

Cellular response to low adhesion nanotopographies

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Abstract: This review focuses on how cells respond to low-adhesion nanotopographies. In order to do this, fabrication techniques, how cells may locate nanofeatures through the use of filopodia and possible mechanotransductive mechanisms are discussed. From this, examples of low-adhesion topographies and sizes and arrangements that may lead to low-adhesion are discussed. Finally, it is hypothesized as to how specifically low-adhesion materials may fit into the outlined mechanotransductive mechanisms.

Keywords: nanotopography, mechanotransduction, filopodia, nanofabrication

Introduction

There are many chemical, physical and geometric cues within the extracellular environment *in vivo* that will provide signals to cells. It has been known for almost one hundred years that cells will react to the shape of their environment, and in 1952 Weiss first used the term contact guidance to describe cell alignment to topography (Weiss and Garber 1952; Curtis 2004). Due to advances in micro-fabrication, researchers have been able to produce accurate micro-scale substrates upon which to study cells. Thus, the effects of micro-topography on cells are becoming well known and include changes in cell adhesion, contact guidance, cytoskeletal organization, apoptosis, macrophage activation and gene expression (Clark et al 1987, 1990, 1991; Wojciak-Stothard, Madeja, et al 1995; Britland et al 1996; Dalby, Riehle, Yarwood, et al 2003). It is further becoming clear that cells will react to their nanoenvironment. *In vivo*, the nanoenvironment may be provided by protein folding and banding. *In vitro* testing of the cells response to defined nanotopographies has drawn on a number of fabrication techniques.

The borrowing of lithographical techniques from the microelectronics industry facilitated research into cell response to the topography of their environment. Initially, photolithography was used, which allowed fabrication of micron scale (width, diameter) features such as grooves and pits with sub-micron depths (Wilkinson et al 2002). Every cell type tested responded to the features by contact guidance (Clark et al 1987, 1991; Wojciak-Stothard, Curtis, et al 1995; Wojciak-Stothard, Madeja, et al 1995; Britland et al 1996). Further examination revealed that contact guidance lead to changes in cell adhesion, migration, cytoskeletal organization and genomic regulation (Dalby, Riehle, Yarwood, et al 2003).

It has now been shown that many kinds of cells also respond strongly to nanoscale topographies (Dalby, Riehle, et al 2002a, 2002b; Dalby, Gadegaard, et al 2004; Price et al 2003; Thapa et al 2003; Ward and Webster 2006). The scope of response, as with μm scale topographies ranges from adhesion (Biggs et al 2007a, 2007b) to genomic regulation (Dalby, Yarwood, et al 2002). This is really very interesting as cells can be influenced in similar ways by their μm and nm scale environments, but the mode of action is probably rather different. At the microscale, cells are clearly influenced by features in the same magnitude of size as themselves, ie, encounter mechanical confinement and robust obstacles. At the

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nanoscale, however, the features are far smaller than the cells themselves and thus must alter cell response through more subtle mechanisms.

So, if nanotopography is more expensive to fabricate and cells respond to microtopography with a large range of responses, why bother pursuing nanotopography as a clinical solution? The answer is that the range of response we can elicit with nanotopography is, in fact, greater and the effects we can produce in cells can range from subtle to strong. A great example of the strength of nanotopographical effect is low-adhesion, as shall be discussed here.

This review will start with a very brief overview of some nanofabrication methods, although it is to be noted that the actual fabrication is not the motivation for the report. The main aim of this report is, however, to present some current, if contentious, thinking on mechanisms of cell response to low-adhesion topographies.

Fabrication

Nanofabrication can be either top-down, starting with a large 'block' and creating small features (eg, lithography) or it can be bottom-up, starting with atoms, molecules or polymers and building into regular features (eg, tunneling fabrication or phase separation). Here, just a few techniques will be described that have been routinely used for cell testing.

Starting with top-down, the most high-resolution top-down fabrication technique is that of electron beam lithography (EBL) where a pattern is generated in a radiation sensitive polymer (resist) by a focused beam of electrons. The achievable resolution for this technique is about 3–5 nm (Vieu et al 2000). The area that can be exposed by an EBL tool without movement of the stage (the field size) is limited by the resolution of feature required. For well-defined 35 nm dots, Gadegaard, Thoms, et al (2003) used a field size of 200 × 200 μm. However, larger areas than this are required for cell culture experiments. To make larger areas of pattern, the sample is moved with high precision under interferometric control to allow tiling of the small pattern to fill a larger area (Wilkinson et al 2002; Wilkinson 2004).

EBL is, however, a rather expensive and time-consuming technique. Another top-down fabrication technique is colloidal lithography, where monodispersed nanocolloids are used as an etch mask from which to create nanofeatures with controlled height and diameter, but random placement (Denis et al 2002a, 2002b; Agheli and Sutherland, submitted). Colloidal lithography allows the rapid production of large areas of nanotopography, but has the pay-off of less controllable feature distribution.

We note at this point that it is envisaged that bottom-up fabrication may play a critical role in production of topographies for cells to react to. An example of currently used bottom up techniques are and polymer demixing where spontaneous phase separation of polymers in a solvent creates nanotopography with reproducible height, but random distribution and diameters (Affrossman et al 1996, 1998, 2000; Affrossman and Stamm 2000). Recently, the Liley group has produced other co-polymer systems that allow better order to be achieved (Minelli et al 2006; Blondiaux et al 2007).

An important step in research for cellular response to nanotopographies (as well as a step closer to mass production of all nanotopographies) and the possibility of improved implant design is the process of producing shims. This technique is borrowed from the DVD manufacture process and involved the plating of Ni onto the master substrates. Firstly, this is done by sputter coating to provide an initial key, next, electroplating is used. Once the master is removed, a negative replica in Ni is left. Polymers can then be injection molded or embossed against the substrate and depending on the design and the polymer used, down to 5 nm fidelity can be achieved. With injection molding, many hundreds of replicas can be produced in a day (Gadegaard, Mosler, et al 2003; Gadegaard, Thoms, et al 2003). The polymers used can either be biodegradable (PCL, PLA) or permanent (eg, polymethylmethacrylate, PMMA or polycarbonate, PC). It is envisaged that in the near future, embossing into metals will be a possibility.

For the described approaches over the past few years, however, there has been considerable research effort into ascertaining the breadth of cellular response to nanoscale features, and again, contact guidance has been observed in many cell types. The physical contact guidance, ie, alignment, however, is on a different scale; that of the filopodia.

Cell filopodia

It was mentioned in the introduction that cells must respond to nanoscale topography on a more subtle scale than the whole cell response to microtopography, it is very likely that filopodia, with tips of approximately 100 nm, are one of the cells' main nanosensory tools. Gustafson and Wolpert (1961) first described filopodia in living cells in 1961. They observed mesenchymal cells migrating up the interior wall of the blastocoelic cavity in sea urchins and noted that the filopodia produced appeared to explore the substrate. This led them to speculate that they were being used to gather spatial information by the cells. When considering filopodial

sensing of topography, fibroblasts have been described as using filopodia to sense and align the cells to microgrooves (Clark et al 1991) and epithelia have been shown to be of importance for alignment to nanogrooves (Teixeira et al 2003). Also, macrophages have been reported to sense grooves down to a depth of 71 nm by actively producing many filopodia and elongating in response to the shallow topography (Wojciak-Stothard, Madeja, et al 1995); in other words, monocyte (rounded) to macrophage (spread) activation occurs.

Cytoskeletal actin bundles drive the filopodia. As the filopodia encounter a favorable guidance cue, they become stabilized following the recruitment of microtubules and accumulation of actin. There is further evidence that nascent focal adhesions then form at the filopodial tips (Dalby, Riehle, Johnstone, et al 2004).

Once cells locate a suitable feature using the filopodia presented on the cells leading edge, lamellipodium are formed which move the cell to the desired site (Dalby, Riehle, Johnstone, et al 2004). These actions require G-protein signaling and actin cytoskeleton. Specifically of interest are Rho, Rac and Cdc42. Rho induces actin contractile stress fiber assembly to allow the cell to pull against the substrate, Rac induces lamellipodium formation, and Cdc42 activation is required for filopodial assembly (Schmitz et al 2000). Rho and Rac are both required for cell locomotion, but cells can translocate when Cdc42 is knocked out. Cells lacking Cdc42 cannot, however, sense chemotactic gradients and simply migrate in a random manner (Jones et al 1998). This, again, presents compelling evidence for filopodial involvement in cell sensing.

There is recent evidence that many types of cells, including mesenchymal stem cells, use filopodia to probe their nanoenvironment (Figure 1) (Dalby, Riehle, et al 2002a, 2002b; Dalby, Childs, et al 2003; Dalby, Gadegaard, et al 2004; Dalby, Riehle, Sutherland, et al 2004a; Hart et al, in press). The smallest feature (thus far) that cells (fibroblasts) have been observed to respond to are 10 nm high polymer demixed islands (Dalby, Riehle, Johnstone, et al 2004).

For low-adhesion materials, however, it is rather the case that filopodia cannot find sites suitable for adhesion. This is likely to be for two reasons. The first and main reason is the surface properties of the materials. Low-adhesion topographies tend to give extremes of environment. If treated with chemicals to normally produce hydrophobicity or hydrophilicity, nanostructures can become super-hydrophobic or super-hydrophilic (Martines et al 2005). This will effect protein interaction with the surfaces and hence cell adhesion;

if a surface does not recruit proteins, cells cannot adhere, if a surface recruits proteins so strongly that conformational shape is altered, cells cannot adhere (Kasemo and Lausmaa 1988, 1994).

Secondly is that of surface area. Transmission electron microscopy of EBL pits in highly ordered arrangement has shown that adhesions cannot form across the pits and that filopodia can only start to form adhesions on the raised, interpit, areas (Dalby, Biggs, et al 2007).

Mechanotransduction

Filopodial sensing of the nanoenvironment and subsequent changes to the cellular cytoskeleton will almost certainly confer mechanotransductive changes to the nucleus and from here alter gene transcription and protein output. Hence microarray studies have regularly shown changes in genome regulation on both microtopographies and nanotopographies (Dalby, Yarwood, et al 2002; Dalby, Riehle, Yarwood, et al 2003). These mechanotransductive signaling events may be chemical eg, kinase based linked to focal adhesions influenced by cytoskeletal contraction. An example would be integrin gathering as an adhesion is formed will activate myosin light chain kinase (MLCK) which will generate actin – myosin sliding (the key event in stress fiber contraction) and in turn will change focal adhesion kinase (FAK) activity. Cytoskeletal involvement in contraction against adhesions will also alter calcium influx and G-protein events. These chemical signaling events are collectively known as indirect mechanotransduction (Burridge and Chrzanowska-Wodnicka 1996).

Another form of mechanotransduction, direct mechanotransduction, is considered to be transduced by the cytoskeletons as an integrated unit. An interesting (although controversial) theory is that of cellular tensegrity, whereby the cells mechanical structure is explained via tensional integrity (Ingber 1993, 2003a, 2003b, 2003c; Maniotis, Bojanowski, et al 1997; Maniotis, Chen, et al 1997; Charras and Horton 2002). Through this tensegrity structure, tensional forces from the extracellular environment (eg, from tissue loading or changes in cell spreading) are possibly conferred to the nucleus and alter genome regulation (Mosgoller et al 1991; Heslop-Harrison et al 1993; Heslop-Harrison 2000; Dalby, Riehle, Sutherland, et al 2004c; Dalby 2005).

We currently support the idea of an integrated cytoskeleton – not necessarily tensegrity, but along those lines – is directly linked to the extracellular matrix through actin anchoring to adhesions and then linked to the nucleus via the intermediate filaments of the cytoskeleton associating

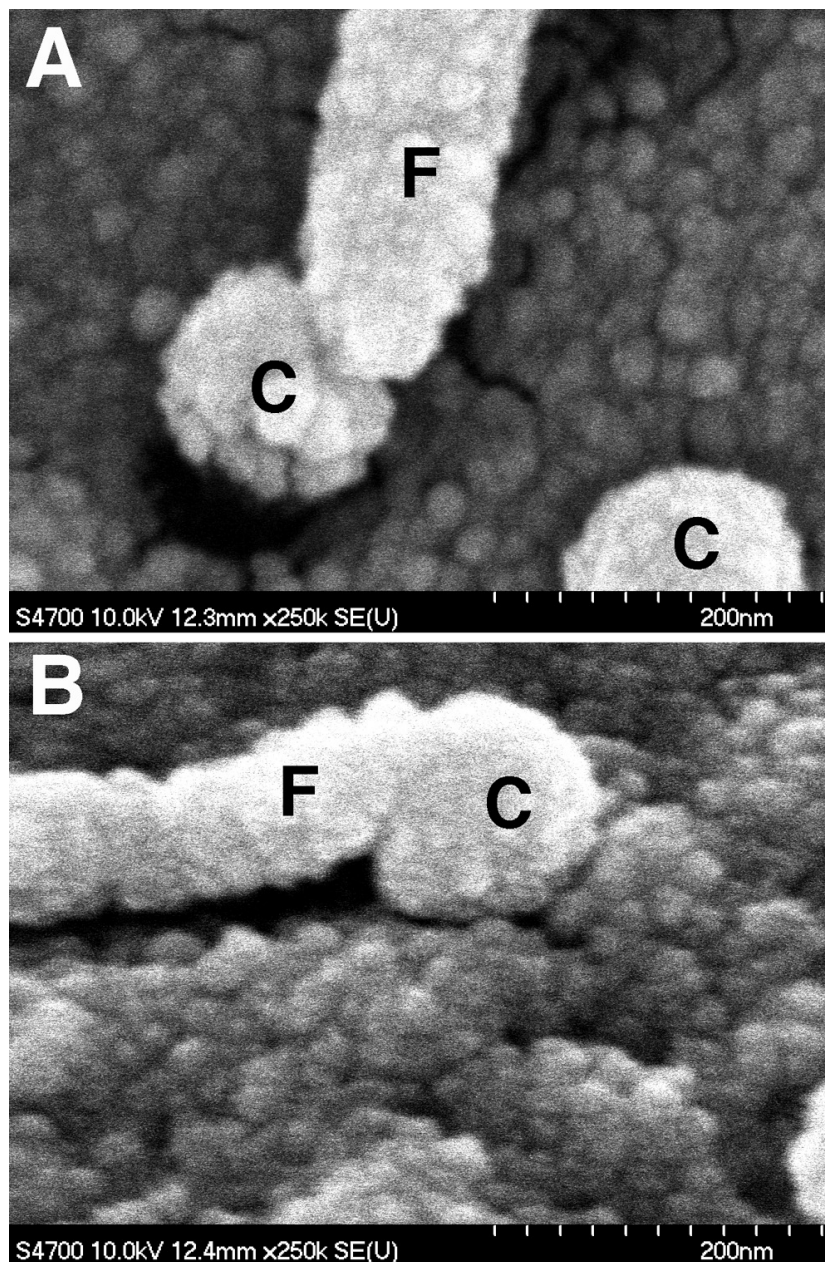


Figure 1 Filopodial sensing of nanocolumns. (A & B) 160 nm high, 100 nm diameter columns originally fabricated by colloidal lithography. Note how the cell filopodia (F) have similar tip dimensions to the columns (C) they interact with.

with lamin intermediate filaments of the nucleoskeleton. It is further known that the telomeric ends of the interphase chromosomes are intimately linked to the lamins (Foster and Bridger 2005). Thus, tension directed through the cytoskeleton may be passed directly to the chromosomes during gene transcription. Changes in chromosomal three-dimensional arrangement (considering the chromosomes in the interphase nuclei to have relative consistency of position (Heslop-Harrison and Bennett 1990) may effect transcriptional events such as access to the genes by transcription

factors and polymerases. Also, changes in DNA tension can also cause polymerase enzymes to slow down, speed up or even stall completely (Bustamante et al 2003). These may be mechanisms by which changes in cell spreading on nanotopographies can change events such as adhesion, proliferation and even differentiation (Dalby, Biggs, et al 2006; Dalby, McCloy, et al 2006a, 2006b) (Figure 2).

Another, possibly connected, theory is that of nano-imprinting into cells by nanofeatures (Curtis et al 2006a, 2006b). This describes a phenomenon that has been clearly

seen in platelets and for which some evidence has been provided in more complex cell types (fibroblasts). For nanoimprinting to occur, the pattern of the topography must be transferred to the cytoskeletal filaments, ie, the topographies produce a template that is favorable/unfavorable to condensation of cytoskeletal polymer chains through invagination (or embossing) of the basal membrane against the topography. There is evidence that this leads to increased 'attempted' endocytosis, ie, the cells recognize the features (in the referenced case, nanocolumns – 160 nm high) as being in the correct size range to try to endocytose and to form clathrin coated pits (Dalby, Berry, et al 2004). Such endocytotic vesicles are moved by actin cables; perhaps it is this mechanism that is causing the topography mimicking actin patterning described by Curtis et al (2006b).

Low adhesion materials

It is fair to say that in biomaterials research there has been major drive to consider how to increase cellular adhesion

and proliferation on materials surfaces, however, this is not always the effect desired. There are many applications where low-adhesion is required such as in stents, catheters, heart valves, acetabulum etc where cell migration and tissue formation are problematic. Whilst it is presently hard to see if topography can have roles where tribological issues are involved, it is possible to foresee that in areas such as catheters and valves topography could play a key role.

Topographies that produce low adhesion in fibroblasts and mesenchymal stem cells have already been patterned inside tubes using polymer demixing to form nanoislands (Gadegaard et al 2004; Berry et al 2005, 2006). The next step has to be to produce the islands on the internal surface in the bulk of the tube polymer. This could perhaps be achieved by embossing the low-adhesion shapes and then precision rolling – in a similar manner to that outlined by Seunarine et al (2006). In a similar way, curved surfaces could be achieved.

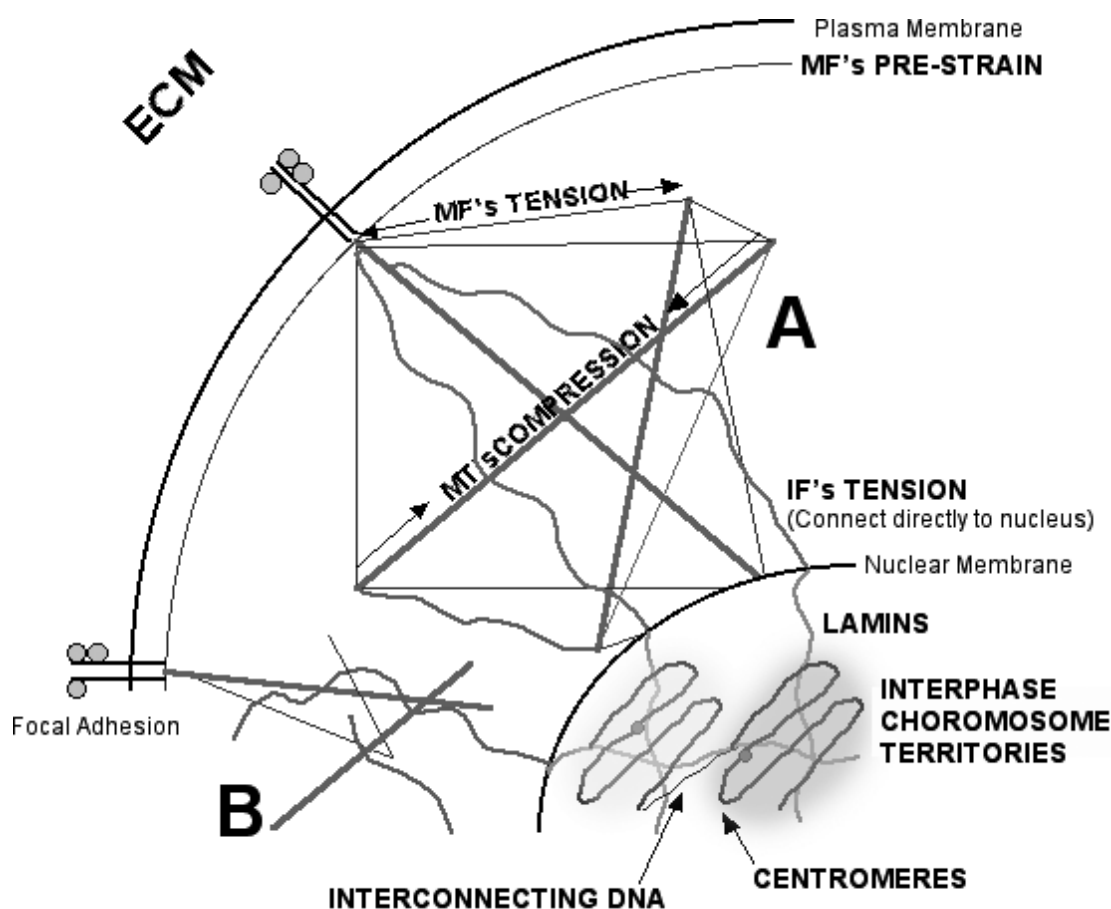


Figure 2 Suggested possible mechanisms for direct mechanotransduction including theories of cytoskeletal tensegrity which relies on the interaction of tensile and compressive elements (A) and percolation (B). Cytoskeletal percolation is a simplified form of tensegrity whereby simple integration is required without the rules of tensegrity. The figure shows continuum from the cellular adhesions to the chromosome territories within the nucleus.

Nanoislands produced by polymer demixing are, in fact, tunable to low or high adhesion (Dalby, Pasqui, et al 2004). Very small islands (<20 nm) promote adhesion in most cell types tested (endothelial (Dalby, Riehle, et al 2002a), fibroblastic (Dalby, Riehle, et al 2002b) and mesenchymal stem (Dalby, McCloy, et al 2006a)). As the islands increase in size, they become less adhesive. Islands produced from a blend of polystyrene/ polybutylmethacrylate were adhesive to fibroblasts at 10 nm high, but almost completely non-adhesive at 50 nm high (Dalby, Riehle, Johnstone, et al 2003).

Topographies produced by colloidal lithography have shown similar trend, with very small nanocolumns (11 nm high) producing increased osteoprogenitor response (Dalby, McCloy, et al 2006a), whilst larger nanocolumns (160 nm high) produced a significant (but far from complete) reduction in fibroblast adhesion (Dalby, Riehle, Sutherland, et al 2004a, 2004b). The Sutherland group has also done some interesting work on topographies fabricated through the colloidal route. They firstly showed 100 nm high columns to reduce epithelial cell adhesion (Andersson, Backhed, et al 2003) and then went further to show, again in line with the polymer demixed nanotopographies, that increasing column size decreased epithelial cell adhesion (Andersson, Brink, et al 2003). In collaboration with Rice and Hunt at Liverpool, they also showed that the 160 nm high nanocolumns imparted properties of low-adhesion on osteoblasts (Rice et al 2003).

Using another fabrication method, that of interference lithography, a recent paper also agreed with these observations of size and adhesion. Choi and others fabricated sharp nanoposts (pointed columns) and nanogratings (very sharp grooves) of different heights. They used 3 size ranges: 50–100 nm high, 200–300 nm high and 500–600 nm high and found decreased adhesion and spreading as size increased (Choi et al 2007).

The notable observation from the above is the theme of tunable height of features, with not much mention about depth. However, there is a very low adhesion pit system that with some cell types can produce almost total non-adhesion. That of nanopits fabricated in a square array by EBL with diameters of approx. 100–150 nm diameters and centre-centre spacing (pitch) in the range of 300 nm. Again, these adhesion effects have been shown to be size dependant. A study that used 3 pit sizes (diameter (nm): pitch (nm) – 35:100, 75:200 and 120:300) and fibroblasts as a cell model showed that as pit diameter increased, filopodial interaction increased, but cell spreading decreased, ie, the

interaction of the filopodia with the pits was preventing adhesion (Dalby, Gadegaard, et al 2004). A previous study by Gallagher et al (2002) with pit diameters of 150 nm showed almost no epitenon attachment. However we know that large pits (in the range produced by photolithography) promote increased cellular response in eg, mesenchymal stem cells (Dalby, McCloy, et al 2006b). Thus, there is a large gap in our knowledge of the transition between non-adhesive and adhesive pits (Figure 3).

This is further complicated by pit symmetry being implicated as important. This was originally mooted by Curtis and others in 2004 (Curtis et al 2004). Subsequent studies have used 120 nm diameter, 300 nm pitch pits in both square and hexagonal arrangement. Both with mesenchymal stem cells (Hart et al, in press; Dalby et al, submitted) and with fibroblasts (Dalby, Gadegaard, et al 2004; Dalby, Gadegaard, Herzyk, et al 2007; Dalby, Gadegaard, Wilkinson 2007), the hexagonal arrangement has been shown to be the least adhesive.

Mechanisms of low adhesion

It seems likely that the ability of a cell to adhere and spread will alter mechanosensitive pathways in a cell. As adhesions form and act as an anchor for the cytoskeleton and the integrins within the adhesions act as a route for cell/substrate signaling (Burridge and Chrzanowska-Wodnicka 1996), it is probable that low-adhesion topographies will firstly act by changing adhesion formation and morphology and as consequence effect mechanosensitive pathways. Indeed, Biggs et al (2007a, 2007b) have shown the total number of adhesions to reduce significantly on the square and hexagonal low-adhesion EBL arrays. Interestingly, however, they showed the proportion of focal complexes (transient adhesions involved in motility), focal adhesions (mature adhesions to which stress fibers anchor) and fibrillar adhesions (very mature, align to the cells endogenous matrix) to remain constant; thus, whilst a reduction in adhesions was seen, a change in morphology was not.

The loss of attachment points for the cytoskeleton will probably result in a reduction of applied tension to the nucleus and a concurrent reduction in integrin related signaling. For the direct mechanosensitive theories (described above), whilst increased cytoskeletal organization, on high-adhesion substrata, would result in increased nuclear tension, reduced organization, on low-adhesion substrata, would result in reduced nuclear tension.

We have investigated this to some extent with low-adhesion topographies. Thus far, it has been seen that reduction of adhesion formation potential and cytoskeletal

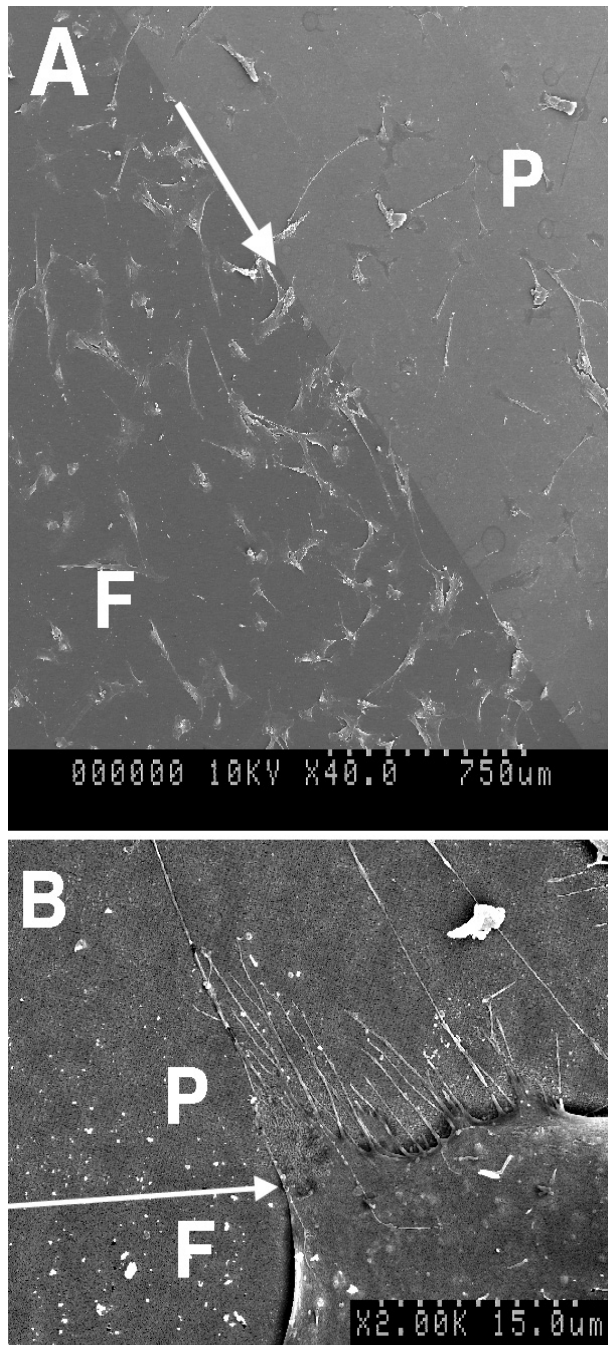


Figure 3 Sensing of low-adhesion borders. In both (A) and (B) there are both flat parts (F) and parts patterned with low-adhesion EBL nanopits (P). (A) Shows that there are many more cells on the planar side of the border, whilst (B) shows a high magnification image of a cell aligning to the border and producing filopodia onto the nanopattern. Arrows show the direction of the planar/topographical borders.

organization changes lamin organization and, via FISH of centromere pairs, relative positions of the chromosomes in the interphase nucleus (Dalby, Riehle, Sutherland, et al 2004c; Dalby, Biggs, et al 2006; Dalby 2005).

Further to this, using human whole genome microarrays and plotting the positions along the chromosome arms of

topographically induced gene changes, it has been proposed that the changes in nucleus morphology caused by culture on low-adhesion topographies, leads to spatial changes in genome regulations along the chromosomes. We proposed that rather like a net, opening or closing areas of the DNA via changing morphology alters the ability of the transcription factors to bind the genes (Dalby, Gadegaard, Herzyk, et al 2007; Dalby et al in press). Whilst speculative, these ideas agree with Forgacs theories of cellular percolation and suggest that the extracellular matrix, through fibrillar proteins, the cell, via integrins and the cytoskeleton, the nucleus through the nucleoskeleton and the DNA via the long interphase chromosomes may be in mechanical continuum.

However, as also mentioned in the section on mechanotransduction, indirect mechanisms must not be forgotten and are critically important. A recent microarray study on two low adhesion materials, columns produced by colloidal lithography and EBL pits showed a number of important canonical pathways to be down-regulated by the topographies. These included transforming growth factor, cytokine pathways, G-protein coupled signaling, calcium signaling, integrin signaling, endothelial growth factor, insulin-like growth factor and cyclic AMP signaling amongst others (Dalby, Gadegaard, Herzyk, et al 2007). All of these pathways being reduced will change (decrease) cellular activity such as growth, proliferation and differentiation.

A further array study again demonstrated that culture on nanopits lead to broad gene down-regulation (Dalby, Gadegaard, Wilkinson 2007). Included in the few significant gene up-regulations, and confirmed by clathrin staining, were changes in endocytotic pathways. This shows that the cells were trying to internalize pits as well as the aforementioned nanocolumns and provides further evidence for nanoimprinting.

Summary

It has been shown that nanoscale topography can both increase and reduce adhesion of a broad number of cell types. It has been suggested, in agreement with traditional views, that indirect mechanotransductive pathways play a key role in changes in cell response to low adhesion. Furthermore, preliminary evidence has been presented that more direct mechanisms may be important. It could be speculated that low-adhesion topographies firstly act by reducing adhesion, which impacts not only of integrin related signaling, but also on nanoimprinting through reduction in sites for adhesion and cytoskeletal anchorage. It could be further speculated that low-adhesion topographies

reduce tension applied to the cell from a well-organized extracellular matrix via the cytoskeleton to the nucleus effectively shutting the cells down to transcription. However, there is a great deal of work that needs to be done yet in order to prove or disprove these theories of cellular response to nanotopographies.

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