this sequence is to *M. musculus* NRF1 mRNA complete cDNAs which was a size of 4040 bp. Regions of homology are shown below, and the probability is 1.4×10^{-27}. This cDNA was later used as a probe

in Northern blot and examined with its specific antibody to detect the location and expression in comparison to the cells which were cultured on the grooved and plain surfaces

```
Query:   60 ATGCTGGGGTTGGCAGTGTCCTCCTCATTCCTCGCACGAT 99
            ||||||||| ||||||||||||||||||||||||||||||
Sbjct: 2176 ATGCTGGGGATGGCAGTGTCCTCCTCATTCCTCGCACGAT 2215

Query: 100  GGCTGACCAGCAGGCTCGGCGACAGGAGAGAAAGCCAAAG 139
            ||||||||||||||||||||||||||||||||||||||||
Sbjct: 2216 GGCTGACCAGCAGGCTCGGCGACAGGAGAGAAAGCCAAAG 2255

Query: 140  GACCGGAGGAA 150
            ||||||||||
Sbjct: 2256 GACCCGAGGAA 2266
```

Band 3)

CTGNTGGCTTATTCGATGGNCGCAGGTCGACTCTAGAGGACTACT

AGTCATATGGATTATTAACCCTCACTAAATGCTGGGGAAAAAAAA

GAGCGAGAGCGCCAGCTATCCTGAGGGAAACTTCGGAGGGAACC

AGCTACTAAATGGTTCGATTAGTCTTTCGCCCCTATACCCAGGTCG

GACNACCGATTTGCACGTCAGGACCGCTACGGACCTCCACCAGAG

TTTCCTCTGGCTTCGCCCTGCCCAGGCATAGTTCACCATCTTTCGG

GTCCTAACGCATACNCTCNTGCTCCACCTCCCCAGCATTTAGTGAG

GGTTAATAATCGGATCCCCGGGTACCGAGCTCGAATTCACTGGCC

GTCGTTTTACAACGTCGTGACTGGAAAA

The closest match found for this sequence is to the Mouse 28S ribosomal RNA which was a size of 4712 bp as well as M. musculus 45S pre rRNA gene, the size of this gene is 22,118 bp. This is due to the homology between the genes. Regions of homology are shown below, and the probability is 1.2 x

10^{-75}. This cDNA was later used as a probe in Northern blot analysis.

emb|X00525|MMRNA02 Mouse 28S ribosomal RNA. Length = 4712

```
Query:   286 GCTGGGGAGGTGGAGCACGAGCGTATGCGTTAGGACCC 249
             || |||||||||||||||||||||||||||||||||||
Sbjct:  1296 GCCGGGGAGGTGGAGCACGAGCGTACGCGTTAGGACCC 1333

Query:   248 GAAAGATGGTGAACTATGCCTGGGCAGGGCGAAGCCAG 211
             |||||||||||||||||| ||||||||||||||||||
Sbjct:  1334 GAAAGATGGTGAACTATGCTTGGGCAGGGCGAAGCCAG 1371

Query:   210 AGGAAACTCTGGTGGAGGTCCGTAGCGGTCCTGACGTG 173
             ||||||||||||||||||||||||||||||||||||||
Sbjct:  1372 AGGAAACTCTGGTGGAGGTCCGTAGCGGTCCTGACGTG 1409

Query:   172 CAAATCGGTNGTCCGACCTGGGTATAGGGGCGAAAGAC 135
             |||||||| |||||||||||||||||||||||||||||
Sbjct:  1410 CAAATCGGTCGTCCGACCTGGGTATAGGGGCGAAAGAC 1447

Query:   134 TAATCGAACCATTTAGTAGCTGGTTCCCTCCGAAGTTT 97
             |||||||||||| ||||||||||||||||||||||||
Sbjct:  1448 TAATCGAACCATCTAGTAGCTGGTTCCCTCCGAAGTTT 1485

Query:   96  CCCTCAGGATAGCTGGCGCTCTCGCTC 70
             |||||||||||||||||||||||||||
Sbjct:  1486 CCCTCAGGATAGCTGGCGCTCTCGCTC 1512

emb|X82564|MM45SRRNA M.musculus 45S pre rRNA gene

     Length = 22,118. Minus Strand HSPs:

Query:   286 GCTGGGGAGGTGGAGCACGAGCGTATGCGTTAGGA 252
             || ||||||||||||||||||||||||||||||||
Sbjct: 15054 GCCGGGGAGGTGGAGCACGAGCGTACGCGTTAGGA 15088

Query:   251 CCCGAAAGATGGTGAACTATGCCTGGGCAGGGCGA 217
             |||||||||||||||||||| ||||||||||||
Sbjct: 15089 CCCGAAAGATGGTGAACTATGCTTGGGCAGGGCGA 15123

Query:   216 AGCCAGAGGAAACTCTGGTGGAGGTCCGTAGCGGT 182
             ||||||||||||||||||||||||||||||||||
Sbjct: 15124 AGCCAGAGGAAACTCTGGTGGAGGTCCGTAGCGGT 15158

Query:   181 CCTGACGTGCAAATCGGTNGTCCGACCTGGGTATA 147
             |||||||||||||||||| ||||||||||||||||
Sbjct: 15159 CCTGACGTGCAAATCGGTCGTCCGACCTGGGTATA 15193

Query:   146 GGGGCGAAAGACTAATCGAACCATTTAGTAGCTGG 112
             ||||||||||||||||||||||||||||||||||
 Sbjct: 15194 GGGGCGAAAGACTAATCGAACCATCTAGTAGCTGG 15228
```

```
Query:   111  TTCCCTCCGAAGTTTCCCTCAGGATAGCTGGCGCT 77
              ||||||||||||||||||||||||||||||||||
Sbjct: 15229  TTCCCTCCGAAGTTTCCCTCAGGATAGCTGGCGCT 15263

Query:    76  CTCGCTC 70
              |||||||
Sbjct: 15264  CTCGCTC 15270
```

Band 4)

ATTNACCCTCACTAAATGCTGGTGGGTGACCCCTTGAGCAGCAAG

GTGACTGGTGCTAACTCCTGCCTTTTCTGATGTTCAGGTGAACTAC

ACCGAGGTTTCCAGGGTCTGCAATCTCTGGCGCAACTATNAATAC

NTCCANGACTCCTGGAAGAGTGTGCTGTCCNTCCTGGACNGGTNT

GTGANACGTCAGGATGTACCGCNTNTCGGTGAGCGNGTCCTGGGC

ACTGGAATGACCCANACATGGTACCAGCCTGGAAAGTGAGGGTTA

ATAATCGGATCCCCGGGTACCGAGCTCGAATTCACTGGCCGTCGT

TTTACAACGTCGTGACTGGGAAAGG

The closest match found for this sequence is to Human alpha-N-acetylgalactosaminidase (NAGA) gene which the size is 13,663 bp. Regions of homology are shown below, and the probability is $8.6e^{-23}$. The fact of this mouse cDNA is mostly matched with human gene is because the human gene bank is more advanced compared with mouse gene databank.

```
Query:   74  GATGTTCAGGTGAACTACACCGAGGTTTCCGGGGTCT 110
             ||   | ||||||||||||||| | | |    |  |||
Sbjct: 6183  GACATCCAGGTGAACTACAGTCTGCTGGCGGACATCT 6219

Query:  111  GCAATCTCTGGCGCAACTATNAATACNTCCANGACTC 147
             ||||  ||||||||||||||||  ||  ||  |||| |||||
Sbjct: 6220  GCAACCTCTGGCGTAACTATGATGACATCCAGGACTC 6256

Query:  148  CTGGAAGAGTGTGCTGTCCATCCTGGACTGGTTTGTG 184
             |||||  |||  |||||  |||||||||  |  |||||||||
Sbjct: 6257  CTGGTGGAGCGTGCTCTCCATCCTGAATTGGTTCGTG 6293
```

```
Query:  185 AAACGTCAGGATGTACCGC 203
            ||  |  |||||  |||  ||
Sbjct: 6294 GAGCACCAGGACATACTGC 6312

Query:  214 GCGGGTCCTGGGCACTGGAATGACCCANACATGGTA 249
            ||  ||  ||||||||||||||||||||||  ||||||||
Sbjct: 6321 GCCGGCCCTGGGCACTGGAATGACCCTGACATGGTA 6356

Query:  250 CCAGCCTGGA 259
            ||||  ||||
Sbjct  6357 CCAGGATGGA 6366
```

gb|M38083|HUMNAGB Human alpha-N-acetylgalactosaminidase mRNA, complete cds. Length = 1821

```
Query:   82 GGTGAACTACACCGAGGTTTCCAGGGTCTGCAATCTCT 119
            ||||||||||||    | |  |      |||||||  ||||
Sbjct:  669 GGTGAACTACAGTCTGCTGGCGGACATCTGCAACCTCT 706

Query:  120 GGCGCAACTATGAATACNTCCANGACTCCTGGAAGAGT 157
            ||||  ||||||||  ||  ||||  |||||||||  |||||
Sbjct:  707 GGCGTAACTATGATGACATCCAGGACTCCTGGTGGAGC 744

Query:  158 GTGCTGTCCNTCCTGGACTGGTNTGTGANACGTCAGGA 195
            |||||||||  |||||  |  ||||  |||    |  |||||
Sbjct:  745 GTGCTCTCCATCCTGAATTGGTTCGTGGAGCACCAGGA 782

Query:  196  TGTACCGC 203
               |||  ||
Sbjct:  783  CATACTGC 790

Query:  214 GCGNGTCCTGGGCACTGGAATGACCCAGACATGGTAC 250
            ||   |  |||||||||||||||||||||  ||||||  |  |
Sbjct:  799 GCCGGCCCTGGGCACTGGAATGACCCTGACATGCTGC 835
```

gb|M62783|HUMAGALNA Human alpha-N-acetylgalactosaminidase mRNA, complete cds. Length = 3598

```
Query:   82 GGTGAACTACACCGAGGTTTCCAGGGTCTGCAATCTCT 119
            ||||||||||||    | |  |      |||||||  ||||
Sbjct: 1066 GGTGAACTACAGTCTGCTGGCGGACATCTGCAACCTCT 1103

Query:  120 GGCGCAACTATNAATACNTCCAGGACTCCTGGAAGAGT 157
            ||||  ||||||  |  ||  ||||||||||||||||  ||
Sbjct: 1104 GGCGTAACTATGATGACATCCAGGACTCCTGGTGGAGC 1141

Query:  158 GTGCTGTCCNTCCTGGACNGGTNTGTGANACGTCAGGA 195
            |||||  |||  |||||  |  |||  ||||  ||  |||||
Sbjct: 1142 GTGCTCTCCATCCTGAATTGGTTCGTGGAGCACCAGGA 1179
```

```
Query:   196 TATACCGC 203
             ||||  ||
Sbjct:  1180 CATACTGC 1187

Query:   214 GCGNGTCCTGGGCACTGGAATGACCCAGACATGGTAC 250
             ||  | ||||||||||||||||||| ||||||  | |
Sbjct:  1196 GCCGGCCCTGGGCACTGGAATGACCCTGACATGCTGC 1232
```

Band 5)

CATTATGCTGAGTGATATCTTTTTTTTTCCCTCTTTCATTCTTAGA

AGCTGGTAGTTATGAAATGACAAATCAACATATAAAGCAAAATG

GAAAATTAGAAGATAACCCTTGCTCTGGTAGTCCTCCGAGGACTA

CATTGTTGGGGACCATATTTTCGCCTGTCTTCAACTTTTTTTCACC

AGCAAATAAAAATGGTAAGTAAAACCCATTTTTTAGGCCCCAGCA

TTTAGTGAGGGTTAATAATCGGATCCCCGGGTACCGAGCTCGAAT

TCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAAAAA

The closest match found for this sequence is to Human partial cDNA sequence, clone x874; which has a size of 697 bp of unknown function. Regions of homology are shown below, and the probability is 1.3×10^{-44}.

```
Query:    56 AGAAGCTGGTAGTTATGAAATGACAAATCAACATATAA 93
             |||||||||||||||||||| |||||||||||||| |||
Sbjct:   482 AGAAGCTGGTAGTTATGAAAAGACAAATCAACATGTAA 519

Query:    94 AGCAAAATGGAAAATTAGAAGATAACCCTTGCTCTGGT 131
             | |||||||||| |||||||||||||||||||| ||||||
Sbjct:   520 AACAAAATGGAAAATTAGAAGATAATCCTTCCTCTGGC 557

Query:   132 AGTCCTCCGAGGACTACATTGTTGGGGACCATATTTTC 169
             |||||||| ||||||| |||||||||||||||||||||
Sbjct:   558 AGTCCTCCAAGGACTACTTTGTTGGGGACCATATTTTC 595

Query:   170 GCCTGTCTTCAACTTTTTTTCACCAGCAAATAAAAATG 207
             |||||||||||||||||||||||||||||||||||||
Sbjct:   596 ACCTGTCTTCAACTTTTTTTCACCAGCAAATAAAAATG 633

Query:   208 GAAAGTAA 215
             |||||| |
Sbjct:   634 GAAAGTCA 641
```

Band 6)

TTCAACGCTNAGACTCGTGTTTACTAATCAAGCTTTTGTGTTTATC

TTCCTTATTTTTACTGAACATCTTCAGTACCTGGGTAATGGAAACC

CACTCTTTTTTCATTGTAATNACTTTGGGGGCTTTTGTGANTNAGG

GAGGGTGGGATAAAAGGTGGCANAAGGAACAAGGTCTTAATTGC

TCTGAAGGTTGGACCCGTGAANCCCTGGCATATACCNGAATANGT

GGCGCCCAGTGGGTGGTGGTTTCTCAATCTCTGGGCCCCAGCATTT

AGTGAGGGTTAATAATCGGATCCCCGGGTACCGAGCTCGAATTCA

CTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAA

No homology is found, therefore, it is a novel cDNA fragment.

Band 7)

GGCCCTTTAACGACGTACGCAGTTTTATCCGGTAAAGCGAATGAT

TAGAGGTCTTGGGGCCGAAACGATCTCAACCTATTCTCAAACTTT

AAATGGGTTAGAAGCCCGGCTCNCTGGCGTTGAAACCGGGCGTTG

AATGCGAATTCCTAATGGGCCACTTTTGGTTAGCAAAACTGGCGC

TGCGGGATGAACCGAACGCCGGGTTAGGGGCCCGATGCCGACGCT

CAGACCCCAGCATTTAGTGAGGGTAAT

The closest match found for this sequence is to 28S ribosomal RNA gene which has a size of 4712 bp. Regions of homology are shown below, and the probability is 9.6×10^{-38}.

```
Query:      2 CGACGTACGCAGTTTTATCCGGTAAAGCGAATGATTAG 39
              |||||||||||||||||||||||||||||||||||||
Sbjct:   1514 CGACGTACGCAGTTTTATCCGGTAAAGCGAATGATTAG1551
```

```
Query:    40  AGGTCTTGGGGCCGAAACGATCTCAACCTATTCTCAAA 77
              |||||||||||||||||||||||||||||||||||||
Sbjct:  1552  AGGTCTTGGGGCCGAAACGATCTCAACCTATTCTCAAA 1589

Query:    78  CTTTAAATGGGTTAGAAGCCCGGCTCNCTGGCGTTGAA 115
              |||||||||||| |||||||||||| ||||||| ||
Sbjct:  1590  CTTTAAATGGGTAAGAAGCCCGGCTCGCTGGCGTGGAG 1127

Query:   116  ACCGGGCGTTGAATGCGAATTCCTAATGGGCCAC 149
              |||||||| |||||||| | |||| ||||||||
Sbjct:  1128  GCCGGGCGTGGAATGCGAGTGCCTAGTGGGCCAC 1161

Query:   190  CCGGGTTAGGGGCCCGATGCCGACGCTCA 218
              | || | ||| ||||||||||||||||||
Sbjct:  1702  CGGGTTAAGGCGCCCGATGCCGACGCTCA 1730
```

Band 8)

CTGNTGGCTTATTCGATGGNCGCAGGTCGACTCTAGAGGACTACT

AGTCATATGGATTATTAACCCTCACTAAATGCTGGGGAAAAAAAA

GAGCGAGAGCGCCAGCTATCCTGAGGGAAACTTCGGAGGGAACC

AGCTACTAAATGGTTCGATTAGTCTTTCGCCCCTATACCCAGGTCG

GACNACCGATTTGCACGTCAGGACCGCTACGGACCTCCACCAGAG

TTTCCTCTGGCTTCGCCCTGCCCAGGCATAGTTCACCATCTTTCGG

GTCCTAACGCATACNCTCNTGCTCCACCTCCCCAGCATTTAGTGAG

GGTTAATAATCGGATCCCCGGGTACCGAGCTCGAATTCACTGGCC

GTCGTTTTACAACGTCGTGACTGGAAAAAAAAAAGGGG

The closest match found for this sequence is with mouse ribosomal RNA genes which has a size of 4712 bp. Regions of homology are shown below, and the probability is 1.1×10^{-75}. This cDNA shows the overlapping area as band 3. This cDNA has been amplified during PCR by the different primer set which picked up the different area from the same gene.

emb|X00525|MMRNA02 Mouse 28S ribosomal RNA. Length = 4712

```
Query:   307 GCTGGGGAGGTGGAGCACGAGCGTATGCGTTAGGACCC 270
             ||  |||||||||||||||||||||||| ||||||||||||
Sbjct:  1296 GCCGGGGAGGTGGAGCACGAGCGTACGCGTTAGGACCC 1333

Query:   269 GAAAGATGGTGAACTATGCCTGGGCAGGGCGAAGCCAG 232
             ||||||||||||||||||||||||||||||||||||||||
Sbjct:  1334 GAAAGATGGTGAACTATGCTTGGGCAGGGCGAAGCCAG 1371

Query:   231 AGGAAACTCTGGTGGAGGTCCGGTAGCGTCCTGACGT 195
             ||||||||||||||||||||||||||||||||||||||
Sbjct:  1372 AGGAAACTCTGGTGGAGGTCCGGTAGCGTCCTGACGT 1408

Query:   194 GCAAATCGGTNGTCCGACCTGGGTATAGGGGCGAAAGA 157
             |||||||||||||||||||||||||||||||||||||||
Sbjct:  1409 GCAAATCGGTCGTCCGACCTGGGTATAGGGGCGAAAGA 1446

Query:   156 CTAATCGAACCATTTAGTAGCTGGTTCCCTCCGAAGT 120
             ||||||||||||||||||||||||||||||||||||
Sbjct:  1447 CTAATCGAACCATCTAGTAGCTGGTTCCCTCCGAAGT 1483

Query:   119 TTCCCTCAGGATAGCTGGCGCTCTCGCTC 91
             |||||||||||||||||||||||||||||
Sbjct:  1484 TTCCCTCAGGATAGCTGGCGCTCTCGCTC 1512
```

emb|X82564|MM45SRRNA M.musculus 45S pre rRNA gene
Length = 22,118

```
Query:   307 GCTGGGGAGGTGGAGCACGAGCGTATGCGTTAGGACC 271
             ||  |||||||||||||||||||||||| ||||||||||||
Sbjct: 15054 GCCGGGGAGGTGGAGCACGAGCGTACGCGTTAGGACC 15090

Query:   270 CGAAAGATGGTGAACTATGCCTGGGCAGGGCGAAGCC 234
             |||||||||||||||||||| ||||||||||||||||
Sbjct: 15091 CGAAAGATGGTGAACTATGCTTGGGCAGGGCGAAGCC 15127

Query:   233 AGAGGAAACTCTGGTGGAGGTCCGTAGCGGTCCTGAC 197
             ||||||||||||||||||||||||||||||||||||||
Sbjct: 15128 AGAGGAAACTCTGGTGGAGGTCCGTAGCGGTCCTGAC 15164

Query:   196 GTGCAAATCGGTNGTCCGACCTGGGTATAGGGGCGAA 160
             ||||||||||||||||||||||||||||||||||||||
Sbjct: 15165 GTGCAAATCGGTCGTCCGACCTGGGTATAGGGGCGAA 15201

Query:   159 AGACTAATCGAACCATTTAGTAGCTGGTTCCCTCCGA 123
             ||||||||||||||||||||||||||||||||||||||
Sbjct: 15202 AGACTAATCGAACCATCTAGTAGCTGGTTCCCTCCGA 15238

Query:   122 AGTTTCCCTCAGGATAGCTGGCGCTCTCGCTC 91
             ||||||||||||||||||||||||||||||
Sbjct: 15239 AGTTTCCCTCAGGATAGCTGGCGCTCTCGCTC 15270
```

Band 9)

TTTTTTCANTTTTTTTTTTGCATGGCCCATTNAACTCTAGANGACT

NCTANTCATATGGATTATTAACCCTCACTAAATGCTGGGGAATCA

GGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCC

AAGGAAGGCAGCAGGCGCGCAAATTACCCACTCCCCAGCATTTAG

TGAGGGTTAATAATCGGATCCCCGGGTACCGAGCTCGAATTCACT

GGCCGTCGTTTTACAACGTCGTGACTGGGAAAACAAAGG

The closest match found for this sequence is to the Human 18S rRNA gene, complete which has a size of 2235 bp. Regions of homology are shown below, and the probability is 1.4×10^{-27}.

gb|K03432|HUMRGEA Human 18S rRNA gene. Length = 2235

```
Query:    69 TGCTGGGGAATCAGGGTTCGATTCCGGAGAGGGAGCC 105
             ||  |||||||||||||||||||||||||||||||||||
Sbjct:   546 TGACGGGGAATCAGGGTTCGATTCCGGAGAGGGAGCC 582

Query:   106 TGAGAAACGGCTACCACATCCAAGGAAGGCAGCAGGC 142
             ||||||||||||||||||||||||||||||||||||||
Sbjct:   583 TGAGAAACGGCTACCACATCCAAGGAAGGCAGCAGGC 619

Query:   143 GCGCAAATTACCCACTCCCCAGC 165
             |||||||||||||||||||| | |
Sbjct:   620 GCGCAAATTACCCACTCCCGACC 642
```

gb|M10098|HUMRGE Human 18S rRNA gene, complete. Length = 1969

```
Query:    69 TGCTGGGGAATCAGGGTTCGATTCCGGAGAGGGAGCC 105
             ||  |||||||||||||||||||||||||||||||||||
Sbjct:   507 TGACGGGGAATCAGGGTTCGATTCCGGAGAGGGAGCC 543

Query:   106 TGAGAAACGGCTACCACATCCAAGGAAGGCAGCAGGC 142
             ||||||||||||||||||||||||||||||||||||||
Sbjct:   544 TGAGAAACGGCTACCACATCCAAGGAAGGCAGCAGGC 580

Query:   143 GCGCAAATTACCCACTCCCCAGC 165
             |||||||||||||||||||| | |
Sbjct:   581 GCGCAAATTACCCACTCCCGACC 603
```

emb|X00686|MMRNA18 Mouse gene for 18S rRNA. Length = 1869

```
Query:    69 TGCTGGGGAATCAGGGTTCGATTCCGGAGAGGGAGCC 105
             ||  ||||||||||||||||||||||||||||||||||
Sbjct:   407 TGACGGGGAATCAGGGTTCGATTCCGGAGAGGGAGCC 443

Query:   106 TGAGAAACGGCTACCACATCCAAGGAAGGCAGCAGGC 142
             ||||||||||||||||||||||||||||||||||||||
Sbjct:   444 TGAGAAACGGCTACCACATCCAAGGAAGGCAGCAGGC 480

Query:   143 GCGCAAATTACCCACTCCCCAGC 165
             ||||||||||||||||||| | |
Sbjct:   481 GCGCAAATTACCCACTCCCGACC 503
```

Band 10)

GCTGCCCGCATGTCGATCTAGAGGNCTACTAGTCATATGGATTAT

TAACCCTCACTAAATGCTGGGGTTAAGCTCTGCGGGCCCAGAGGG

CTCCTTCAGGTGCTGCAAGTAATCGTAATCGTCGTCNAANNACAC

GCCGTACTTCCTCTGCTCGGCCCGTCTTTCCCCAGCATTTAGTGAG

GGTTAATAATCGGATCCCCGGGTACCGAGCTCGAATTCACTGGCC

GTCGTTTTACAACGTCGTGACTGGGAAACAAAAGG

The closest match found for this sequence is to *Homo sapiens* cig64 mRNA, partial sequence which has a size of 272 bp. Regions of homology are shown below, and the probability is 3.8×10^{-09}.

```
Query:    17 GATTATTAACCCTCACTAAA 36
             ||||||||||||||||||||
Sbjct:     4 GATTATTAACCCTCACTAAA 23

Query:   150 TTTAGTGAGGGTTAATAATC 169
             ||||||||||||||||||||
Sbjct:   239 TTTAGTGAGGGTTAATAATC 258

Query:   172 TCCGATTATTAACCCTCACTAAA 150
             ||||||||||||||||||||||
Sbjct:     1 TCCGATTATTAACCCTCACTAAA 23

Query:    36 TTTAGTGAGGGTTAATAATCCATATGACTAGTAG 3
             |||||||||||||||||||||||||||||||||
Sbjct:   239 TTTAGTGAGGGTTAATAATCCATATGACTAGTAG 272
```

Band 11)

AGTGCATGCCATCTTGGGAGTTCTAAGCCTCCTACCTTGGCCTCCT

GGATGCTAGAACAGGCCTAAGCTCCACACTCTGTGTCCAAAGGAA

GATGTAAGTTTTTCACTAGAAAGATAAATAATGCTGCACAGAAAG

ATCAAAAGACATTTGACCCAGCATGGTGGCACACATCTTTATCTC

AGCACTGTTCAGGGAACTGAGTCAACACTGTTCTGGGATAGCAAT

CGGATCCCCGGGTACCGAGCTCGAATTCACTGGCCGTCGTTTTACA

ACGTCGTGACTGGGAA

The closest match found for this sequence is *M. musculus* c-kit gene exon 1, the size of this gene is 4490 bp. Regions of homology are shown below, and the probability (P value) is 8.6×10^{-23}. This cDNA has been further investigated with its antibody and antisense later. (see Chapter 5).

```
Query:   177 TAAAGATGTGTGCCACCATGCTGGGTCAAA 148
             ||||||||||||||||||||||||||  ||
Sbjct:   200 TAAAGATGTGTGCCACCATGCTGGGTTCAA 229

Query:   155 GGGTCAAATGTCTTTTGATCTTTCTGTGCAGCA 123
             ||  ||||||||||||||||||||||||||||||
Sbjct:   223 GGTTCAAATGTCTTTTGATCTTTCTGTGCAGCA 255

Query:   122 TTATTTATCTTTCTAGTGAAAAACTTACATCTT 90
             |||||||||||||||||||  ||||||||||||
Sbjct:   256 TTATTTATCTTTCTAGTGAAAAACTTACATCTT 288

 Query:    89  CCTTTGGACACAGA 76
              ||||||||||||  |
 Sbjct:   289  CCTTTGGACACATA 302
```

Band 12)

AGTGCATGCCATCTTGGGAGTTCTAAGCCTCCTACCTTGGCCTCCT

GGATGCTAGAACAGGCCTAAGCTCCACACTCTGTGTCCAAAGGAA

GATGTAAGTTTTTCACTAGAAAGGTGTCCCCTGCTGCAGCTGCTA

AGGCACGCAAATATGGTGCTGCTGGCCTTGGAGGCTTC

CTAGGAGCCAGGCCATTCCCAGGTGGAGGAGTTGCAGCAA

GACCTGGCTTTGGACTTTCTTGAGTCAACACTGTTCTGGGATAGC

gb|U08210|MMU08210Mus musculus elastin mRNA, complete cds. Length = 2651. Regions of homology are shown below, and the probability (P value) is 8.6 x 10^{-22}.

```
Query:    115 GTGTCCCCTGCTGCAGCTGCTAAGGCACGCAAATA 149
              |||||||||||||||||||||||||||||||||||
Sbjct:   2326 GTGTCCCCTGCTGCAGCTGCTAAGGCAGCCAAATA2360

Query:    150 GCCACTAGGATGGTGCTGCTGGCCTTGGAGGCTTC 184
              |||||||||||||||||||| ||||||||||||||
Sbjct:  2361 CTAGGAGCCATGGTGCTGCTGGCCTTGGAGGTGTC 2395

Query:    185 GGCCATTCCCAGGTGGAGGAGTTGCAGCAAGACCT 219
              |||||||||||||||||||||||||||||||||||
Sbjct:  2396 GGCCATTCCCAGGTGGAGGAGTTGCAGCAAGACCT 2430

Query:  220   GGCTTTGGACTTTCT 234
              |||||||||||||||
Sbjct:  2431 GGCTTTGGACTTTCT 2445
```

Band 13)

ATCTTCCTTATTTTTACTGAACATCTTCAGTACCTGGGTAATGGAC

TCTTTTTTCATTGTAATACTTTGGGGGCTTTTGTGATAGGGAGGG

TGGGATAAAAGGTGGCAAAGGACAAGGTCTTAATTGCTCTGAAG

GTTGGACCCGTGAACCCTGGCATAACCGAAAGTGGCNCCCAGTGG

GTGGTGATCGGATCCCCGGGTACCGAGCTCGAATTCACTGGCCGT

CGTTTTACAACGTCGTGACTGGGAAAAAAA

This sequencing has 'no known matches' on the database and therefore is a novel sequence.

Band 14)

GCTTGCATGCCTGCAGGTCGACTCTAGAGGATCTACATGTCATTA
TGGATATTTTATTTTCAAGGCCTTCTAAACTCTAATTTGGTTCGCC
AGCTGTCGGATTTCCATCCATTTCCTCGTCTTCACTCTCAGAGTCA
CTGTCATACTCCCCAGCATTTAGGTGAGGGGTTAATAATCGGATC
CCCGGGTACCGAGCTCGAATTCACTGGCCGTCGTTTTACAACGTCG
TGACTGGGAAAAAAA

This sequencing has 'no known matches' on the database and therefore is a novel sequence.

Band 15)

TGCAGGTCGACTCTAGAGGATCTACATGTCATTATGGATAGCCAA
ACCCCTTTGGTCACCTTCTGCTTTGATAAGTTGTGTACGCAGCTCT
TCTACCTGAGCAAGACGTTCTCTATTGCTTTCAGATGACTCAGCA
GCCTGCGTGAGACTCCAATTCCGTTCAGTTGGCTCTCCCGGTTTCA
CTTGTCGCAGCTTTCTTTTATACTCCTCATTCTCACTGCTTTACTC
TGTTGGATCCCCGGGTACCGAGCTCGAATTCACTGGCCGTCGTTT
TACAACGTCGTGACTGGGAAAACC

This sequencing has 'no known matches' on the database and therefore is a novel sequence.

NORTHERN BLOT ANALYSIS

The sequencing results show the wide range of different genes expressed as a response of cells to their surface topography. Northern blot analysis was carried out to confirm these differentially expressed genes. The total RNA was isolated from P388D1 macrophages after allowing them to adhere to the grooved surface for 4 or 24 hours. The total RNA was separated by electrophoresis in 1% formaldehyde agarose/MOPS gels and blotted to nitro-cellulose. The different DNA fragments received from DD-PCR were used as probe with ^{32}P labelling to hybridised mRNA in Northern Blotting analysis (see figure 4-4, figure 4-5).

When using 18S, 28S and 45S rRNA fragments as probes, of course, no differences were detected by Northern Blotting analysis. This may due to the high level of ribosomal RNA in cultured cells. Due to the size (25 mm x 35 mm) of the structure used in this study, only a limited number of cells could be handled, this leads to the difficulty with Northern blots. Therefore, antibody staining were used to confirm those genes, see chapter 5.

Figure 4-4 shows that when P388D1 macrophage like cells were cultured on a grooved surface, *tctex* mRNA level increased more than in the cells grown on planar surface at 4 hrs, although after 24 hours culturing, it is hard to tell the differences. This also proved that P388D1 macrophage like cells could react to the grooved surface after few hours by elongation along the direction of the grooves.

Figure 4-5 shows that α-N-acetylgalactosaminidase mRNA level is higher when P388D1 macrophage like cells were cultured on grooved surface after 4 hours comparing with the cells culturing on planar surface.

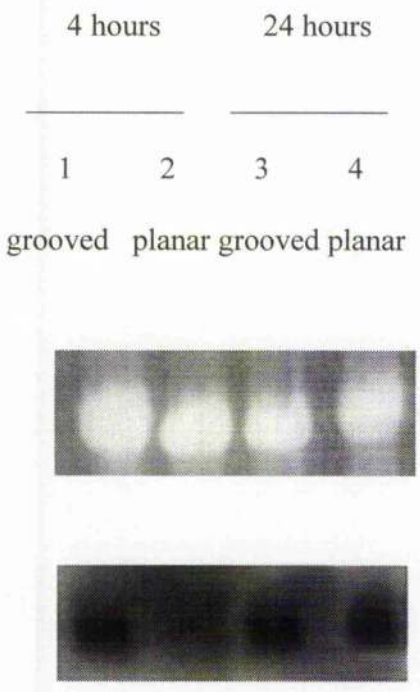
ANTISENSE EXPERIMENT

Tctex-1 *(t complex testis expressed 1)* is a protein encoded by a member of the multigene family in the *t* complex region that is involved in male sterility and development of the germ cells. *Tctex-1* has been demonstrated very recently that it is a light chain component of the cytoplasmic dynein complex, and was recently recognised as a strong fyn binding protein in the yeast system, and this interaction was localised to the first 19 amino acids of fyn.

In order to invest the role of *tctex-1* which is involved in microtubule-associated movement. Phosphorothioate-modified antisense against *tctex* oligonucleotides were used as described in Chapter 2, to block the *tctex-1* translation pathway, to address whether *tctex*-1directly controls cell spreading and elongation along their microgrooved substrata.

P388D1 macrophage-like cells were cultured on the grooved quartz structure (12.5 μm wide; 2.5 μm deep). Antisense against *tctex-1* was added alternatively at the final concentration of 2 μM and 10 μM and incubated at 37°C. Cell length (cell major axis) were measured after fixing and staining the cells with Coomassie blue. The results show that with antisense *tctex1* treatment, cell

length was affected, cell length was significantly reduced when compared with a control without antisense *tctex-1* treatment (Figure4-6).

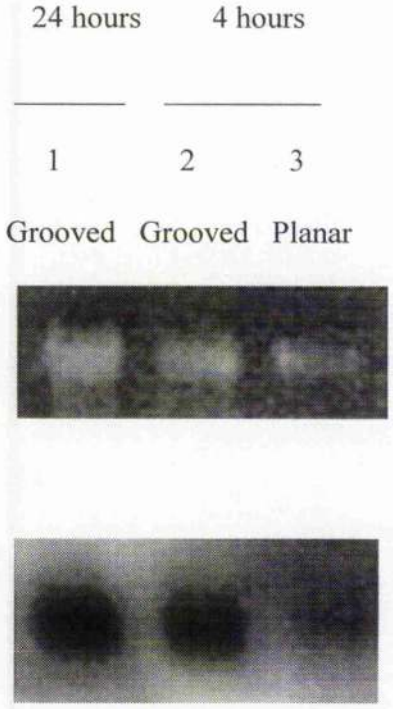


a. 28S RNA

b. *tctex* mRNA

Figure 4-4. Northern blot analysis of *tctex* mRNA expression

About 2 µg total RNAs of P388D1 were separated on a 1% agarose /formaldehyde gel, blotted and probed with a *tctex* probe (lower panel) that I reamplifed and cloned from DD-PCR. *tctex* mRNA levels were compared with their 28s ribosomal level (upper panel, 28S RNA were labelled with EtBr.). Lower panel shows that when p388D1 macrophage-like cells were cultured on grooved surface after 4 horus, *tctex* mRNA level increase faster than those for cells cultured on planar surfaces as marked above, although after 24 hours, it is hard to tell the differences.



a. 28s RNA

b. *α-N-acetylgalactosaminidase* mRNA

Figure 4-5. Northern blot analysis of *α-N-acetylgalactosaminidase* expression

About 2 μg total RNAs of P388D1 were separated on a 1% agarose /formaldehyde gel, blotted and probed with a α-N-acetylgalactosaminidase DNA probe that I reamplified and cloned from DD-PCR (lower panel b.) α-N-acetylgalactosaminidase mRNA levels were compared with their 28S ribosomal level (upper panel a, total RNAs were labelled with EtBr). Lower panel shows that when p388D1 macrophage-like cells were cultured on a grooved surface after 4 hours, *α-N-acetylgalactosaminidase* mRNA level increase faster than those cells cultured on a planar surface.

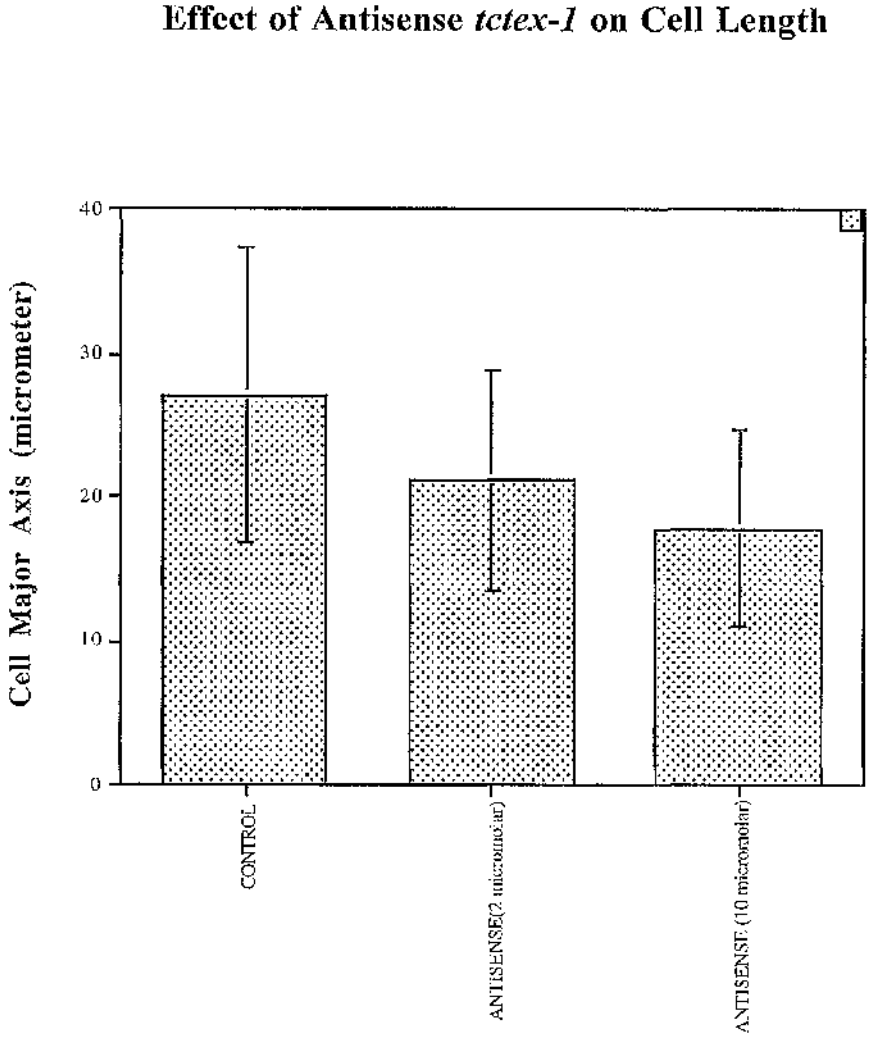


Figure 4-6. Effect of antisense *tctex* on the length of epitenon cells cultured for 24 hours on microgrooved structure (12.5 μm wide; 2.5 μm deep). With antisense *tctex-1* treatment at both final concentration of 2 and 10 μM, cell length has significantly decreased (t-text, $P<0.05$ at 2 μM and $P<0.01$ at 10 μM compared with control). Error bars indicate standard deviation. Results are means, N=100.

DISCUSSION

With 90 combinations of T and P primer sets, a large number of different fingerprints were obtained depending on the primer sets. Differentially expressed bands appeared in two ways: presence in a lane and absence in the other, or the band from one lane being much more intense than in the other. In this study, the differentially expressed bands that only appeared in the samples from grooved surfaces were excised from P388D1 cells after 4 hours culture. Although the results from culturing cells for 1 hour on different surfaces also show the differentially expressed bands, they were less clear. Thus the focus was on P388D1 for four hours after adhering to the grooved surfaces.

Furthermore, there are also certain bands which were observed in the control lane and absent in grooved lanes. It would be interesting to examine those bands as well, since it could help us to understand how cells react to smooth surfaces compared to grooved surfaces by loss of expression. Some useful information might be gained from this study.

Although DD-PCR was also applied to epitenon cells comparing between the cells which were cultured on a grooved surface or on a flat surface, mRNA fingerprinting result shows that different genes were stimulated by their surface topography as early as one hour. But cloning and sequencing have not been carried out due to lack of time. It would also be interesting to investigate this point.

It has been noted that there are four sequences cloned from the grooved surface which have no homology in the DNA data bank. Although there is no overlap found between them, it is still possible that they are from the same novel gene since the size of these four novel peptides are rather small. It is not surprising to find new novel DNAs, since the DNA data bank is still growing. It would be interesting to clone the whole cDNA of those genes although, unfortunately it has not been possible due to constraints of time.

The results from Northern Blotting shows there are 8 out of the 15 differentially expressed genes which are detected. Among these 8 DNAs, 4 are homologous with ribosome RNA, which is common in the sample. This is not surprising. Unfortunately, the slight changes at mRNA level could not be detected here. As discussed later, mRNA fingerprinting is a relatively new technique, which still needs improvement such as DNA contamination. (also see Introduction).

c-kit and *NRF-1* cDNA fragments failed to detect any signals. An alternative to Northern Blot is antibody staining and such experiments were carried out (see Chapter 5). Since it was reported that Northern Blot signals were detected only by using longer cDNA clones as probes (Liang *et al.*, 1993), many differentially expressed bands identified by Delta RNA fingerprinting will correspond to mRNAs of average or low abundance. Such mRNAs may not be detectable on a Northern Blot of total RNA. It is critical that any RNA sample is DNA free, but often, even though the RNA sample was treated with DNase, there could still be a residual amount of DNA contamination after DNase treatment of the RNA, and this could explain why there are some false positives (Liang *et al.*, 1993, 1995).

As mentioned before, only the more intense bands observed were isolated from the polyacrylamide/urea gel. There are however, a lot of faint bands whose intensities cannot reliably be compared. More information could be gained if they could all be analysed by cloning, sequencing and Northern Blot. For example they might cover slightly overexpressed genes, such as actin and microtubules, both of which are likely to be involved in contact guidance. Because loading on each track even when obtained from measured concentrations of DNA vary by at least 15% it is possible to be misled by apparent differences between 2 tracks. Thus I have tended to look for 'all or none' effects.

CHAPTER 5

IN SITU HYBRIDIZATION AND ANTIBODY STAINING TO DETECT CHANGES IN GENE EXPRESSIONS

INTRODUCTION

c-*kit* kinase Receptor

I had tentatively identified *c-kit* as one of the differentially expressed genes when cells are grown on different surface topography. Due to difficulties in Northern Blot analysis for this gene's, I carried out antibody staining to compare the gene expression on either smooth or grooved surfaces.

Studies in bone marrow-derived mast cells (BMMC) show that the c-kit receptor activation initiates a cascade of molecular events. Subsequently, receptor autophosphorylation and phosphorylation/activation of downstream signaling molecules occurs (Rottapel *et al.*, 1991; Reith *et al.*, 1991). It has been demonstrated that kit can induce the adhesion of BMMC to fibronectin (Vosseller *et al.*, 1997).

The receptor tyrosine kinase c-kit is thought to mediate its diverse effects on different cell lineages by association and activation of distinct second messenger systems. Features characteristic of these receptor tyrosine kinases are an

extracellular domain with five immunoglobulin-like domains and a kinase which is divided into two subdomains by an insert sequence of variable length.

c-kit Receptor signalling through its PI-3-Kinase-binding side

It is reported that tyrosine at residue 719 of the c-kit receptor is essential for binding of the p85 subunit of phosphotidylinositol (PI) 3- kinase and for c-kit-associated PI 3 kinase activity in COS -1 cells (Serve *et al.*, 1994, 1995). One of the immediate events after binding of the kit ligand to the receptor is its association with the 85Kda subunit (p85) of the phosphotidylinositol (PI-3) kinase and the activation of the enzyme (Vosseller *et al.*, 1997).

PI 3-kinase catalyzes the phosphorylation of phosphotidylinositol at the 3' position of the inositol ring and consists of two subunits of 85 and 110 kDa, both of which have been molecularly cloned (Whitman and Cantley, 1988; Auger *et al.*, 1989). The 85-kDa subunit of PI-3-kinase contains two SH2 domains (Escobedo *et al.*, 1991b; Skolnik *et al.*, 1991) and, by itself does not exhibit enzymatic activity but, confers activity onto the 110kDa catalytic subunit in mammalian cells (Carpenter and Cantey, 1990; Hiles *et al.*, 1992). PI-3-kinase associates with receptor tyrosine kinases of the PDGFR subfamily through the SH2 domains of the p85 subunit (Escobedo *et al.*, 1991a). The tyrosine phosphate binding sites for p85 in the kinase insert region of PDGFR have been identified.

Due to the special role of c-kit in mediating cell responses including proliferation, migration, differentiation and adhesion to extracellular matrix, I decided to carry out antibody staining for c-kit to examine its expression and location. Apart from looking at c-kit antibody staining, PI-3-kinase p110 subunit antibody staining and blocking PI-3-Kinase activity by using its specific inhibitor wortmannin were also carried out.

NRF1

By using mRNA fingerprinting methods, I found that the NRF-1 gene is expressed when P388D1 macrophage-like cells are cultured on grooved substrata. This has excited my interest since nuclear respiratory factor 1 (nrf-1) is a transcription factor that is likely to play an important role in the co-ordination of the expression of nuclearly and mitochondrially encoded mitochondrial polypeptides, and thus the regulation of mitochondrial biogenesis (Virbasius and Scarpulla, 1993, 1994; Evans and Scarpulla, 1988, 1989).

It has shown that the functional NRF-1 recognition sites are present in nearly all of those nuclear genes that function in mitochondria. The sites from each gene are transcriptionally active and form indistinguishable DNA-protein complexes with NRF-1 (Evans and Scarpulla, 1990). By regulating the genes, NRF-1 may help co-ordinate the expression of nuclear and mitochondrial genetic systems in response to cellular energy demands. In addition, functional NRF-1binding sites are present in the genes for tyrosine aminotransferase and

the translation initiation factor eIF-2α (Chou *et al.* 1992). These two proteins participate in the rate-limiting steps of their respective pathways of tyrosine catabolism and protein synthesis. Thus, NRF-1 may integrate a number of metabolic processes by regulating the genes encoding key enzymes (Virbasius *et al* 1993). In a recent database search up to 50 different mammalian genes that contain nrf-1 binding sites are not only important for the regulation of mitochondrial biogenesis, but also for the regulation of a variety of other cellular processes (Evans *et al*, 1988).

RESULTS

Immunofluorescence

c-*Kit* Antibody Staining

P388D1 macrophage-like cells

c-kit antibody staining in P388D1 macrophage-like cells cultured on grooved fused silica (Quartz) substrata were specifically localised along groove/ridge boundaries. Figure 5-2 shows P388D1 cells, after 3 hours culture, on a planar quartz surface, stained with c-kit (C-19, polyclonal rabbit IgG) antibody and Texas Red sheep anti-rabbit secondary antibody. Bright dots of staining can be observed dispersed randomly throughout the cells which were cultured on a planar surface. In contrast, when the cells were cultured on microgrooved quartz structure (1.5 μm deep; 12.5 μm wide) for the same length of time a specific staining pattern was observed, localised along the groove/ridge boundaries (see Figure 5-1). Figure 5-5 shows as a negative control of P388D1 macrophage-like

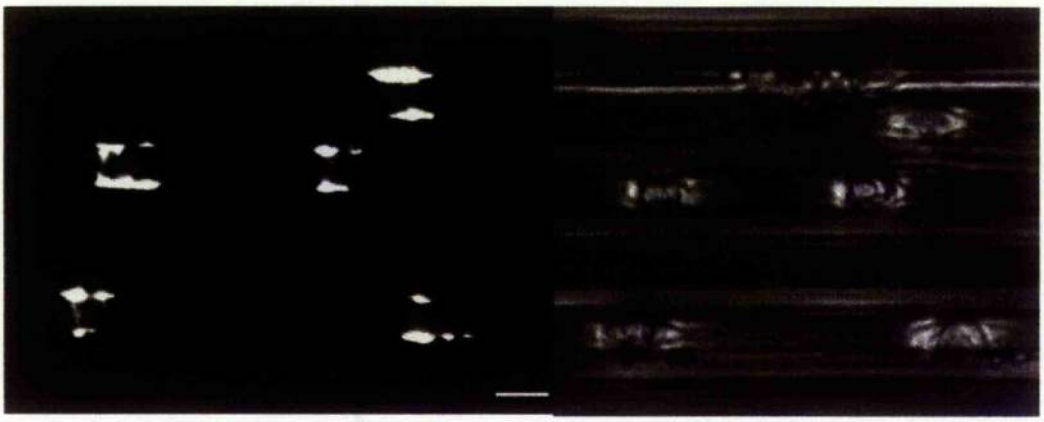
cells which were cultured on grooved (1.5 μm deep; 12.5 μm wide) structure, the cells were stained only with the second antibody. There are no special pattern or bright spots.

Epitenon Cells

Epitenon cells were cultured in the same way as P388D1 macrophage-like cells. The cells were cultured on either non-grooved or grooved (1.5 μm deep; 5 μm wide) structure for 4 hours. Cells were then fixed and stained with c-kit antibody as first antibody and Texas Red sheep anti-rabbit as the secondary antibody. It shows that c-kit has been expressed along the groove/ridge area as seen in figure 5-3 and this suggests that grooved surface can stimulate the c-kit expression. This figure shows that, compared with the cells cultured on planar surface, those cells cultured on grooved surface have over expressed c-kit. Figure 5-4 shows that when cells were cultured on non-grooved substrata, bright dots of staining can be observed dispersed randomly throughout the cells. In contrast, Figure 5-6 shows a negative control of epitenon cells which were cultured on grooved (1.5 μm deep; .5 μm wide) structure, the cells were stained only with the second antibody. There are no special pattern or bright spots observed.

PI3-Kinase antibody staining

Antibody against PI3-Kinase subunit p110δ was used in cultured P388D1 macrophage-like cells which were cultured on non-grooved or grooved (1.5 μm deep; 2.5 μm wide) structure. Texas Red anti mouse antibody was used as the second antibody. Figure 5-7 shows that PI3-Kinase is expressed through the cells, specific patterns along the groove/ridge area were observed. Figure 5-8 shows when the cells were cultured on planar surface, PI-3 kinase were also expressed. Figure 5-9 shows a negative control of P388D1 stained only with the second antibody. There are no special pattern or bright spots.



A B

Figure 5-1. (A) Distribution of c-kit receptor in P388D1 macrophage-like cells on a grooved quartz substrate of 1.5 μm deep; 12.5 μm wide. Cells were stained with c-kit (C-19) antibody and Texas Red anti-rabbit secondary antibody. (B) Phase contrast image of (A) P388D1 macrophages on grooved substrata. Scale bar represents 10 μm.

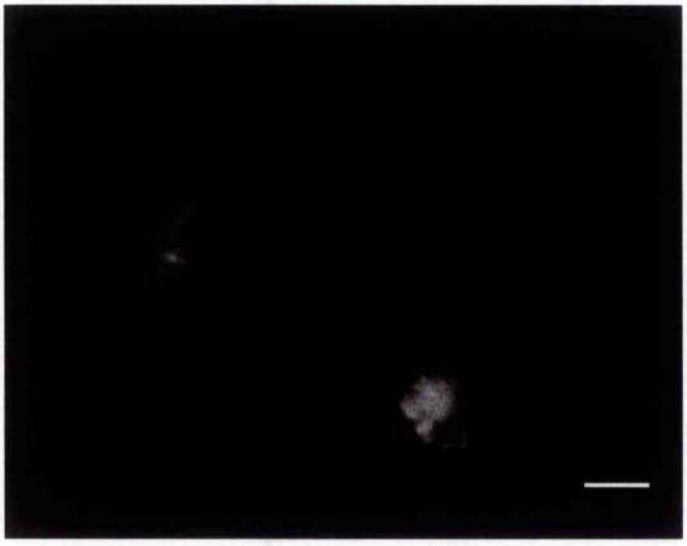


Figure 5-2. Distribution of c-kit receptor in P388D1 macrophage-like cells on a flat quartz substrate. Cells were stained with c-kit (C-19) antibody and Texas Red sheep anti-rabbit secondary antibody. Scale bar represents 10μm.

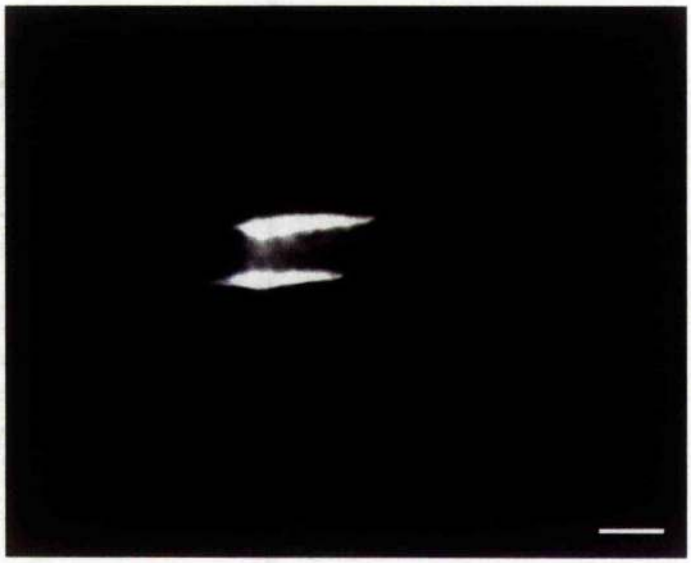


Figure 5-3. Distribution of c-kit receptor in Epitenon cells on a grooved quartz substrate. Cells were stained with c-kit (C-19) antibody and Texas Red anti-rabbit secondary antibody. Scale bar represents 10 μm.

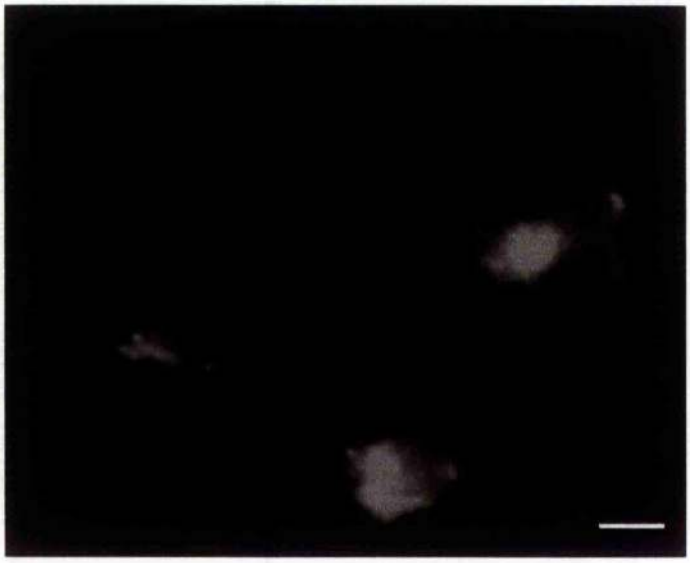


Figure 5-4. Distribution of c-kit receptor in Epitenon cells on a flat quartz substrate. Cells were stained with c-kit antibody and Texas Red anti-rabbit secondary antibody. Scale bar represents 10 μm.

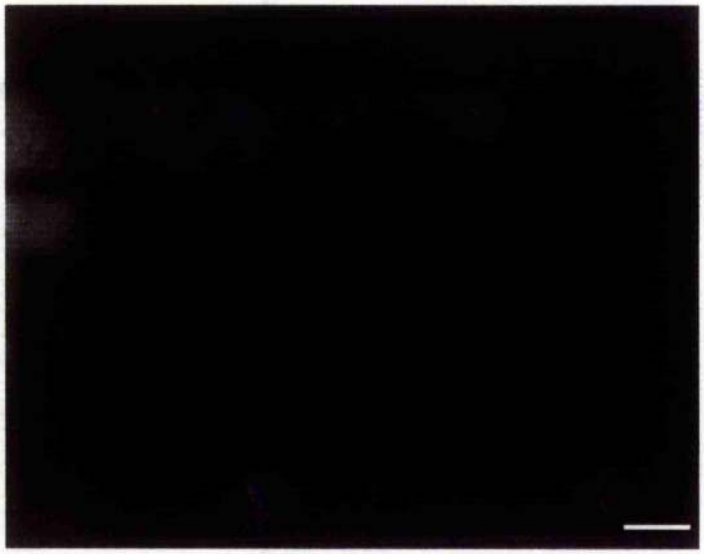


Figure 5-5. Negative control for staining of c-kit receptor. P388D1 macrophage-like cells are shown, on a grooved quartz substrate, stained only with the secondary antibody (Texas Red). Grooves are 1.5 μm deep and 12.5 μm wide. Scale bar represents 10 μm.

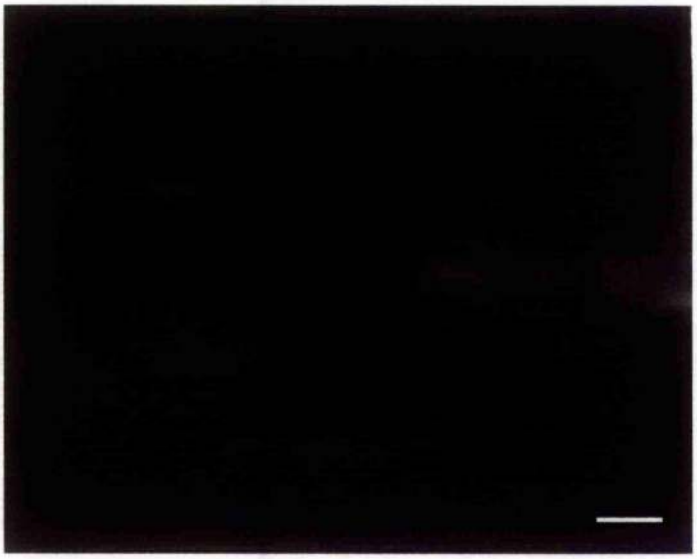


Figure 5-6. Negative control for staining of c-kit receptor. Epitenon cells are shown, on a grooved quartz substrate, stained only with the secondary antibody (Texas Red). Grooves are 1.5 μm deep and 5 μm wide. Scale bar represents 10 μm.

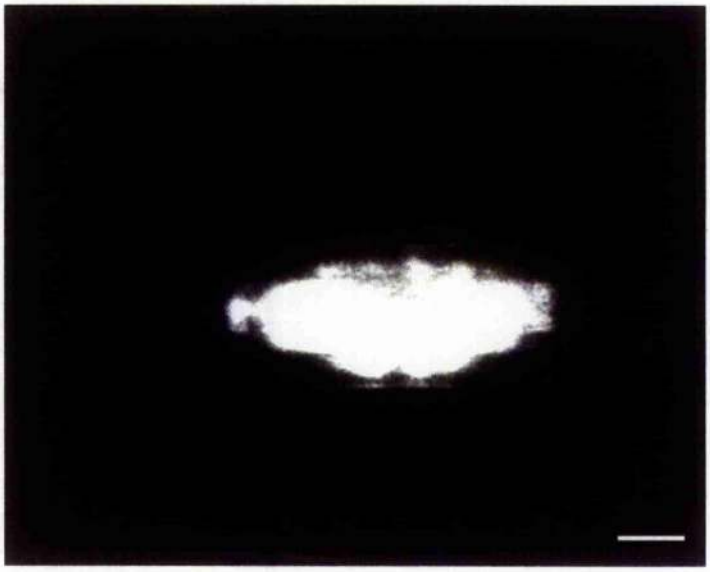


Figure 5-7. Distribution of PI-3 kinase in P388D1 macrophage-like cells on a grooved quartz substrate. The cells were stained with PI-3 kinase p110 subunit antibody and Texas Red sheep anti-mouse secondary antibody. Scale bar represents 10 μm.

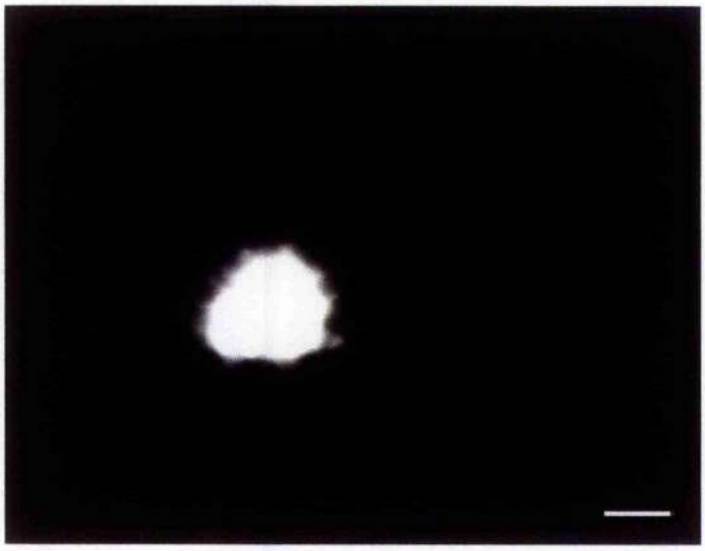


Figure 5-8. Distribution of PI-3 kinase in P388D1 macrophage-like cells on a flat quartz substrate. The cells were stained with PI-3 kinase p110 subunit antibody and Texas Red sheep anti-mouse secondary antibody. Scale bar represents 10 μm.

Inhibition of PI-3-kinase activity by Wortmannin

To investigate a possible role for PI-3-kinase in the reaction of cells to their surface topography, I examined the effect of treatment with the PI3-Kinase inhibitor, Wortmannin, on cell shape and elongation at 4 hours in P388D1 and Epitenon cells. As shown in Figure 5-9 and figure 5-11, the cell area on both P388D1 macrophage-like cells and Epitenon cells was significantly reduced in the presence of 100 nM Wortmannin. The cell area was not significantly changed at the presence of 10nM Wortmannin. Figure 5-10 and figure 5-12 shows that the cell orientation to the grooves changes when cells were cultured on a grooved surface. When cells were cultured on flat surface, cells spread randomly over the surface, this has not been examined in this study.

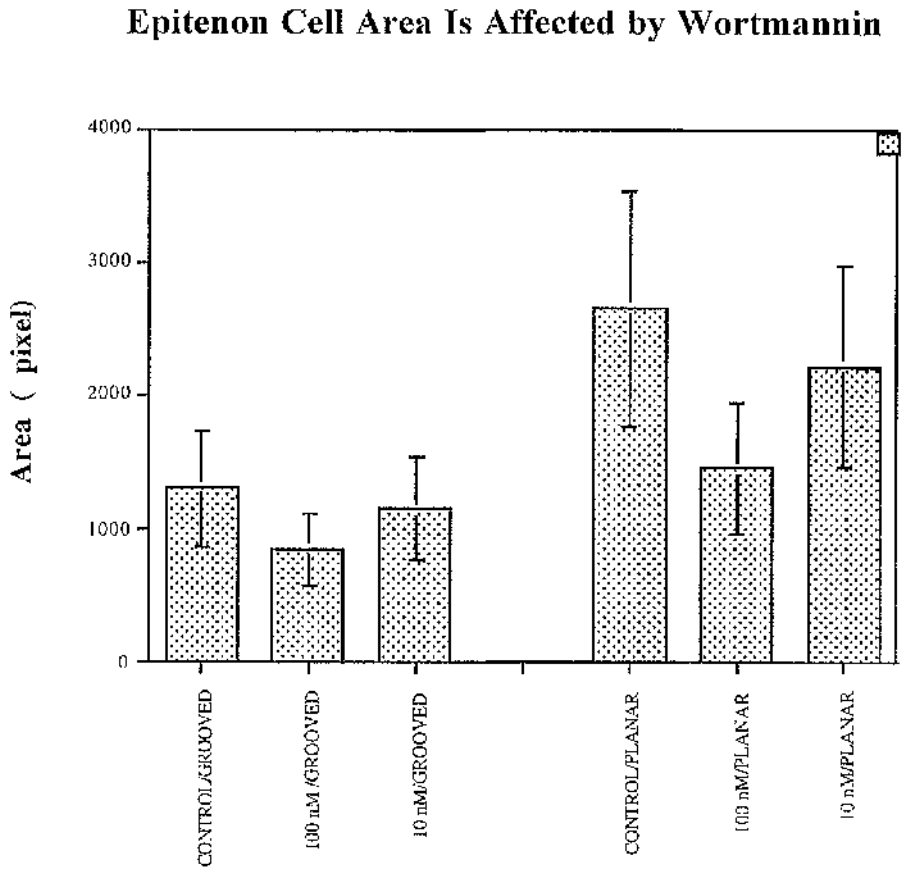


Figure 5-9. Effect of Wortmannin (at 10 nM and 100 nM) on Epitenon cells which were cultured on either non-grooved or grooved surfaces. Wortmannin was added directly into the medium after the cells were seeded. After four hours incubation, cells were fixed and stained with Coomassie Blue and cell areas were measured with the program as described in the text. This figure shows that cell areas at both grooved and planar surfaces were significantly reduced with Wortmannin inhibition at 100 nM concentration (t-test $P<0.01$ in each case compared with control without Wortmannin), but non-significantly at 10 nM concentration of Wortmannin. Furthermore, cell areas were also reduced when culturing cells on grooved surface compared with cells cultured on planar surface. Error bars indicate one standard deviation. Results are means, N=100 (each sample).

Epitenon Cell Orientation Is Affected by Wortmannin

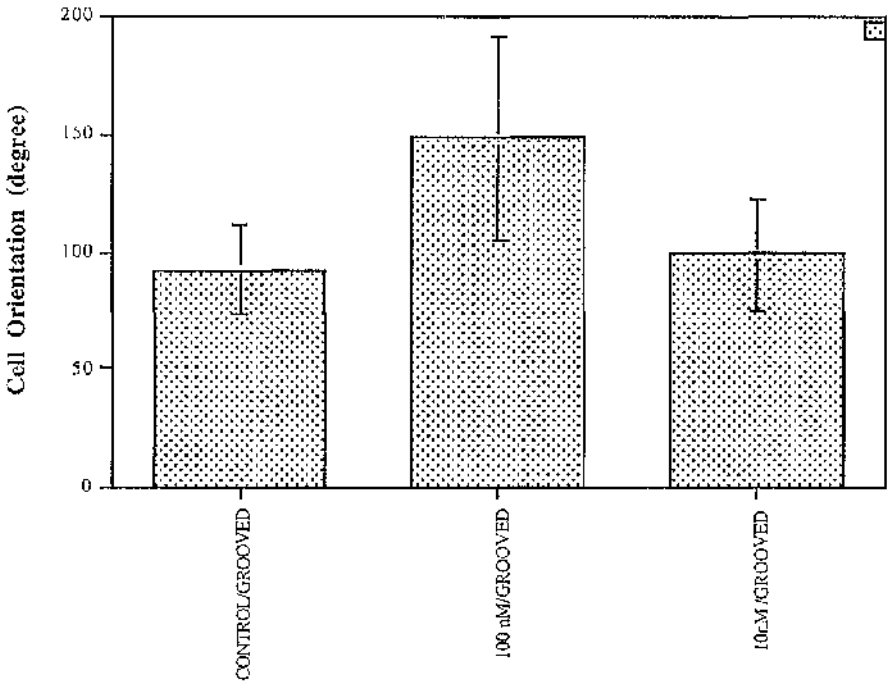


Figure 5-10. Effect of wortmannin (at 10 nM and 100 nM) on Epitenon cells which were cultured on either non-grooved or grooved surfaces. Wortmannin was added directly into the medium after the cells were seeded. After four hours incubation, cells were fixed and stained with Coomasie Blue and cell orientation was measured with the program as described in the text. This figure shows that cell orientation were significantly reduced with Wortmannin at 100 nM concentration (t-test $P<0.01$ compared with the control without wortmannin treatment) and non-significant at 10 nM concentration of Wortmannin when cells were cultured on grooved surface. Error bars indicate one standard deviation. Results are means, 90° orientation is complete orientation, N=100.

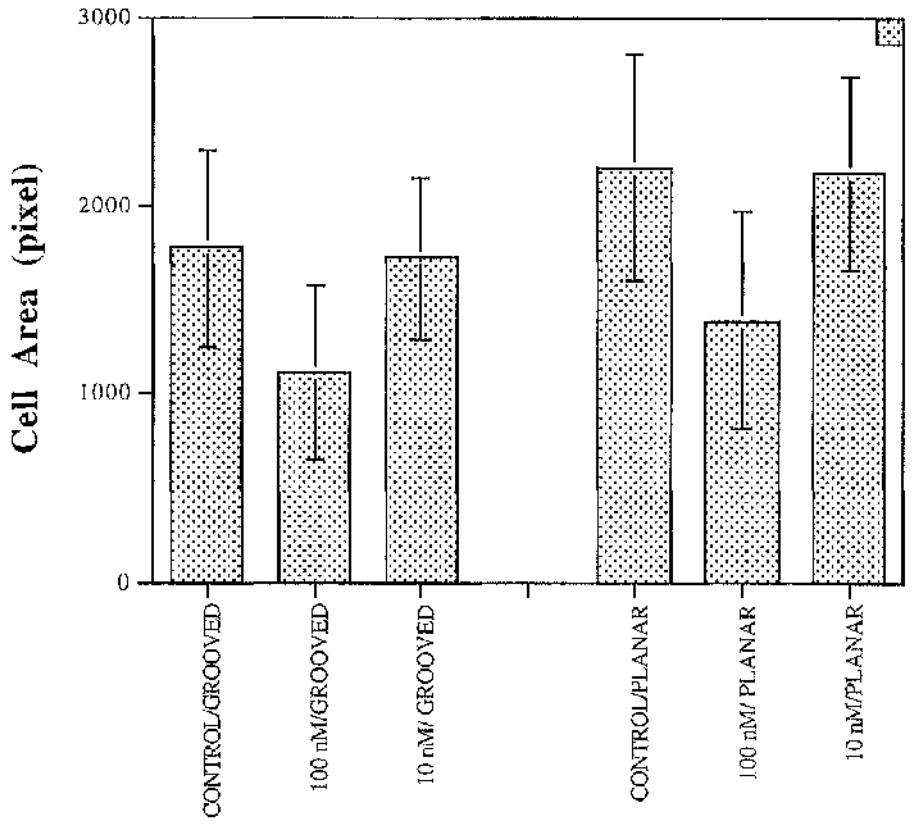


Figure 5-11. Effect of wortmannin (at 10 nM and 100 nM) on P388D1 macrophage-like cells which were cultured on either non-grooved or grooved surfaces. Wortmannin was added directly into the medium after the cells were seeded. After four hours incubation, cells were fixed and stained with Coomasie Blue and cell areas were measured with the program as described in the text. This figure shows that cell areas were significantly reduced on planar or grooved surfaces with Wortmannin at 100 nM concentration in both grooved and non-grooved surface (t-test $P<0.01$ in each case compared with control without Wortmannin) and non-significant at 10 nM concentration of Wortmannin. Furthermore, cell areas were also reduced when culturing cells on grooved surface compared with cells cultured on planar surface. Error bars indicate one standard deviation. Results are means, N=100 in every samples.

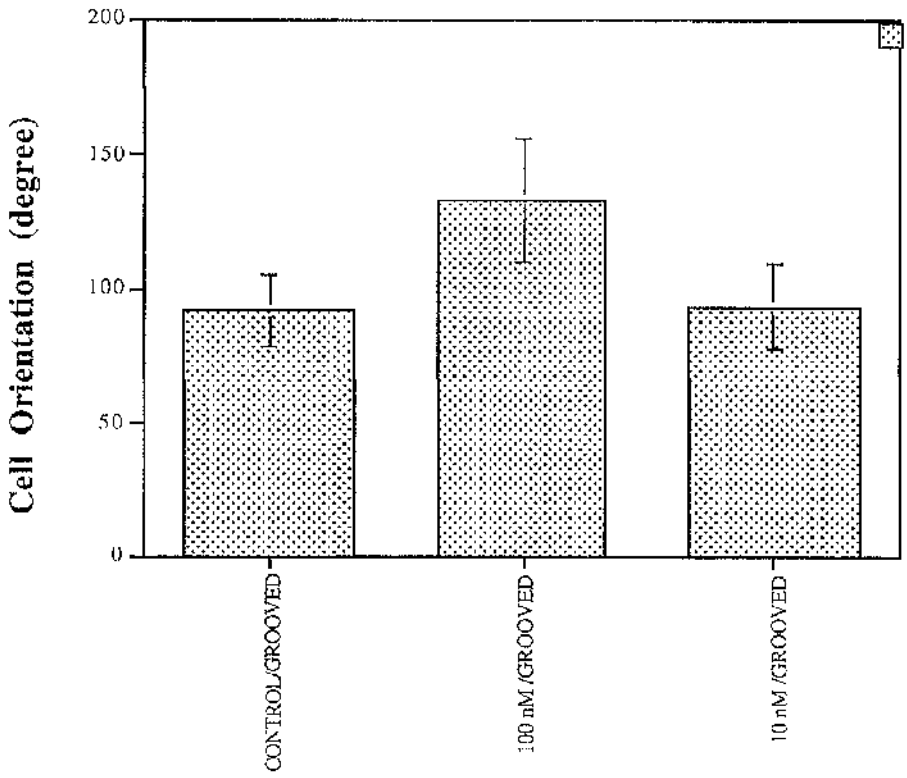


Figure 5-12. Effect of wortmannin (at 10 nM and 100 nM) on orientation of P388D1macrophage-like cells which were cultured on either non-grooved or grooved surfaces. Wortmannin was added directly into the medium after the cells were seeded. After four hours incubation, cells were fixed and stained with Coomassie Blue and cell orientation was measured with the program as described in the text. This figure shows that cell orientation was reduced significantly with Wortmannin inhibition at 100 nM concentration (t-test $P<0.01$ compared with the control) and non-significant at 10 nM concentration of Wortmannin when cells were cultured on grooved surface. Error bars indicate one standard Deviation. Results are means, 90° orientation is complete orientation, N=100.

NRF-1 Antibody Staining

Antibody against NRF-1 was applied in P388D1 macrophage-like cells which were grown on non-grooved and grooved (280 nm deep; 2 μm wide). The results are shown in figure 5-13; 280 nm deep; 5 μm wide in figure 5-16 as shown in figure 5-16) structure. Figure 5-13, figure 5-15 and figure 5-16 show that NRF-1 was expressed through out the cells and slightly more intensely along the groove/ridge area in comparison with the cells cultured on a flat surface (Figure 5-14) when Texas Red anti-rabbit antibody was used as the second antibody.

In situ hybridization in tendon section

After obtaining good evidence for c-kit expression on a grooved surface, I then decided to look at the c-kit expression *in vivo* in normal macrophages in tissue, since there is always a gap between *in vivo* and *in vitro* studies. It was impossible to look at early times after the implant of grooved material, which was made by Mr. J. Crossan in rats. The earliest times I used in this study was 72 hours. A DNA probe against c-kit messenger RNA was made with biotin labelling to detect c-kit mRNA expression. Figure 5-18 shows that c-kit m-RNA was expressed in macrophages after 72 hours after seeding on a grooved PDS (polydioxanone suture) implant. I did not use flat PDS as a control, although it is unlikely that there is no c-kit expression on such a surface. This is discussed below.

Figure 5-13. Distribution of NRF-1 in P388D1 macrophage-like cells on a grooved quartz substrate. Cells were stained with NRF-1 antibody and Texas Red anti-sheep secondary antibody. Scale bar represents 10 μm.

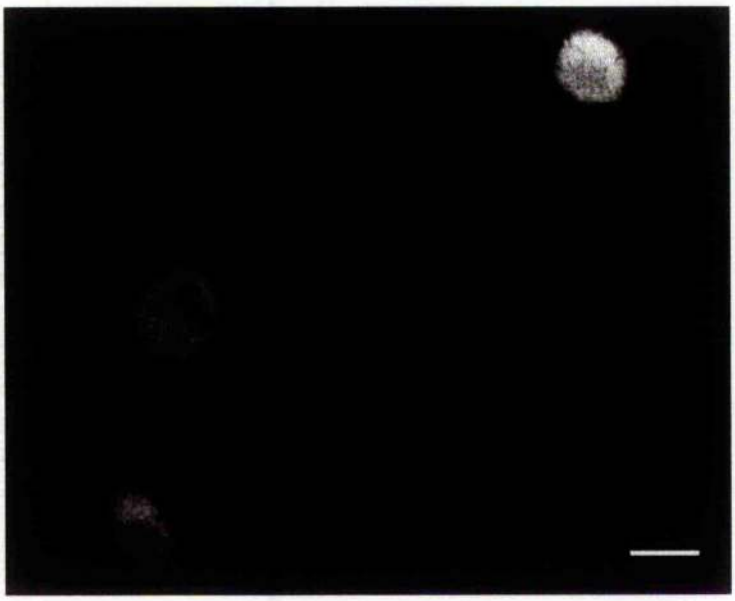


Figure 5-14. Distribution of NRF-1 in P388D1 macrophage-like cells on a flat quartz substrate. Cells were stained with NRF-1 antibody and Texas Red sheep anti-mouse secondary antibody. Scale bar represents 10 μm.

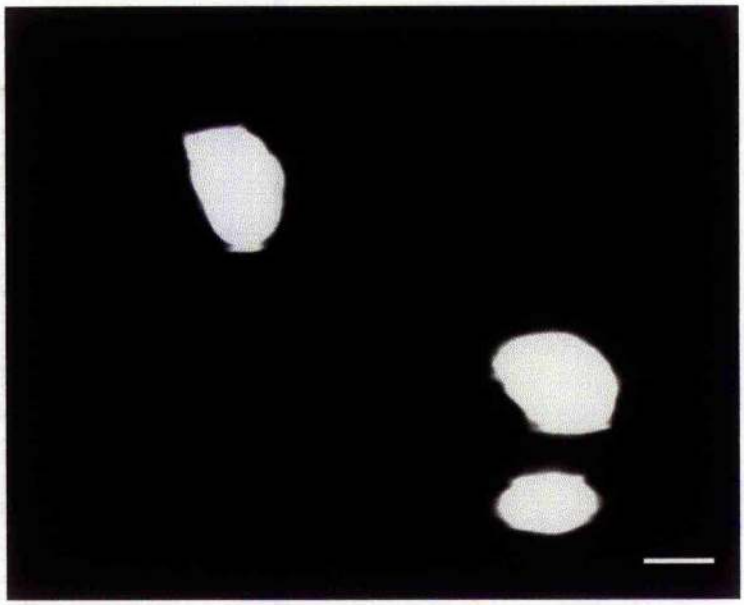


Figure 5-15. Distribution of NRF-1 in P388D1 macrophage-like cells on a grooved quartz substrate. Cells were stained with NRF-1 antibody and Texas Red sheep anti-mouse secondary antibody. Scale bar represents 10 μm.

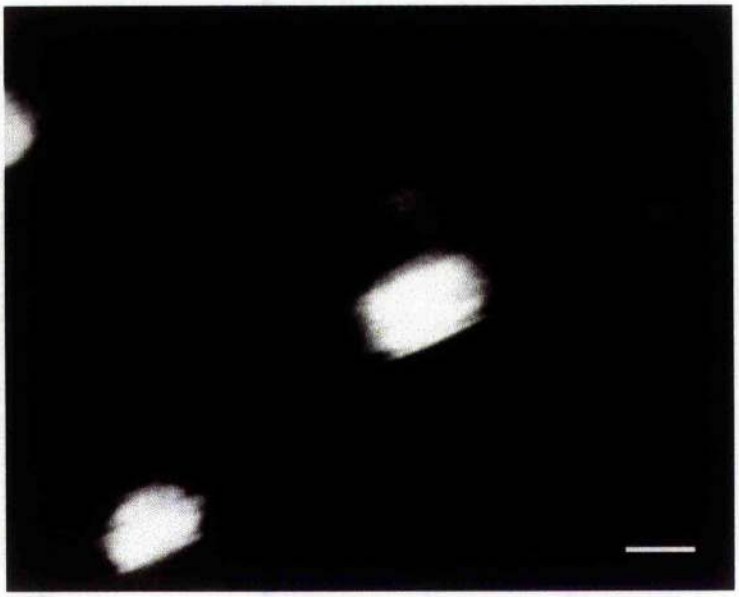


Figure 5-16. Distribution of NRF-1 in P388D1 macrophage-like cells on a grooved quartz substrate. Cells were stained with NRF-1 antibody and Texas Red anti-sheep secondary antibody. Scale bar represents 10 μm.

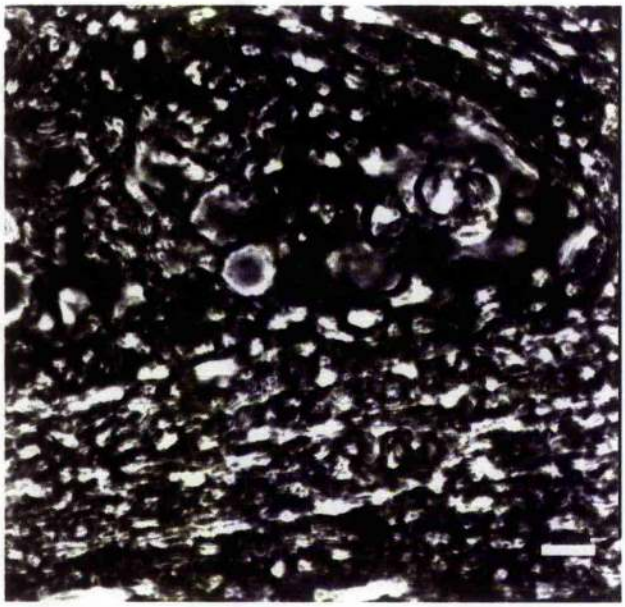


Figure 5-17. Phase contrast of tendon section after 3 days of implant seeded. Scale bar represents 10 μm.

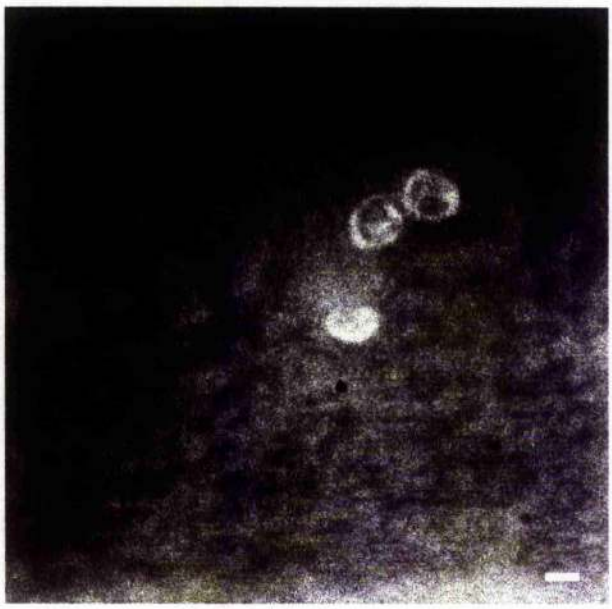


Figure 5-18. In situ hybridisation of c-kit in tendon section after 3 days of implant seeded. The bright cells are macrophages. Scale bar represents 10 μm.

DISCUSSION

Immunofluorescence

From the results of c-kit antibody staining, it appears that the expression of c-kit tyrosine kinase receptor is increased in cells on grooved surfaces and located along the groove ridge boundaries. This suggests that this protein may be related to contact guidance or in general, to cell adhesion. In this study, both the P388D1 macrophage-like cells and epitenon cells I used are very sensitive to the surface topography which they encounter. The details of how sensitive these two types of cells are was examined by Wojciak *et al* (1996) who used a wide range of structures whose size ranged from 44 nanometers deep to 5 micrometers deep and different widths. In my study it has not been possible to examine the effect of the different sizes on these cell types.

It is not very clear how c-kit regulates its signal pathway, especially which second messengers are involved in this signal pathway. It has been shown that c-kit mediates in the activity of PI-3 kinase through its PI-3-kinase binding side. This leads to cytoskeletal rearrangements and adhesion to fibronectin in bone marrow mast cells (BMMC) (Vosseller *et al.*, 1997).

It is reported that in bone marrow mast cells secretory enhancement and adhesion is stimulated directly by c-kit through protein kinase C, which mediates adhesion to fibronectin, secretory enhancement, membrane ruffling and

filamentous actin assemble (Vosseller *et al.*, 1997). Although this study is demonstrated in BMMC, the theory has been supported by a study of Wojiack *et al* (1994). They grew P388D1 macrophage-like cells on a microgrooved silica and stained with F-actin antibody, and found that F-actin filaments were oriented along substratum microstructures.

Wortmannin as a inhibitor of PI-3 kinase

PI-3 kinase has been proven to be a critical second messenger in kit signalling in BMMCs. In this study, I examined the effect of PI-3 kinase inhibitor in the response of cells to their surface topography. Wortmannin is one of two inhibitors of PI-3 kinase which can bind to the p110 subunits of PI-3 kinase and block enzyme activity (Yano *et al.*, 1993). Another inhibitor of PI-3-kinase is LY294002 (Vlahos *et al.*, 1994), which has a inhibitory effect similar to that of wortmannin.

The results show that inhibition of PI-3 kinase with wortmannin also inhibited cell spreading and reduces the cell elongation. It would be interesting if the activity of PI-3 kinase during cells reacting to their special surface topography was measured perhaps by the method described by Rittenhouse (1995). This part of the work has not been done by me due to difficulties in technique and lack of time, but it is going to be very interesting in the future to look at since it is very likely that PI-3 kinase activity could be increased by a c-kit mediated signal pathway as cells encounter their surface topography.

Knowledge gained from this study of the particular, cell types P388D1 macrophage-like cells and epitenon cells, should be valuable in understanding kit-mediated responses in contact guidance or topography guidance, and cell spreading in relation to topography. More importantly, this study demonstrates for the first time that the signal system is involved in contact guidance. It suggests that surface topography can regulate the signal system by increasing the factors and enzyme activity.

Role of c-kit in tendon healing

A small part of *in vivo* work was also carried out in implant seeded tendon section (see above P117). Since my colleagues were working on grooved and planar PDS used as implant in tendon repair, this naturally interested me to look at c-kit expression in tendon repair with PDS as implant. Biotin labelled probe against c-kit mRNA was used to detect c-kit expression in tendon section by in situ hybridisation. It was originally planned to examine the results gained from the RT-PCR message RNA fingerprinting which was carried out *in vitro* with rather high passages cell lines (more than 15 passages), since there is always a gap between *in vivo* and *in vitro* study. It is not surprising that not much information was gained by this study since this was a new technique used for the first time. Still, I found that c-kit was well expressed in macrophages 3 days after implant seeding. It is also going to be interesting to look at the

different time point, for example, to look at one day and 2 days or even one week.

In situ hybridisation with biotin labelled c-kit as a probe was also examined in P388 D1 macrophage-like cells and Epitenon cells *in vitro*, although due to the high background, a specific location from the staining has not been observed.

NRF-1 in Contact Guidance

Like most factors which have been discovered in recent years, NRF-1 is a relatively newly discovered factor, whose all cellular functions are not yet all known. Since there are up to 50 different mammalian genes that contain nrf-1 binding side, this suggests that NRF-1 is not only important for the regulation of mitochondrial biogenesis, but also for the regulation of a variety of other cellular processes (Evans *et al*, 1988). From the evidence gained in my study, NRF-1 may play a role in contact guidance.

It is likely that NRF-1 is involved in an energy pathway involved in contact guidance or in cell adhesion, since studies on contact guidance have shown that grooved topography can stimulate cell proliferation, accelerate cell movement and cell elongation (Curtis *et al.*, 1988, Wojciak *et al.*, 1996). Development of cellular morphologies presumably involve an energy requirement. The detailed function that NRF-1 plays in this cellular process is not clear.

CHAPTER 6

The lyn TYROSINE KINASE AND REACTION TO TOPOGRAPHY

INTRODUCTION

It has previously been observed (Karen McDonald, Ph.D thesis) that tyrosine phosphorylation is switched on by growing cells on a microgrooved surface. Further work suggested that only src family kinases is involved in this matter. Further experiments were then carried out (Curtis *et al.* unpublished) and the results showed that only lyn and fyn proteins had the same sort of localisation on the groove/ridges. I continued this work by examining the function of *lyn* and its mRNA level during the response of cells to topographic cues.

Lyn is a member of the src family of intracellular membrane-associated tyrosine kinases (Yamanashi *et al.,* 1987). While the amino-terminus of each kinase is unique, this family shares significant structural homology in several domains (the *Src* Homology (SH) 1 or kinase domain and two protein interaction domains, SH2 and SH3). Alternative splicing at the 5' end of the *lyn* gene results in the expression of p53 and p56 isoforms of the protein (Stanley *et al.*, 1991; Yamanashi *et al.*, 1991b; Yi *et al.*, 1991). Furthermore, *lyn* has been implicated in the phosphorylation of a number of signalling molecules, including phosphoinositol-3-kinase, ras GTPase activation protein (GAP),

phospholipase C (PLC) γ2 and mitogen-activated protein (MAP) kinase (Cichowski *et al.*, 1992; Yamanashi *et al.*, 1992; Corey *et al.*, 1993; Pleiman *et al.*, 1993).

In this study, I used antisense against *lyn* to examine whether lyn directly controlled cell behaviour or cell adhesion. Use of antisense oligonucleotides is a recently developed technique (Sczakiel *et al.*, 1994) The mechanism of action of antisense oligonucleotide is thought to be a selective blockade of the expression of a particular gene via nucleotide sequence-specific hybridisation and blockade of translation of its mRNA. This hybrid formation causes a steric or conformational obstacle for protein translation. As a result the production of a specific protein is inhibited without affecting the translation of other genes. In this experiment, antisense *lyn* is used to inhibit the expression of the *lyn* gene to determine if loss of function results.

It would be expected that the expression of *lyn* gene might be affected by substrata which can alter the distribution of cytoskeletal elements. If tyrosine kinase lyn is extremely active and switched on by growing cells on the microgrooved structure, should *lyn* mRNA level be increased as well? To answer this question, I cultured epitenon cells on both microgrooved fused silica and flat silica substrata. Total RNAs were extracted and used in Northern Blots. *lyn* gene was also amplified and used as a probe in Northern Blot analysis.

RESULTS

Effect of Antisense *lyn* on Cell Morphology

Antisense *lyn* Start Kit (Biognostic GmbH, Gottingen, Germany) was used (see Chapter 2). To address whether *lyn* directly controls cell spreading and elongation along their microgrooved substrata, phosphorothioate-modified antisense oligonucleotides were used, to block the *lyn* translation pathway. Epitenon cells were cultured on two sizes of the grooved structure fabricated in quartz (2 μm wide; 2.5 μm deep and 5 μm wide; 2.5 μm deep). Antisense *lyn* and random sequence antisense were added either after 1 hour or after 24 hours incubation at 37°C. This allows differentiation between effects during attachment and extension from those on fully extended cells. Cell length and width were measured after fixing and staining the cells with Coomassie blue. The results show that with antisense *lyn* treatment, both cell length and cell width were great affected, cell length was significantly reduced when compared with a control without antisense *lyn* treatment at both 24 and 48 hours (Figure 6-1).

Figure 6-2 shows that, cell width was also affected by adding *lyn* and random antisense. Cell width increased with antisense *lyn* treatment when culturing cells on the grooved structure (2 μm wide; 2.5 μm deep) for 24 hours and similarly, cell width increased after treated with antisense *lyn* after 48 hours culture on grooved substrata (5 μm wide; 2.5 μm deep). With random antisense treatment,

no significant difference was found. Increase in cell width means that the cells are more rounded ie have reacted less to topography.

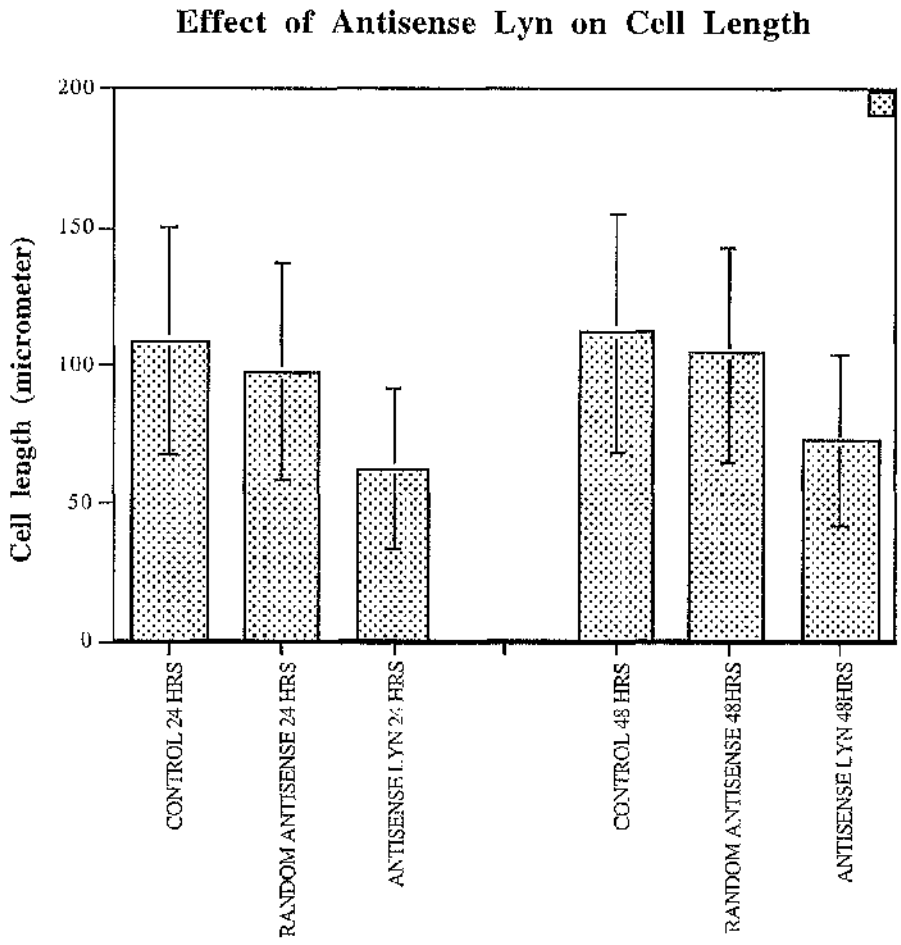


Figure 6-1. Effect of antisense *lyn* on the length of epitenon cells cultured for 24 and 48 hours after treatment with antisense *lyn* and random antisense. This figure shows that with antisense *lyn* treatment, cell length has significantly decreased (t-test $P<0.01$ compared with the control; $P<0.05$ compared with random sense treatment) after 24 and 48 hours culture, whereas with random antisense treatment, there is no significant effect. Error bars indicate one standard Deviation. Results are means, N= 105.

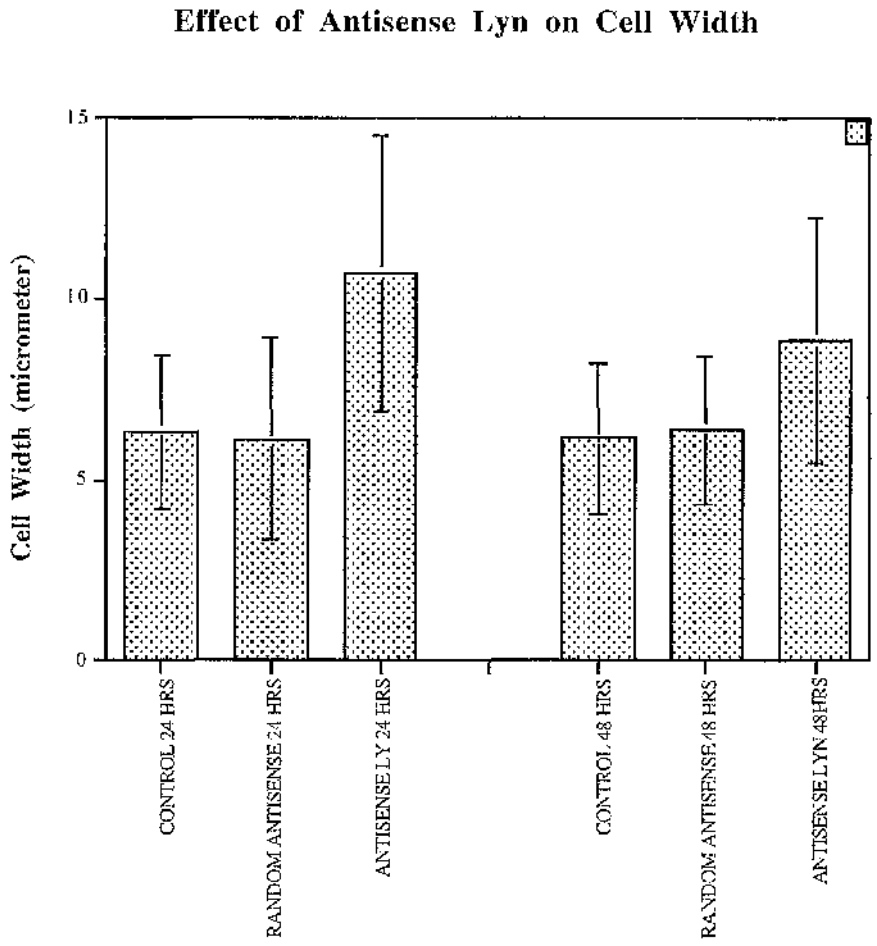


Figure 6-2. Effect of antisense *Lyn* on the width of epitenon cells cultured for 24 and 48 hours on grooved structure after treated with antisense *lyn* and random antisense. This figure shows that with antisense *lyn* treatment, cell width has significantly increased ($P<0.01$ compared with control) after 24 and 48 hours culture, whereas with random antisense treatment, there is no significant effect. Error bars indicate one standard Deviation. Results are means, N=105.

Effect of antisense *lyn* on Cell Migration

Epitenon cells were plated on patterned substrata with groove of 2 μm depth and 12.5 μm width. Cells were video recorded every 1 frame per min for 48 hours by using a Panasonic Neuvicon video camera. Images were stored on a Panasonic Model AG-6730 time-lapse video-recorder. The average speed of cell locomotion defined as total length of cell path/time of recording was measured by analysis of the video in the NIH program.

It has been found that cells spread on grooves and move faster than cells on plain substrata (Wojciak-Stothard *et al.*, 1996). In this experiment, I only compared the cells on grooved substrata with and without antisense added. Cells with antisense *lyn* treatment migrate significantly slower along groove/ridge edges than control cells with no antisense added (Table 6-1).

Reamplification of *lyn* gene

To examine *lyn* mRNA level from cells grown on flat substrata in comparison with the cells grown on grooved substrata, I amplified the *lyn* gene by RT-PCR. The sequence of *lyn* cDNA was checked in a Gene bank, and primers against it were made. cDNA was reverse-transcribed from total RNA which was extracted from p388D1 macrophage-like cells. cDNA was then used as a template in PCR. The PCR reaction mix was first through 94 °C for 1 minute, then went through 30 cycles of 94 °C for 55 second, 58 °C for 55 second and 72°C for 1 minute and 10 second, and extending at 72°C for 10 minutes. The

PCR products were then checked in a 2% agarose gel with the presence of 100 bp DNA maker. The reamplified cDNA fragment was then cut out from the agarose gel, cleaned (see the methods) and used as a probe in Northern Blot analysis (Figure 6-3).

NORTHERN BLOT ANALYSIS

Total RNA was extracted from P388D1 macrophage-like cells which were cultured on microgrooved silica (Quartz) and flat quartz surface for four hours and twenty four hours. The RNA was separated by electrophoresis in a 1% formaldehyde-agarose/MOPS gel, blotted to nitro-cellulose and hybridised with ^{32}P-labelled random-primed amplified *lyn* gene probes. The filter was then exposed to Fuji X-ray film for 1 - 3 days. Sizes were determined with respect to an RNA ladder (Gibco BRL). Northern analysis shows no obvious difference between cells which were cultured on grooved and flat surface at four hours and twenty four hours (see figure 6-4). Since the signal was weak due to the limited number of cells that could be handled in this study, it was difficult to compare samples to see if this gene has been overexpressed.

Epitenon cells	Rate of cell migration (μm/h)	SD
Control (without antisense treated)	35.78	8.23
Antisense *lyn* treated (2μM)	23.73	6.87

Table 6-1. Mean speed, persistence parameter for cell movement of epitenon cells grown on patterned substrata with grooves 12.5 μm width and 2.5μm depth. Cells were treated with antisense lyn in comparison with control as described in text. The rate of cell migration was slowed down significantly (t-test, $P<0.01$ compared with control without antisense treated) when antisense *lyn* was applied. 43 cells were measured each here.

Figure 6-3. Amplification of lyn gene. Total RNA was extracted from P388D1 macrophage-like cells. cDNA was reverse transcribed from RNA and then used as template in PCR reaction with the presence of primers (5'-CTC AAG AGT GAT GAA GGT GGC A-3'; 5' TAC AGG AGA ATT CCG AAG GAC C-3'). The PCR reaction was first through 94 °C for 1 minute, then through 30 cycles of 94 °C for 55 seconds, 58 °C for 55 seconds and 72°C for 1 minute and 10 seconds, and extending at 72°C for 10 minutes. The PCR products were then checked in a 2% agarose gel with the presence of 100 bp DNA maker as seen in lane 4. Lane 1 is the reamplified lyn cDNA fragment of the size of 360 bp. This band from lane 1 was then cut off and cleaned as described in chapter 2. It was then used as probe in Northern Blot analysis.

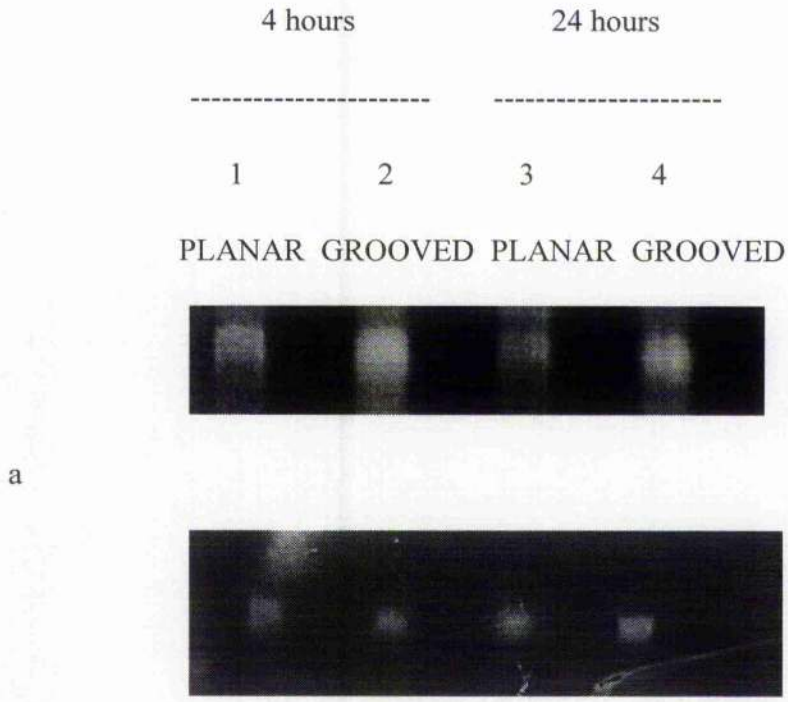


Figure 6-4. Northern blot analysis of *lyn* mRNA level from Epitenon cells cultured on either non-grooved or grooved structures. Total RNA was extracted from Epitenon cells which were cultured on microgrooved silica (Quartz) and flat quartz surface at the time point of four hours and twenty four hours. The RNA was separated by electrophoresis in a 1% formaldehyde-agarose/MOPS gel, blotted to nitro-cellulose and hybridised with ^{32}P-labelled random-primed amplified *lyn* gene probes. The filter was then exposed to Fuji X-ray film for 1 - 3 days. Lane 1 represent cells cultured on flat surface for 4 hours and lane 2 is at same time point but cultured on grooved surface, same with lane 3 and 4 in the same order except the cells were cultured for 24 hours.

DISCUSSION

Effect of Lyn in Contact Guidance

The antisense *lyn* kit used in this study was purchased from Biognostik (Biognostike, GmbH, Gottingen, Germany). It has been impossible to find out the detailed sequence which they had designed against *lyn* mRNA but, it is said to form stable duplexes between antisense and target RNA. The concentration we used in the study is that suggested by the kit instruction of 2 μM for the final concentration, which is rather low although it is reported that higher concentration could affect the cells more but also increase the chances of toxicity.

Detectable antibody staining of *lyn* on epitenon cells on grooves could be seen as early as 20 minutes after plating (Adam Curtis personal communication). This suggests that *lyn* plays a role in contact guidance. Antisense *lyn* experiment further proved this role. Since the half life of the free target RNA is 146 seconds (Sczakiel and Goody, 1994), due to the very slow rate of duplex formation, a significant inhibition in a realistically short times is not expected. For this reason, I chose 24 and 48 hours as the time point to examine the cell morphological changes.

As described previously in this project, it has also found that antibody against fyn (member of the *src* family of tyrosine kinase) showed the same localisation as lyn. An antisense *fyn* experiment was also carried out and the same effects

were observed. Antisense *fyn* reduced the rate of migration of epitenon cells on grooves of 88 nanometre depth, and decreased the length of the same cells on grooved structure following 24 and 48 hours culture (Adam Curtis, personal communication).

The results suggest that *lyn* antisense oligonucleotides can be efficiently taken up by epitenon cells. Cell length and width were both affected with antisense treatment in comparison with non-specific antisense treatment and controls (see figure 6-1, figure 6-2). This observation supports the study of antibody against lyn which shows the same localisation along the groove/ridge when growing cells on the grooved structure compared those cultured on the flat surface, as described above. This result also suggests that lyn could play a role in the signalling mechanism responsible for the phosphorylation pattern observed when cells respond to their surface topography, although the detailed pathway is not very clear.

Northern Blot

Northern Blot results shows that tyrosine kinase *lyn* mRNA level does not change after epitenon cells were cultured on a grooved and flat surface for both 4 and 24 hours. As discussed in Chapter 4, it was not possible to get a large number of cells which were cultured on a grooved structure. It is probable that the reason for this is due to the small amount of its messenger RNA used, it is not possible to detect any small changes in its messenger RNA level.

CHAPTER 7

GENERAL DISCUSSION

TOPOGRAPHY

In this work, epitenon fibroblasts and P388D1 (macrophage-like) cells were used, both of which have been shown to be very sensitive to microgrooved surfaces. Meyle *et al* (1991) observed after 2 hours, 20% of macrophages and 30% of fibroblasts reacted with alignment and after 2 days, 30 % of macrophages and almost 100% of fibroblasts were aligned. I found that there are always about 30% or less of the macrophages which remain attached and rounded.

As suggested by Clark *et al* (1987, 1990), the depth of the grooves may be of great importance for cellular orientation. They observed that the alignment increased with depth and decreased with pitch. Wojciak *et al* (1996) showed that P388D1 could be oriented when the depth of structure is great than 44 nanometers at the width of 10 micrometers, although it is rather difficult to say yet which exact size could lead to a maximum cell orientation and elongation. The sizes I used were adequate for the cells to spread and elongate.

All the fused silica (quartz) structures used in this study were made using photolithography. Photolithography of substrates followed by plasma etching may result in chemical heterogeneity between the etched and unetched regions

of the substrate. However, my materials were blanket-etched ie after etching the grooves. To do this, the resist was removed and the whole structure exposed to plasma for a short time, to equalise chemistry. As a control, I used the planar fused silica which had not been etched, but it is believed this can only cause minor differences and should not affect the reliability of gene expression. There is little reason to expect a difference between planar blanket-etched silica and planar silica because both are exposed strongly to oxidising environments in cleaning.

As described above, since the concept of contact guidance was first introduced by Weiss in 1934, several theories about the mechanisms of the action of the topographic cues have been raised. Weiss *et al.* (1941, 1945) suggested that contact guidance might account for features of morphogenesis, and the phenomenon might be explained by the oriented adsorption of macromolecules produced by the cells or derived from serum. Curtis and Varde (1964) argued that the range of diameters of fibers on which cells were guided was too large for any obvious orientation of surface-adsorbed molecules to occur. Thus they suggested that the cells were reacting to features of surface topology. Dunn and Heath (1976) suggested that the underlying mechanism was the unbendablility of the microfilaments. At almost the same time, Ohara and Buck (1979) suggested that there might be a preplan of focal contacts aligned in the cell ready to respond to in the direction of the substrate topography. Since focal contacts

are connected to the cytoskeleton this might well align the cells to the substratum.

More recently, much work has been done using a wide range of very precisely specified microtopographies. Curtis and Wilkinson (1998) suggested that since topographies produced reactions to the directions of maximal stress there might be some involvement of stretch reception. While Dunn and Heath's theory and Curtis and Wilkinson's theory involve the actin cytoskeleton, consistent with my finding of c-kit activation, tctex is involved with microtubles suggesting that they may also be involved with topographic reactions.

CHANGES IN GENE EXPRESSION

It is well known that surface topography is an important factor in controlling cell shape, orientation and adhesion of mammalian cells (Curtis and Varde 1964, Brunette, 1986a; Curtis and Clark, 1990; Dunn and Brown, 1986). Cell shape was found to be tightly coupled to DNA synthesis and growth in nontransformed cells (Folkman and Moscona, 1978). Their findings suggest a mechanism that is important in the growth control of mammalian cells.

Numerous studies (Farmer *et al.*, 1983; Ben-Ze'ev *et al.*, 1986,1987,1991; Chou *et al.*, 1995,1996) have shown that cell shape controlled by adhesion is closely related to gene expression, for example, cytoskeletal gene expression. It is known that topography changes cell shape. Initial studies on the regulation of

expression of α- and β-tubulin showed that disruption of microtubules by colchicine results in a rapid inhibition of new tubulin synthesis due to a decreased ability *in vitro* to translate cytoplasmic tubulin mRNA (Ben-Ze'ev, 1986). Moreover, the disruption of the microtubular network by vinblastine, which induces the formation of tubulin paracrystals, causes a slight increase in tubulin synthesis. (Bruttene *et al.*, 1993).

Cell spreading on a substrate also affect the expression of intermediate filaments. The same group (Ben-Ze'ev *et al.*) compared the level of synthesis of cytoskeletal proteins in cells cultured in a monolayer with that of cells in a suspension. They found that the transition from a flat to a spherical cell shape configuration is accompanied by a specific and marked repression in the synthesis of vimentin (the protein of mesenchymal type intermediate filaments) (Ben-Ze'ev, 1983). Thus, the organisation and the expression of vimentin appears to be linked and probably related to the extent of cell spreading on the substrate.

The actin cytoskeleton is a major structural element in determining cellular morphogenesis and cell contact formation. Actin filaments and their associated proteins, also known as stress fibres, play a major role in cell adhesion. Wojiack *et al* (1995) also showed that F-actin content could increase up to 60% by growing macrophages on microgrooved substrata compared with the same type of cells growing on a flat surface. The relationship between extracellular matrix

components such as fibronectin and its transmembrane receptor and the cytoplasmic cytoskeletal elements α-actin, vinculin and talin (which are all actin-filament-associated proteins) at adhesion plaque or focal contacts is very complex, and some details remain unclear (Ungar *et al.*, 1986; Burridge *et al.*, 1988,; Schaller *et al.*, 1994; Chen *et al.*, 1995).

Numbers of studies have shown the close relationship between cell shape, cytoskeleton and gene expression. The mechanism of cell contact guidance is still not understood, but it is reasonable to search for the changes in gene expression which are induced by surface topography, for instance, microgrooved substrata. In order to investigate the interesting possibility of changes in gene expression, I used messenger RNA fingerprinting to detect differential expression messenger RNA. I extracted total RNA from the cells growing on smooth and grooved substrata, and compared in a fingerprinting gel. As I describe in chapter 3 and chapter 4, the differentially expressed bands were observed from both macrophages and epitenon cell types.

Messenger RNA fingerprinting is a method developed in the early 1990s, and this method has been widely used to compare gene expression between tumour and normal tissues. This is the first time that this method has been applied in the cell engineering field. As mentioned before, very few studies have been done at a molecular level. Only one study done by Chou *et al.*, used Northern blotting and examined fibronectin mRNA levels between cells growing on a grooved and

flat surface. Therefore, my study is the first to discover that a wide range of different genes expressed were stimulated by topographic cues such as grooves. Such findings are discussed later.

In this study, only two cell types were used to investigate gene expression towards their surface topography. The cells demonstrated fairly similar reactions to topography. Both cell types can be guided by grooved surface which results in the cells align along the direction of the grooves. This poses the question of whether different cell types use the same set of genes when reacting to topography. However, since it is not very clear just how close the relationship between the different cell types and their gene expression towards surface topography, it will be very useful to study different cell reactions in the future. Whatever the eventual result on gene expression induced by surface topography will be, this study could be used as a good beginning for future direction of research.

POSSIBLE ROLE OF c-kit

The c-kit receptor, a receptor tyrosine kinase, is a transmembrane protein with its ligand-binding domain on the outer surface of the plasma membrane. It encodes a receptor tyrosine kinase closely related to the platelet-derived growth factor (PDGF) receptor subfamily and macrophage colony stimulating factor

receptor (CSF-1R) (Besmer *et al.*, 1986; Yarden *et al.*, 1987; Qiu *et al.*, 1988) as well as to the immunoglobulin superfamily. Together with its ligand steel factor (SLF), c-Kit is a key controlling receptor for a number of cell types including haemopoietic stem cells, mast cells, melanocytes, and germ cells. c-Kit is the gene product of the W locus in mice (Huang *et al.*, 1990), and its ligand SLF is the product of the *sl* locus (Chabot *et al.*, 1988; Geissler *et al.*, 1988). It is known that it contains an extracellular domain which often binds a ligand and an intracellular domain, which contain a tyrosine-specific protein kinase. Upon binding of the ligand to the extracellular domain of a transmembrane receptor kinase, the kinase becomes activated; this leads to autophosphorylation as well as the phosphorylation of cellular substrates (Majumder *et al.*, 1988). It is clear that the activity is crucial for the transmission of a signal from the cell surface to the nucleus; however, the detailed steps, specific substrates and second messenger systems, involved in this process are less well understood (Majumder *et al.*, 1988). Recent studies also indicate that *c-kit* receptor signalling through its phosphatidylinositide-3'-kinase-binding site induced adhesion of bone marrow-derived mast cells (BMMC) to fibronectin, secretory enhancement, membrane ruffling and filamentous actin assembly and adhesion to extracellular (Vosseller *et al.*, 1997). We can then conclude that topography alone can stimulate *c-kit* expression which leads to a cluster of signal transduction and affects cell behavior on topography guidance.

The changes in gene expression include over-expression and reductions in expression in these investigations. In the signal transduction pathway, upstream receptor binding to its ligand in turn will active their downstream enzyme and lead to a phosphorylation cascade which could act across a few other signal pathways and affect their downstream proteins. Thus, once an upstream molecule was upregulated, several downstream proteins could be affected as well. For example, I discovered that c-kit could be upregulated by topography cues, which in turn bind to one of PI-3-kinase regulatory subunit p85. Activated PI-3- kinase is essential for mediating Rho family which could lead to membrane ruffling. PI-3-kinase is also associated with several signalling proteins, including growth factor receptors such as PDGF receptor, receptor tyrosine kinases of the Src family and GTP-binding proteins of the Ras and Rho families (Rodriguez *et al.*, 1994; Zhang et al., 1994; Toliask *et al.*, 1995). PI-3-kinase has also been shown to be involved in the regulation the actin cytoskeleton by growth factor (*Kurniz et al.*, 1995; Hu *et al.*, 1995; Wennstrom *et al.*, 1994). However, the detailed molecular events involved in this process are unclear. It is not going to be too surprising if more molecules will be discovered in the future which are regulated by contact guidance, since upstream protein molecules and downstream protein molecules are closely related to each others.

NOVEL cDNAs

From the results I obtained, there are four cDNA fragments (peptides) which appear to be novel DNAs. The sizes of these four novel DNA fragments are

413bp, 312bp, 203bp and 274bp. Although there is no overlap found between them, it is likely that they might originate from the same gene since their size are rather small. Due to the time I have left, it is not possible for me to investigate further isolation of these whole DNAs. For example, cloning the whole cDNAs and sequencing them.

POSSIBLE ROLE OF LYN

In this study, a possible role for lyn tyrosine kinase in the mechanism of cell contact guidance was discovered. Tyrosine kinase lyn was found to be very important in contact guidance which includes both cell morphology and cell motility. Previously, in vitro studies have shown that tyrosine kinase lyn was specifically expressed along groove/ridge boundaries when cells were cultured on micro- and nano-grooved substrates. The direct role for tyrosine kinase lyn involvement in contact guidance had not previously been demonstrated.

The antisense *lyn* tested not only prevented cell elongation on grooved structures, (as discussed in chapter 6), but also prevented cell movement along the grooved structures. This suggests that tyrosine kinase lyn plays a very important role in topographic cues or contact guidance. Although, it is not possible to demonstrate that *lyn* mRNA levels change during cell reaction to different surface topographies.

The fyn tyrosine kinase ($p59^{fyn}$) has also been shown to have the same localisation of tyrosine phosphorylation as that described previously. Like lyn and any other scr family members, fyn consists of four domains: an N-terminal domain where myristylation occurs, allowing for association with cell membranes; SH3 and SH2 domains which bind to proline-rich and phosphotyrosine-containing sequences, respectively, and facilitate interactions with other proteins; a catalytic kinase domain containing a tyrosine autophosphorylation site; and a negative regulatory tyrosine residue at the C - terminus (Superti-Furga and Courtneidge, 1995).

Antisense fyn experiment have also been carried out, and antisense fyn reduced the rate of migration of epitenon cells cultured on grooves of 80 nanometer depth and decreased the length of the same cells on grooved structures, following 24 and 48 hours culture (Adam Curtis, personal communication).

ERRORS

Finally, although it is unlikely, the results that I obtained from this study may be vitiated by some errors: Namely 1) Unsuspected nanofeatures of the topography which have not been discovered. 2) RT-PCR handling errors. To avoid getting false results, I have repeated RT-PCR three times at least to get the same mRNA fingerprinting results. From the recovered DNAs, I cloned and each time required three identical results. This probably means that all the results presented here are reliable and repeatable.

FUTURE WORK

The surface topography to which cells are exposed plays a important role not only in cell shape, proliferation but it also alters gene expression. The mRNA level of fibronectin is increased significantly on grooved surface (Chou *et al.* 1995). The most important general result of my experiments is that there are extensive changes in gene expression induced within three hours in macrophage-like cells and epitenon cells by culture on grooved topography as opposed to flat topography. We can conclude that topography alone will stimulate different gene expression and different cell behaviour.

In addition, there are other important questions for future work. These include whether different cell types react identically or very differently form the cells we have used. There is also the question about possible differences with different types of topography.

This work has been continued in the laboratory recently. It can be predicted that with more studies carried out, there will be other interesting discoveries in the future. Hopefully, with a more detailed understanding of molecular mechanism involved in cell engineering, it should enable future implants to be designed that reproducibly produce desired cell responses.

CHAPTER 8
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