Effect of Six-Sided Pillars on Epithelial Cell Behaviour

by

Mandana Nematollahi

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ABSTRACT

A desirable attribute of implants penetrating epithelium is the inhibition of inward migration of epithelial cells. This inward epithelial migration can be inhibited by the ingrowth of connective tissue on grooved substrata. Very few studies have focused on the direct effect of geometrically more complex topographies on epithelial migration.

We examined the migration, morphology, cytoskeletal organization and proliferation of epithelial cells cultured on a novel complex topography comprising square floors surrounded by six-sided pillars and compared these properties to those of cells cultured on smooth control surfaces. Relative to smooth surface, cells had a reduced velocity but a higher persistence in their direction of migration on the pillar substrata. Vinculin staining demonstrated that cells formed adhesions on pillar tops, in gaps and on the walls. Attachments on pillar tops were mature plaques while the adhesions in gaps and on walls were significantly smaller. Overall there were more mature adhesions on pillars compared to smooth surfaces, which may account for the reduced speed of migration as well as the limited distance of migration on this substratum.

At 3 hours, actin stress fibers readily formed on pillar tops, gaps and walls, however, by 6 hours the stress fibers were predominantly found on pillar tops.

At 6 hours, the positioning of actin stress fibers on pillar tops differed significantly ($p \leq 0.001$) from the positioning of microtubules (MTs) which had a tendency to form in the gaps of the six-sided pillars; very few microtubules were found on pillar tops.

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LIST OF ABBREVIATIONS

ADP   adenosine diphosphate
ATP   adenosine triphosphate
CS buffer   citrate saline
ECM   extracellular matrix
FAK   focal adhesion kinase
G-actin   globular actin
GFP   green fluorescent protein
GTP   guanidine triphosphate
HA   hydroxy apatite
IRM   interference reflection microscopy
IF   intermediate filaments
MTs   microtubules
MEM   minimal essential medium
MF   microfilaments
PBS   phosphate buffered saline
SEM   scanning electron microscopy
SLA   sand blasted grit and acid etched
TEM   transmission electron microscope
VASP   vasodilator-stimulated phosphoprotein

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This thesis and the manuscript are the principal work of the candidate, Mandana Nematollahi. However, the thesis supervisor and senior author of the manuscript, Dr. D.M. Brunette , and postdoctoral fellow, Dr. D.W. Hamilton, offered both editorial comments and contributed advice and suggestions with the setup of the experiments throughout this thesis. The candidate and senior author, as well as the thesis supervisor agree that the contributions of the respective parties are as stated above.

CHAPTER 1

INTRODUCTION

I. Overview of tissue engineering

Tissue engineering is an emerging field of biomedicine that incorporates the advancements of cellular and molecular biology with biomaterial, chemical and mechanical engineering to provide a viable alternative to replace, restore and augment deficient tissues within the body. Three key issues that need to be addressed in a tissue engineered construct are cells, scaffolds including biomaterials, and the integration of the device with the surrounding cells and tissues [22, 34 and 35].

Currently there are three strategies that are employed in engineering tissues to ensure the proper placement and ingrowths of cells, namely conductive approach, inductive approach and cell transplantation. Conductive approaches employ the biomaterial in a passive manner to allow the growth and regeneration of existing tissues. An example is the use of barrier membranes in guided tissue regeneration where exclusion of the gingival epithelium and connective tissue from reconstruction sites occurs while other desired host cells are allowed to repopulate the area. The second major approach involves activating cells in close proximity to the area of interest with specific biological signals such as BMPs. One limitation with this approach is that the inductive factors for a particular tissue may not be known. The third approach is to directly transplant the cells

grown on biomaterials in the laboratory. This approach truly demonstrates the multidisciplinary approach of tissue engineering where clinicians, bioengineers and cell biologists all collaborate together [30]. One of the challenges facing tissue engineering is that once the cells are guided to certain positions, they need to be maintained in those spots as the biomaterial and the cells age together. Therefore, employment of biomaterials that are biocompatible and capable of confining the cells in their optimal positions within the construct would be of great value to the survival of the tissue-device complex.

II. Literature Review

1. Dental implants

The predominant consideration in endosseous implant dentistry has focused on the bone to implant interface, since implant anchorage requires the implant to directly contact the bone tissue. However, as implants extend above the bone into the soft tissue, it is necessary to ensure the proper attachment of soft connective tissue and epithelium to the implant interface.

Biological width is a physiological complex that represents the dimension of the tissues surrounding teeth and dental implants. It is composed of sulcular depth, junctional epithelium, and connective tissue attachment. Electron and light microscopy reveal that epithelial structures similar to those attaching epithelium to teeth are found around dental

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implants. The histologic dimensions of the epithelium and connective tissue comprising biological width around dental implants are similar to the same tissues around teeth. Thus the implantogingival tissues have a barrier function similar to that of dentogingival tissues and the integration of the implant necessitates the integration of all three tissues [17, 40 and 41].

2. Modes of failure of dental implants

Implant failures are classified as early and late failures after osseointegration. Most common factors contributing to the early failures of osseointegrated implants are poor bone quality and quantity, systemic conditions, parafunctional habits such as bruxism, heavy smoking, inadequate surgical technique, inappropriate choice of prosthesis and improper implant designs. There are other unidentified causes for early implant failures, which make the field even more challenging. Several causes of late implant failures reflect early problems, such as excessive loading of the implants and bruxism which will lead to the micro motion of the inserted implant and hence lead to the formation of fibrous tissue at the implant interface as opposed to the desired osseointegration [22]. Poor oral hygiene which leads to peri-implantitis is another cause of the implant failures. Generally, the success of a dental implant is related to the proper integration of the surrounding bone, junctional epithelium and connective tissues with the artificial device. Migration, proliferation and differentiation of osteoblasts onto the dental implant surface will result in the process best known as osseointegration, which is considered critical for the success of dental implants. Failure to achieve and maintain the proper integration with

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bone will result in the loss of the device. In order to prevent bacterial penetration that jeopardizes the initial healing process and long term survival of the dental implants, the formation of an early and long lasting proper barrier that is capable of biologically protecting the implant is mandatory [46]. Junctional epithelium that directly contacts the surface of the implant needs to form an adequate seal with the implant surface. However, due to the inherent capability of epithelial tissue to proliferate and migrate on surfaces, the epithelium at the border of the incision crosses over the fibrin clot bridge that rapidly forms after abutment installation. Upon reaching the surface of the implanted component, it moves in a corono-apical direction giving rise to a junctional epithelium about 2 mm long. The sulcular and junctional epithelium are phenotypically different. These differences indicate that the epithelial tissue is subjected to morphological and functional changes while migrating and proliferating apically [40, 41]. The role of the connective tissue in preventing the downward migration of epithelium has been clearly demonstrated in animal models [13-15].

Failure to prevent this apical migration and proliferation results in different failure modes such as marsupialization, permigration, avulsion and inadequate seal that leads to bacterial infection [46].

The healing of connective tissue on the implant surface involves formation and adhesion of fibrin clot to the implant surface and adsorption of extra-cellular matrix (ECM) proteins and subsequent connective tissue cells to the implant surface. After maturation, the connective tissue portion located between the epithelium and the marginal bone has

been found poor in cells and in vascular content but rich in collagen fibers [40, 41]. Improper attachment of connective tissue to the dental implant interface results in capsule formation on the implant surface with collagen fibers oriented parallel to the implant surface. This capsule formation designates the failure of the implant to integrate with the soft tissue, local blood supply is compromised and a persistent fluid space between the tissue and the implant is produced which precludes stable implant fixation [32]. To ensure the proper attachment of the aforementioned tissues to the implant surface, various macroscopic and microscopic (topographic) designs have been devised on implant surfaces.

3. Macroscopic implant surface designs

Implants have various shapes and designs; parallel, tapered, conical, hollow, solid, threaded and non threaded. Some companies have introduced other features such as vents, grooves and indentations to replace threads [20]. These are known as the macroscopic features of implant surface.

Achievement of better bone anchorage because of the increased surface area due to modifications in the implant surface design has been the focus of biomaterial engineers. In vivo studies have shown that screw shaped implants show better bone formation compared to T shaped and cylinder shaped implants [22].

4. Microscopic implant designs (surface topography)

A large number of surface treatments methods are available to alter surface topography of implants, including, machining, particle blasting, acid etching, combination of blasting and acid etching resulting in SLA surfaces.

Ti plasma spraying and hydroxy apatite (HA) plasma spraying are other ways to produce surface topography, however they will also introduce chemical changes to the surface topography. These chemical changes together with the physical features of surface topography elicit responses in cell behavior.

The topography of a surface is defined in terms of texture (form and waviness) and roughness of the surface [40, 41]. Cellular interactions are not only defined by macroscopic features of the implant, but also by microscopic features of an implant surface (surface topography). The microscopic features of implants have prominent effects on cellular interactions. Some of the effects of topography on different aspects of cell behavior will be discussed here:

A) Cell selection

Due to cellular preferences for various topographic features such as roughness, these topographic characteristics may be varied to promote attachment of certain cell types in

different regions of the implant surface. For example, it is known that fibroblasts shun rough surfaces a phenomenon that was termed rugophobia by Rich and Harris in 1981 and prefer smooth topographies while macrophages prefer rougher topographies (rugophilia) [10].

B) Cell Adhesion

The majority of mammalian cell types are anchorage dependant; they need to adhere to a surface in order to migrate, proliferate, differentiate and express certain genes [4]. In order to accomplish all the aforementioned tasks, cells need to selectively adhere to the extracellular matrix (ECM) through receptors on the cell membrane. Four classes of receptors namely cadherins, immunoglobulins, selectins and integrins have been identified. It is through active engagement of a selective number of membrane receptors that cells convey information about their surrounding microenvironment intracellularly via activation of a series of signaling cascades which determine the fate of the cell [4]. Preference of cells for various surface features differs among different cell types [10]. It has been postulated that proper manipulation of substratum topographical characteristics will increase the success rate of implants through selective cell adhesion and optimum tissue device interactions [27].

C) Topographic or Contact guidance

Contact guidance or topographic guidance refers to the ability of cells to orient themselves, their cytoskeleton and their direction of migration according to the features

of the substratum. This phenomenon was first discovered by Harrison in 1914 and was extensively studied by Weiss in 1951 who then coined the term as contact guidance [10]. Contact guidance has been shown among various cell types such as macrophages, neutrophils [20], epitheliocytes [31,39,44 and 45] , fibroblasts [4], astrocytes [19,43] and various structures such as glass, silica, polymers, polyvinyl chloride and epoxy and on a variety of surface features such as grooves, holes/ pits, steps, fibers, tunnels/tubes, discontinuous surfaces and pillars [25].

Several explanations for the possible underlying mechanisms of contact guidance have been proposed. The earlier suggestion postulated that the inflexible microfilaments or stress fibers act as a reference standard for detecting the curvature of the substratum [21]. Ohara and Buck [38] suggested that inflexible linear focal contacts govern the alignment of cells on grooves with narrow pitches. Brunette [8] later showed that focal contacts were capable of bending around the ridges and therefore the alignment of cells is not a result of rigidity of focal contacts. He suggested that cell alignment is not an all or nothing affair, rather surface topography alters the possibility of successful attachments and therefore the cells orient themselves based on where the successful attachments form. Oakley *et al.* observed that microtubules were the first element to align and therefore suggested that microtubules are the primary cause of cell alignment on groove substrata [37]. In a study in 2005, Hamilton *et al.* studied cell alignment on discontinued edge surfaces and proposed that the stability of focal adhesions on the walls of the DES is the cause of cytoskeletal and cellular alignment and cell migration through the gaps of the structures [26].

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5- Cell cytoskeleton

A) Actin

The Actin cytoskeleton plays a pivotal role in a range of functions such as motility, cell shape maintenance, cytokinesis and intracellular transport processes in animal cells.

In non muscle cells actin is distributed in the cytoplasm and concentrated on the cell cortex. The function of the cell cortex is to resist deformation caused by extracellular forces and to maintain plasma membrane integrity [37]. However, the cell cortex is deformable enough to allow for the formation of actin associated cellular processes such as filopodia and lamellipodia.

Actin microfilaments are 7 nm thick filaments. Actin in this form is also known as Filamentous-actin (F-actin). Actin monomers are dumbbell shaped globular actin also termed Globular-actin (G-actin). Each monomer is associated with one molecule of ATP or ADP and each subunit has an intrinsic polarity. According to Bremer and Aebi [5], because of the intrinsic polarity of the monomers, upon their binding together the resulted filament also has an intrinsic polarity with ends termed as minus and plus ends.

Motile cells must be coordinated in actin polymerization and de-polymerization in order to maintain their polarity and three dimensional architectures. In vivo, the organization of actin is accomplished through a series of complex interactions between actin and series of actin binding proteins. These binding proteins can be categorized under binding proteins

that interact with G-actin or F-actin. The proteins that interact with F-actin have subtypes such as, proteins that sever filament, cap filament and proteins that connect actin filaments with other actin filaments and other proteins of cellular components.

Actin filaments are organized into special types of structures within a cell that serve specialized purposes. Filopodia are finger like structures 0.2-0.5 µm in diameter and 5-35 µm in length consisting of parallel actin filaments. Lamellipodia are sheet like structures with variable breadth ranging from 0.1-0.5 µm which contain cross linked actin meshwork. Both filopodia and lamellipodia are found at the leading edge of the cell and play pivotal role in cell migration. Stress fibers are the third subgroup of intracellular actin structures. They are long bundles of actin filament that traverse the width of the cell and are linked to the extracellular matrix through focal adhesions. They are associated with myosin I and II proteins and form the main contractile apparatus of the cell. Generation of force through this contractile apparatus results in the growth and maintenance of focal adhesions [37].

B) Microtubules

Microtubules are polymers of α-tubulin and β-tubulin monomers. The α-β tubulin monomers share 36-42 % of their amino acid sequence. The α-β dimer binds 2 GTP molecules, one per monomer. The α-β tubulin dimers are 4-5 nm in diameter and 8 nm long. The α-β assembly is such that it gives an intrinsic polarity to the protofilament and microtubules. The α subunit faces the plus end and the β subunit faces the minus end of

the protofilament. The assembled microtubule filaments are 24 nm in diameter and vary in length.

Microtubules are involved in a series of functional roles in a cell such as cell shape maintenance, mitosis and intracellular transport.

A subset of microtubules named tyrosinated microtubules are involved in maintaining cellular polarity through stabilizing cell lamellipodium [24]. Other suggested roles for microtubules are maintaining polarity of the cell through relaxing and dissociating selective focal adhesions in cell rear and aiding cellular detachment in selective areas that leads to cellular translocation [4,24].

C) Specialized adhesion sites

C.1. Adhesion sites based on appearance in Interference Light Microscopy (IRM)

i. ECM contacts

Extracellular matrix contacts appear light grey or white areas in IRM indicating distances of 100-140 nm of separation from the substratum. They are typically located under the cell centre. Electron microscopy showed that ECM contacts are composed of actin filaments and strands of extracellular matrix [1, 29].

ii. Close contacts

They appear as broad grey areas in IRM and are separated 30 nm from the substratum. They are distributed under the peripheral regions of the leading lamellae in spreading cells. Close contacts may be distributed within focal contacts under the leading lamellae [1, 29].

iii-Focal contacts

They appear as black areas in IRM and are separated 10-15 nm from the substratum. They are 2-10 μm long and vary in width between 0.25-0.5 μm and 0.1-2 μm. They are located under the peripheral sites of the leading lamellae and near edges of non-spreading regions of the cell margin in moving and stationary cells [1, 29].

C.2. Cell adhesion sites based on molecular structure

i- Focal adhesions

Focal adhesions are sites at which extracellular and intracellular forces are applied. Their interactions with intra and extra cellular components are mediated through the presence of some anchoring and adaptor proteins. The cytoplasmic domains of the beta subunit of integrins play a major role in the intracellular and extracellular interactions and establishment of connections [4].

Focal adhesions are dynamic structures. According to the age of the formed adhesion and components of the adhesion complex they are classified as focal complexes and focal adhesions. The following description of adhesion dynamics was observed in pig aortic endothelial cells.

The earliest adhesions are called early focal complexes; 0.5-1 μm^2 in size consisting of $\alpha v \beta 3$ integrin and phosphotyrosinated proteins, closely followed by paxillin and talin. At later times vinculin and α-actinin along with focal adhesion kinase (FAK) and vasodilator-stimulated phosphoprotein (VASP) are incorporated into the developing focal complex, however, tensin and zyxin are absent from the focal complexes regardless of their age [4,49].

Assembly, growth and maturation of focal adhesions require induction of mechanical force. The mechanical force changes the size and shape of focal complexes and the molecular composition of these adhesion sites through incorporation of additional proteins into these adhesions, thus maturation of the focal complex into a focal adhesion will result in the incorporation of zyxin and tensin. Another difference between focal complex and focal adhesions is that each is associated with a different type of actin filament. The focal complexes are associated with rapidly branching actin filaments in the lamellipodium while the mature focal adhesions which are 2-5 μm in size are associated with the straight bundles of actin filaments known as stress fibers that may contain actin associated proteins such as myosin II. Together actin and myosin form the contractile machinery of the cell and through activation of this system tension is generated within the cell that is later transmitted to the ECM through integrins in the focal adhesions [4, 49].

ii- Fibrillar adhesions

Another subtype of adhesions is termed fibrillar adhesions. Time lapse observations of cells with GFP-tensin have shown that there is a continuous flow of tensin from focal adhesions to fibrillar adhesions and hence concluded that the latter are derived from focal adhesions. Fibrillar adhesions differ in their characteristic morphology and composition from focal adhesions. They are 1-10 μm in size; appearing as elongated fibrils or array of dots under the more central areas of the cell. These adhesions are associated with fibronectin fibrils. Fibrillar adhesions may be involved in ECM reorganization. Their primary integrin receptor is $\alpha 5 \beta 1$ integrin which is primarily bound to fibronectin and unlike focal adhesions that are comprised of paxillin and vinculin, main component of fibrillar adhesions is tensin with little or no phosphotyrosine [4].

6. In vitro and in vivo studies on microfabricated surfaces

A. Single steps

Single steps or cliffs are the simplest structures that have been used. Wojciak-Stothard *et al.* showed that a variety of cells responded to steps. They found that macrophage like cells respond to steps as small as 30 nm while endothelia react to steps as high as 100 nm in height [48].

B. Pits and holes

Dow *et al.* used pit-type structures to trap macrophages [25]. In theory this type of structure could control the positioning of macrophages around implant surfaces. Macrophages play a pivotal role in evoking immunological reactions to the implanted biomaterial and therefore, the ability to control their function and position on the implant surface provides an interesting opportunity to control these reactions.

C. Grooves

Effects of grooved surfaces have been extensively studied on different cell types. In 1983 [6], Brunette showed that epithelial cells on titanium coated vertical walled and v shaped 3-60 um deep grooved substrata were markedly oriented in the direction of the grooves compared to that of smooth controls. The orientation index was highest for grooves with smallest repeat spacing. SEM and TEM observations showed that cell processes were well capable of bending around and closely adapting to groove edges.

Another study by Brunette in 1986 [7], showed that fibroblasts were contact guided by major grooved substrata. Additional minor grooves that were present as the secondary features on these substrata were also capable of guiding the cells in the absence of any other influencing feature. However, when both grooves were present, fibroblasts preferentially oriented themselves to the major grooves. Brunette also showed that epithelial and fibroblasts were aligned to grooved substrata with depths as shallow as 0.5

um, and grooves with lower feature spacing were more efficient in aligning cells [9]. Hong *et al.* [28], studied the effects of grooves and different adhesive substrata on epithelial cell shape, proliferation and morphology. Neutral proteinase and plasminogen activator secretion were found to correlate with cell shape, rounder cells secreting greater amounts of proteinase compared to the more spread ones.

In an *in vivo* study, Chehroudi *et al.* showed that on percutaneous implants with V shaped grooves, epithelial cells were impeded in their migration up to 10 days, however, on the smooth portion of the implant, epithelial cells migrated apically and reached the base of the implant. Chehroudi *et al.* showed that in vitro epithelial cells were markedly oriented along the axis of titanium coated 10 um deep grooves. More epithelial cells were attached to the grooved titanium surfaces than to adjacent smooth controls. In percutaneously placed implants, E cells were found to be tightly adhered to the implant surface through hemidesmosomes. Histomorphometric measurements showed that there was a shorter epithelial attachment and longer connective tissue attachment in the grooved portion compared to the smooth portion after 7-10 days of implantation. This study showed that horizontally placed grooves are capable of impeding epithelial apical migration compared to smooth controls [11-13].

Chehroudi *et al.* examined the effects of vertical and horizontal grooves with different depths and spacing on epithelial cell behavior in vivo. They showed that vertical grooves accelerate the migration of epithelium compared to that of horizontal grooves.

However the mechanism of epithelial inhibition differed between deep and shallow horizontal grooves. On deeper grooves it was indirectly caused by the obliquely inserted fibroblasts while on shallower grooves it was the direct result of contact guidance since no evidence of obliquely inserted fibroblasts was seen [14].

Cell shape plays a pivotal role in different aspects of cell behavior such as proliferation, differentiation and gene expression [3, 24]. Chou *et al.* showed that cultured gingival fibroblasts on V shaped grooves were significantly elongated and oriented along the grooves and cell height was 1.5 fold greater on grooved substrata compared to that of smooth controls. They showed that the amounts of secreted fibronectin on the grooved substrata were increased 2 fold for all times examined and the stability of fibronectin mRNA was altered compared to that of smooth surfaces. Also the presence of grooved substrata caused a two fold increase in the amount of assembled fibronectin in the extra cellular matrix compared to that of smooth controls [16].

Oakley et *al.* examined the effect of grooved substrata on singles, pairs and clusters of porcine epithelial cells. Cell contacts increased cell spreading, and surprisingly cell contact increased alignment on grooved substrata. Actin and microtubules were initially aligned along the walls and ridge groove edges. Clusters of cells on grooved substrata showed great variability in their cytoskeletal arrangements compared to that of single cells on grooves, indicative that local topographical effects can be overridden by cell-cell contacts [36].

D. Rough surfaces

Cochran *et al.* [18] studied attachment and growth of gingival fibroblasts, periodontal ligament fibroblasts and an epithelial cell line to smooth and rough titanium surfaces. Both fibroblast cell types had more cells attached to smooth and smooth titanium controls than any of the rough titanium surfaces. Epithelial cells didn't attach well to any surface, however, after a lag period they only proliferated on the smooth surfaces and not on any of the rough titanium surfaces. Baharloo *et al.*[2], further showed that rough titanium surfaces decrease spreading of epithelial cells up to 28 days with a more pronounced effect on SLA surface. Cell attachment strength was defined by an increase in number and size of focal adhesions. Cells on smooth surface showed an increased number and area, therefore a stronger adhesion compared to rougher surfaces. TEM membrane proximity measurements showed that cells on the smooth surface had less distance with the substratum and the distance increased as the surface roughness increased. Kim et al. showed that connective tissue attachment was significantly higher and fibrous capsulation was thinner on rougher topographies compared to the polished ones, concluding that rougher implant surfaces are associated with more stable connective tissue attachments [2].

E. Pillars

Lim *et al.* [33] showed that cultured retinal pigment epithelial cells on fibronectin coated polydimethylsiloxane (PDMS) circular pillars presented more prominent vinculin and stress fiber formation on smooth controls compared to the pillars. Significant inhibition of cell cycle progression was observed on PDMS pillars compared to the pillars. IL-6 mRNA and secreted proteins induced by IL-1β were markedly down regulated on PDMS pillars compared to smooth controls. It was postulated that disruption of early focal contact formation was the plausible explanation for the alterations in cell behavior. Another study by Su *et al.* [42] showed that fibroblasts cultured on square shaped pillars with different heights affected fibroblast morphology dramatically and the fibroblast morphology changed in proportion to height of the substrata features. They concluded that geometries of the microenvironment strongly affect cellular morphology and migration. A study on polystyrene micron sized pillars by Frey *et al.* showed that fibroblasts had more branched shapes on pillars compared to smooth controls. The cells had an increased linear speed and a decreased directional stability on pillars compared to smooth controls. Using FAK -/- cells, they showed that FAK is essential for cell migration on pillar structures. Focal adhesions showed a lower turnover and a higher stability on pillars compared to smooth controls [23].

F. Discontinuous substratum

A study by Hamilton et al. on discontinuous edge surfaces (DES), comprising of squares of various width and depths with gaps in the corners, showed that the existing gap between the square edges act as guiding cues for fibroblasts and epithelial cells , causing them to align and migrate diagonally through the squares [26].

III. Aims and hypotheses of the thesis

Extensive work on various cell types and their responses to grooves of different shapes and dimensions have been done [6-16]; however very few studies have investigated the effects of pillar substratum on cell behavior [19,32,42 and 43]. In my study, I used a novel six sided pillar substrata to investigate the effects of complex pillar geometry on different aspects of cell behavior. According to the study by Frey *et al.* on fibroblast behavior on pillar substrata [23], I hypothesized that cells will have altered speed of migration, directional persistence and distance of translocation on pillar substratum compared to smooth.

Due to the capability of discontinued edge surfaces to guide cells [26], I also hypothesized that presence of gaps on the pillar structure will guide the cells through the gaps. Another hypothesis made, was that increased surface area on pillar substratum compared to the smooth will result in increased number of adhesion sites in cells causing relative immobility of cells on the pillar surface compared to smooth.

To test the aforementioned hypotheses, the aims of my study include the following:

1- To investigate direct effect of novel complex pillar geometries on epithelial cell migration and understand the underlying mechanisms for any difference in the migration of epithelial cells time lapse movies on pillar substrata and smooth control surfaces will be made and using a macro. Quantitative measurements on migration variables such as speed of migration, time on the run, number of stops, persistence, direction of migration and finally translocation distance will be measured.

2- To evaluate formation of focal adhesions on six sided pillars and smooth surfaces by staining for vinculin; a marker of these adhesions. This idea was prompted from a study by Hamilton *et al.* on discontinuous edge surfaces (DES) [26]. They showed that focal adhesions are determinants of cellular orientation and migration. Formation and maturation of focal adhesions on the corners of the square boxes eventually guided cells through the gaps of the DES structures. Since cellular translocation and directional migration are results of equilibrium between the formation of new focal adhesions at the cell leading edge and dissociation of selective focal adhesions at cell rear. This is to test the hypothesis that an increase in the surface area of the substratum will increase the number and size of focal adhesions compared to smooth controls and possibly result in relative immobility of the cell.

3- To observe the development of actin fibers on pillar and smooth surfaces with time. Since actin cytoskeleton together with binding proteins from the myosin

family is considered as cell's machinery apparatus, it plays a pivotal role in cellular translocation.

4- To observe microtubular changes in cells and their relationship to the maintenance and growth of focal adhesions. Maximum speed of migration of cells on a surface is result of maintaining the ratio between cell adhesiveness to the substratum and contractility below 1. Any increase in cell adhesiveness or drop in cell contractility will result in the immobility of the cell on a surface.

Microtubules are involved in selective dissociation of focal adhesions at the rear of the cell which eventually result in cell detachment and consequently cellular translocation. Therefore it would be interesting to observe microtubule dynamics on the pillared and smooth surfaces.

Since microtubule and actin dynamics are closely related, changes in one may result in alterations in the arrangement of the other therefore a comparison between the arrangements relative to each other will be made.

5- To understand the effects of pillar substrata in nuclear changes, quantitative data on nuclear area, cell proliferation and qualitative as well as quantitative changes in cellular morphology will be analyzed.

A study by Lim et al. [33] showed that early disruption of focal adhesions will result in changes in cell growth and cell cycle and IL production.

Cellular adhesion to a substratum activates intracellular signaling cascades that will result in changes in cellular gene expression, protein synthesis, cell growth and proliferation. Mechanically induced changes in nuclear shape can affect the activation of transcriptional factors within the nucleus and nuclear size has been correlated with cell growth [49].

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CHAPTER 2

EFFECT OF SIX-SIDED PILLARS ON EPITHELIAL CELL BEHAVIOR[11]

Introduction:

The long term integrity and success of any percutaneous device relies on the proper positioning and maintenance of tissues on the device tissue interface [27].

A desirable attribute of implants that penetrate the epithelium is the ability to impede epithelial inward migration on the implant-tissue interface because such epithelial migratory behavior on the interface of the implant-tissue complex jeopardizes the long term success of the device-tissue complex [6, 7, 27, 29 and 37]. Failure to provide a tight seal between the epithelial tissue and the implant surface promotes the development of bacterial infection leading to the failure of the implant.

Two plausible approaches to inhibit downward epithelial migration are to employ topographies that directly inhibit epithelial migration and/or encourage the firm attachment of connective tissue to the device surface in a manner analogous to the way sharpey's fibers prevent epithelial inward migration [9-13 and 19].

The recognition that substratum topography affects diverse aspects of cellular behavior such as cell orientation, cytoskeletal organization and direction of migration (a phenomenon named topographic or contact guidance) has lead to fabrication of implants with specific topographic features in the hope of improving function. Microfabrication techniques enable surface topography to be purposely and precisely varied and examined for its effects on diverse aspects of cellular response.

Several studies have employed microfabricated grooves of micrometer scale to investigate the response of epithelial and connective tissue to varying substratum features. For example, Chehroudi *et al.* studied the effects of vertical and horizontal grooves with a depth of 3 μm, 10 μm and 22 μm and feature spacing of 30 μm on connective tissue and epithelial cell migration in vitro and in vivo [9-13]. These studies showed that deeper horizontal grooves inhibit epithelial apical migration at times up to 10 days. However, at later times, the inhibition is largely the result of connective tissue ingrowth rather than the direct effect of topography on epithelial cells.

Arrays of cylindrical pillars and columns have been used to investigate the behavior of astrocytes, fibroblasts and retinal epithelial cells and astroglial cells , however, very little is known of the direct effect of more complex pillar topographies in the micron range on epithelial cell behavior and migration [14, 15 , 21 and 34].

In this study we cultured epithelial cells on a complex topography comprising square shaped floors surrounded by four six-sided pillars with the top surface of the pillar

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making a 135° angle with the wall of the pillars. These pillars provide an increased surface area for focal contact formation compared to that of smooth controls. The surfaces were designed with smooth surfaces immediately adjacent to the pillared topography so that cellular behavior between smooth controls and pillar substrata could be compared directly under identical conditions.

Time lapse observations showed that cells had a reduced velocity but a higher persistence in their direction of migration on the pillar substrata. The majority of epithelial cells covered the pillars, while some migrated through the gaps. The more numerous and more mature adhesions that formed on the pillars are probably responsible for the reduced migration velocity and limited distance of cellular translocation on the pillars compared to smooth controls. Actin and microtubules were predominantly diagonally aligned through the gaps. Cells spread and proliferated significantly less on pillars compared to smooth controls.

Materials and methods:

A. Surface fabrication and preparation of replica surfaces

The microfabrication techniques used in this study were originally developed by Camporese et al. for the fabrication of high-quality photomasks for solar cells [4]. Substrata were produced in the laboratories of the Centre for Microelectronics, Department of Electrical Engineering, at University of British Columbia. The appearances and dimensions of the microfabricated hexagonal pillars are given in figure 1

and table I respectively. Epoxy replicas of six-sided pillars and smooth controls were cast [5].

B. Cell culture

Epithelial cells from the porcine periodontal ligament were isolated and cultured as described by Brunette *et al.* [3]. Cells were cultured on tissue-culture plastic in alpha minimal essential medium (Stem cell Technology, Vancouver, BC, Canada) supplemented with antibiotics (penicillin G,100 mg/ml (Sigma, St. Louis, MO); gentamicin, 50 mg/ml (Sigma); fungizone, 3 mg/ml (Gibco, Grand Island, NY); and 15% fetal bovine serum (Flow, Mclean, VA) at 37° C in a humidified atmosphere of 95% air and 5% CO_2. Cells were removed from the growth surface using trypsin solution [0.25% trypsin (Gibco), 0.1% glucose , citrate saline buffer (pH 7.8)] and seeded onto the substrata at $2x10^4$ cells/ ml to ensure that the epithelial cells do not cluster and form lateral adhesions as it was the aim of the study to investigate the behavior of single cells.

C. Epithelial cell spreading and morphological analysis

To observe spreading and morphology, epithelial cells were grown on pillared substrata for 1, 2, 3, 6 and 24 hours. At the end of each time, cells were rinsed in warm citrate saline (CS) buffer to remove any unattached cells and then fixed in 3.7% formaldehyde for 10 min. The samples were then washed with PBS 5 times and fixed in 0.5 % osmium tetroxide in 0.1 M phosphate buffer (pH=7.2) for 1 hr. Preparation for the SEM analysis

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was performed as previously described [23]. The samples were viewed using a Cambridge 260 Stereo scanning electron microscope at 4 kV accelerating voltage.

D. Measurement of cell area

In each of two separate experiments, fifty cells were selected randomly for measurement at each time point. Using Image J software, SEM images were outlined and the cell projected area was measured.

E. Cytoskeletal observation and quantification

For F-actin staining, the cells were fixed in 4% paraformaldehyde and rinsed three times for 15 min with PBS. The samples were then incubated with TRITC-Phalloidin (Sigma Chemicals) at a dilution of 1:200 in PBS for 1 hour. After incubation, the cells were rinsed three times for 15 min with PBS, and then mounted on a slide in 8% wt./vol. Mowiol (Calbiochem, Mississauga, Ontario) in 0.2 M Tris HCl pH 8.6 with 20% vol./vol. methanol.

For observing microtubules using β-tubulin labeling, all samples were fixed in ice-cold acetone and permeabilized with 0.5% Triton X-100 (Sigma) for 5 minutes. Blocking of nonspecific binding was performed using 1% bovine serum albumin (BSA, Sigma) and the samples were incubated with monoclonal anti- β -tubulin [clone KMX-1, (Chemicon, Temecula, CA)] at a dilution of 1:200 for 90 minutes. This was followed by 3 washes in 1%BSA in PBS, and the samples were then incubated with sheep anti-mouse IgG

conjugated to Texas red (Molecular Probes Inc., Oregon,WA) at a dilution of 1:200 for 1 hour. All antibodies were diluted with PBS containing 0.1% BSA to further prevent any non specific blocking.

For observation of focal contacts using vinculin staining, cells were rinsed in PBS buffer, permeabilized with 0.1 % Triton, and then fixed in 4% paraformaldehyde. Samples were prepared using the same technique as above, using instead mouse anti-human vinculin (Chemicon International) at a dilution of 1:100 , followed by a goat anti-mouse IgG conjugated to Texas Red (Molecular Probes Inc.), at a 1:200 dilution for 1 hr.

In all cases, labeling was visualized on Zeiss Axioscope 2 using a 63x oil objective under rhodamine optics, and images were captured using a Q Imaging Q ICAM MONO 10 bit camera and Northern Eclipse software (Empix, Mississauga, Ont.).

To prepare optical sections of the samples, cells were visualized on Nikon Confocal Microscope C1 using a 60x objective under TRITC and DAPI filters. Images were imported to Photoshop 7.0 for labeling and sizing.

F. Cell proliferation and nuclear shape analysis

For proliferation assays, cells were seeded at an initial concentration of 2.0×10^4 cells/ml and cultured for 1, 3, 5, and 7 days. At each time, cells were removed from the cultured surface with trypsin and counted in an electronic cell counter (Coulter Z1).

For each time, two replicate surfaces were analyzed. Three readings from each replicate were used and experiments were repeated three times. For nuclear shape analysis, cells were seeded at 2.0 x 10^4 cells/ml and stained at 1,2,3,6 and 24 hours. The surfaces were rinsed with PBS buffer and stained with propidium iodide. Cells were imaged with an epifluorescent microscope (Zeiss Axioscope, Germany) equipped with a rhodamine filter. Random fields were selected and images were captured digitally with Northern Eclipse software (Empix, Mississauga,Ont.) and used for nuclear shape analysis with Image J. Randomly selected images were calibrated on image J to convert area from square pixels to square μm. The outline of each nucleus was traced and the projected area of each outlined cell was calculated. Images of nuclei of forty cells from three repeat experiments were analyzed.

G. Fluorescent time-lapse digital videomicrography

The movement of fluorescently labeled cells was documented by time-lapse digital videomicrography. Suspended cells were stained with Cell Tracker Orange (Molecular Probes Inc.), at a concentration of 0.0004% for 30 min, and plated on the pillar substrata at a concentration of 10^5 cells/ml. Cells were incubated for 24 hours to ensure full spreading,. Then individual surfaces were secured with silicon grease on glass slides and sealed in a previously sterilized Pentz chamber (Bachofer, Reulintgen, Germany). Cell growth medium was added through the silicon rubber seal by sterile syringe. The Pentz chamber was placed on a stage incubator (Bachofer) that was set at 37 °C and perfused with 5% CO_2 throughout the experiment. Images were taken automatically at 5 min

intervals over a period of 24 hours with a digital video camera (Q imaging, Retiga 1300) connected to a computer running Northern Eclipse software (Empix Imaging Inc., Mississauga, Canada). For data analysis, 20 cells on each surface were chosen and the centre of each cell was traced over the course of the time lapse observation. The video was digitized (1 frame/3 h real time) and analysis performed on a Macintosh computer using the public domain NIH image. The *xy* coordinates of the cells were determined interactively using NIH image. The time-lapse videos were analyzed using a designated spreadsheet created by Dr Mathis Riehle at the Centre for Cell Engineering (freely available for download at http://www.gla.ac.uk/centres/cellengineering/mathis) and by visual observations.

H. Statistics

Comparison of various parameters between pillar surfaces and smooth controls for morphological changes, cell number and projected nuclear area were determined by the student t-test, with $\alpha=0.05$ as the level of significance. Statistical comparisons of categorical data between pillar and smooth surfaces for cytoskeletal elements were determined by the χ^2 (Chi squared) test. The data were set as 2x2 contingency tables .A contingency table's degrees of freedom (df) can be calculated as (r-1)(c-1) where r is the number of rows and c is the number of columns. For 1x2 contingency tables, df is calculate by (k-1) where k is the number of values or categories on the variable.

The χ^2 test only determines whether two variables are independent. It doesn't provide further detail about the degree of their dependence.

Cramer Phi is an index used for contingency tables larger than 2x2 and can be derived from the following formula: (where N = the total number of observations, and k = the smaller of the number of rows or columns): Cramer's phi = $\sqrt{\chi 2/(N(k-1)}$ [30].

Cramer Phi measures the strength of the dependence. The value of Cramer Phi is between 0-1. For example, a Cramer Phi value of 0.12 indicates a much weaker dependence value between to variables compared to a value of 0.60.

Therefore, to measure the degree of association between different actin and microtubule arrangements at 6 and 24 hours; 1-distribution of actin on pillar tops, gaps and walls, 2- distribution of actin on pillar tops alone, 3-distribution of MTs on pillar tops, gaps and walls 4- distribution of MTs in gaps and on walls alone, Cramer phi was assessed.

Results:

A. Pillar dimensions:

SEM observation of the epoxy replicas demonstrated that they closely replicated the original Si wafers (Figure 1).

The dimensions of the pillars were measured by scanning electron microscopy. Table I summarizes the measurements of the six-sided pillars. The different spatial aspects of pillars are referred to as top surface of the pillar, walls of the pillars, square floors and gaps. The region described as gaps refers to the spaces between adjacent pillars.

B. Time-lapse observations:

Cells on pillars traveled a distance of 51.3±2 μm in 32 hours, while cells on the smooth traveled an average of 1795.7±73μm in 32 hours (table II). On pillars significantly lower number of cells moved at different times compared to that of smooth controls, an indication of tendency of cells on pillars to adhere tightly to the substrata (figure 2 A,B). Average velocity measurements made at 5 min intervals, showed that cells on pillars traveled at a reduced speed compared to smooth (10.8±4μm/hr and 371.5±149μm/hr respectively)(table II). Nevertheless, cells showed a higher persistence in movement on pillars compared to smooth controls (0.31±0.20 μm/ 5min and 0.14±0.1 μm/ 5min respectively)(Figure3,4 and table II).

Cells on smooth surfaces spread extensively and didn't show any preference in direction of their movement. In contrast, cells on the pillar substrata showed a tendency to align within the gaps (Figure 3B). During the course of migration through the gap, the cells extended filopodia to the perimeter of the adjacent pillars, in some instances, cells climbed onto the pillar otherwise they continued to migrate through the gap.

Cells that had settled on the square floor, either climbed onto the pillar top or extended filopodia through gaps. Attachments on the walls were temporary and dissociated when the cell moved to the top of the pillar. Once on the top, cells covered the pillars and the number of the pillars covered by cells, increased.

C. Epithelial cell spreading and morphology on pillars:

One hour after seeding, 24.5±11.3% of cell bodies were found on top of the pillars and 66.7±8.4 % in the bottom of the pillars on the square floor, that is in effect a well formed by 4 adjacent pillars, and 8.8±5.6 % in the cross shaped depression created by 4 neighboring pillars.

At 1 hour, the majority of cells on pillars were spherical (Figure 5A). Cells found on top of the pillars, showed extended filopodia and occasionally lamellipodia on the top surface, on the walls of the pillars and in gaps. Some filopodia reached to the square floor. The gaps guided the extension of filopodia diagonally.

Cells, settled in the square floor showed extended lamellipodia within the square, through gaps and on the walls of the pillar. At 1 hour, majority of cells on smooth surfaces were spherical, with filopodial extensions at the cell periphery.

At 2 hours, the majority of cells had spread to present a prominent lamellipodia. The cells seeded on the floor, extended their filopodia and lamellipodia on the walls of the pillars, some reaching the top surface of the pillar (Figure 5B). At 2 hours, majority of cells on smooth substrata radially spread.

At 3 hours, the cells that had reached the top of the pillar spread to the adjacent pillar, covering two pillars at this time (Figure 5C). Cells on smooth substrata had prominent cell body with lamellipodia extended around the cell periphery.

At 6 hours, the number of covered pillars increased. The majority of cells covered 4 pillars, with some cells covering as many as 6 pillars. Cells showed a variety of morphologies on pillars relative to smooth controls. The two most common cellular morphologies resulting from the covering of 4 pillars were hexagonal and cross shaped (Figure 5D, E). Cells on the smooth structures had completely flattened on the smooth surfaces with a circular morphology.

At 24 hours the cells covered an increased number of pillars, with a few cells covering up to 8 pillars. The same morphology of cells on smooth surfaces at 6 hours resumed to 24 hours.

Interestingly, cells have a tendency to cover the adjacent pillars, not the opposite ones (adjacent pillars are defined as any two pillars that share a corner of the floor). This is due to the closer proximity of two adjacent pillars compared to the opposing pillars.

At the junction of the topography and the adjacent planar surfaces, epithelial cells showed the ability to climb up on or off the top surface of the pillars (Figure 5G).

D. Morphological analysis of epithelial cells on six-sided pillars and smooth controls:

Cells on the pillars were significantly more elliptical than cells on smooth control surfaces at 1, 3, 6 and 24 hours ($p \leq 0.05$). The circularity values decreased on pillars, reaching their lowest value at 24 hours (0.17 ± 0.06), while the values on smooth controls increased with the highest value at 6 hours (0.33 ± 0.14) with a decline at 24 hours.

The cells on pillars were significantly more elongated than the cells on smooth controls ($p < 0.05$) at 2, 3 and 6 hours. Fit ellipse measurements reached their highest value at 24 hours on pillars and smooth (3.14 ± 2.4 and 2.6 ± 1.29 respectively).

Projected cell area measurements showed that cells were significantly more spread on the smooth control surface compared to pillars at all times ($p \leq 0.05$). On both structures, the cell surface area increased with time (figure 10C).

E. Cytoskeletal element configuration on pillars and smooth

Cytoskeletal element configuration was assessed by examining the arrangement and distribution of F-actin, microtubules and vinculin, a component of focal adhesions.

i-F-actin configuration:

At 1 hour, the majority of cells demonstrated distinct positive phalloidin staining for F-actin on both pillar and smooth surfaces (Figure 6 A, E).

At 2 hours, the predominant actin configuration on the smooth controls was the presence of actin condensations and cortical actin at cell peripheries, while on the pillar only 40 % of cells showed cortical actin and actin condensations that were closely associated with the edges of pillar tops (Figure 6B, F). Very few cells had aligned actin filaments in the gaps.

At 3 hours, both cortical actin and diagonally aligned stress fibers were present (Figure 6 C, G). In a small subset of cells, the simultaneous presence of circumferential actin fibers and fine actin filaments were observed on pillar and smooth substrata. As indicated by cell height measurements at 3 hours, aligned filaments were formed at 2-3 μm below the cell's dorsal surface, while actin filaments located in the plane above the aligned zone, displayed a rather unorganized meshwork (Figure 7 A-F).

At 6 hours, numerous prominent stress fiber bundles which traversed the width of the cell were the distinctive actin configuration in cells on pillars and smooth controls.

At 24 hours, numerous stress fibers throughout the cell body were observed on both substrata. On pillars, however, actin stress fibers were diagonally aligned through the

gaps, on the floors and pillar tops and alignment was evident throughout the cell body. The filaments on smooth surfaces had a random orientation with no noticeable alignment (Figure 6 D, H).

ii. Microtubule configuration:

At 1 hour, microtubules appeared as a randomly oriented meshwork on both pillar and smooth substrata, although significantly more cells lacked any evident MT network on pillars compared to smooth ($p < 0.05$).

On pillars, approximately 40% of the cells showed a simultaneous presence of MTs that diagonally aligned themselves to the gaps and MTs that were arrayed radially on top of the pillars. On the smooth substrata however, the microtubules were radially oriented throughout the cell.

At 2 hours, more cells simultaneously showed radial array of MTs on pillar tops and aligned MTs to the gaps. On smooth surfaces the same pattern as that observed at the first hour was seen.

At 3 hours, on pillars, the same arrangement as 2 hours was seen, however, the number of cells with simultaneous aligned MTs in gaps and radial arrayed MTs on pillar tops declined with time. On smooth surfaces, the same MT arrangement from the first hour continued through to 3 hours.

At 6 and 24 hours, the main MT arrangement was the diagonal alignment of MTs to the gaps, and presence of MTs that conformed closely to the walls of the pillars bending around the corners of the pillars (Figure 8 A-D). The number of cells showing aligned MTs in gaps was 56% at 6 hour and 69% at 24 hour (p≤0.025). MTs on the top surface of the pillars presented a radial array on the pillar top (Figure 8D).

On smooth surfaces, the same pattern of MT seen at 1-3 hours, was found at 24 hours, only the individual microtubules became more distinct at the later times compared to the first hour (Figure 8 E-H).

iii.Comparison of actin and MT configuration:

Actin and microtubule arrangements differed, actin stress fibers were straight and present on the tops of pillars while the microtubules were predominantly found in the gaps and bent around the corner of the walls of the pillar. Three categories were defined for actin and MTs distribution at 6 hours and 24 hours; 1)actin/MTs on pillar tops, 2) actin/MTs in gaps, 3) actin/MTs on pillar tops and in gaps. X^2 test showed a significant difference between actin formation on pillar tops and the preference of microtubule formation in gaps at 6 and 24 hours (p≤0.001). To measure the strength of the significance, Cramer phi test was calculated. The correlation between the mentioned actin and MT arrangements was 58% meaning that the reason for the different distribution of these two elements are 58 % due to the inherent differences of actin microfilaments and MTs.

Using confocal microscopy, actin was aligned throughout the cell body on the z axis at 6 hours while there was an apparent segregation of the microtubule alignment at 6 hours on the z axis; with diagonal alignment starting at 2-3 μm below the dorsal surface of the cell.

iv.Vinculin configuration:

At 1 hour, significantly more cells on pillared surfaces lacked any apparent focal contact associated vinculin staining compared to cells on smooth surface (70% vs. 29 %, p<0.0001). On pillars, 21% of cells had punctuate (dot-shaped) vinculin staining on tops and in gaps, indicative of late focal complexes (figure 9A). Nine percent of cells showed the presence of mature focal adhesions at this time. (Focal complexes are morphologically distinguishable from focal adhesions in a cell; they are much smaller and are more peripheral compared to the larger and more central focal adhesions). On smooth controls, punctuate focal contacts appeared at this time with radial arrays and no preferred orientation in the cell periphery.

At 2 hours, some cells showed simultaneous presence of mature focal adhesions on pillar tops and punctuate focal contacts in gaps. A few cells showed diagonally aligned focal adhesions to the gaps of the pillars (Figure 9B). The number of cells with no vinculin staining in adhesive structures decreased with time.

At 3 hours, some cells simultaneously had mature focal adhesions on pillar top and aligned mature adhesions in gaps (Figure 9C). Some cells showed mature adhesions on pillar tops and edges. Few cells showed dot-like focal complexes at the bottom of the

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square boxes. Cells exhibited distinct plaque focal adhesions at the cell periphery at 2-3 hours on smooth substrata.

At 6 and 24 hours, an increased number of cells showed mature focal adhesions on pillar tops and in gaps. Predominant vinculin configuration was diagonally aligned plaque (mature) focal adhesions on pillar tops and in gaps (Figure 9D). On smooth surfaces , in addition to peripheral plaque focal adhesions, central adhesions became evident at 6-24 hours (Figure 9E-H).

An interesting observation is that cells on pillared substrata showed a simultaneous presence of focal complexes and focal adhesions within an individual cell at different areas of the pillars at different times, however the number of cells with more mature focal adhesions relative to focal complexes within cells declined with time. On smooth surfaces, each cell at earlier times showed the presence of focal complexes, and at later times number of cells with focal complexes declined and an increasing percentage of cells showed mature focal adhesions. However, on smooth substrata, at no point did cells show simultaneous presence of focal complexes and focal adhesions within one cell .

F. Nuclear area and cell number:

The projected area of cell nuclei was significantly lower on pillars compared to that of smooth controls at 1, 2, 3 and 24 hours ($p < 0.05$). The mean values for the projected nuclear area, remained almost constant for the first 3 hours, with a marked increase at 6 hours and a sudden decline at 24 hours on both structures (figure 10A).

Cell number increased during the course of 7 days on both pillars and smooth controls, however, cell number increased significantly more on smooth controls compared to that of pillars (p<0.05) (figure 10B).

Discussion:

A desirable attribute of a percutaneous device would be the ability to impede epithelial apical migration in order to increase the success rate of these devices. Microfabrication allows the study of cell behavior on wide range of precisely specified surface features and dimensions.

Various micrometer range grooved substrata have been employed to examine the behavior of connective tissue and the indirect effect of this tissue on impeding epithelial apical migration. However, direct effects of more complex geometries on epithelial migration have not been investigated. This study has employed six-sided pillar topographies to examine whether these geometries are capable of altering epithelial migration. To understand the mechanisms by which pillars affect cell behavior such as migration, cell morphology, cytoskeletal organization, proliferation and nuclear area were examined.

A. Migration:

Epithelial cells moved more slowly on pillar substrata compared to smooth controls. Since the speed of migration is a result of equilibrium amongst four critical steps of cell migration namely, lamellipodium formation, adhesion of the lamellipodium to the substratum, generation of enough traction through cytoskeletal system and detachment of the rear of the cell. Any of these steps may be paramount in determining the rate of migration speed under certain conditions [20, 22, 28 and 32]. In this study, time lapse observations showed that cellular protrusion at the cell front formed regularly and, provided that successful attachments formed, cells then spread. However, after the cell had spread, there was a delay before cell would de-adhere from the surface and proceed with its movement. One possibility for the reduced migration on pillar substrata is that cells adhered to the substrata rather strongly, that enough traction could not be generated to de-adhere the cells from their substratum. Another possibility is that cell attachments at the rear were rather strong imposing a delay in cellular de-adherence from the substratum. In fact, the attachments at the cell rear end seemed to be rather strong, because mature focal adhesions were abundantly located in the cell rear. Palecek *et al.* showed that cells on highly adhesive substrates cells show regular lamellipodia extension and retraction with little cell movement. Highly adhesive substrates impede cell migration by disrupting cell rear release [26].

Sultzman *et al.* found that neutrophils moved more rapidly on substrata with micron array holes with edge to edge spacing of 6 μm to 14 μm compared to smooth controls. They

suggested that cells use the edges of the holes as mechanical foot holds to gain the necessary traction for their movement, therefore enabling an increased speed of migration on these substrata [31]. The cause of the differences between our results and theirs may lie in the differences between cell types, substratum geometry and the feature spacing used in the two studies.

Epithelial cells showed a higher persistence in their direction of migration on pillar substrata compared to smooth controls. Persistence is defined by the frequency of change in cell's direction of movement. Random cell movement results in lower persistence. Hamilton *et al.* showed that discontinued edge surfaces are capable of guiding fibroblasts through gaps at the corner of the boxes. They showed that focal contacts that form at the corner of the boxes eventually mature and align through the gaps, thereby directing the cells, a phenomenon they termed'' gap guidance''[17]. Similarly in this study the presence of gaps between the pillars could serve as an orientation cue and explain the higher persistence of epithelial cells on the pillar substratum. On smooth controls, the absence of any orientation cue resulted in random migration. The movement of cells on pillar tops however followed the pattern of random movement as seen on smooth surfaces, however, if successful attachments were formed within the gaps, then cells would reorient and migrate through the gaps. As suggested by Brunette et al. in 1986, effects of surface topography are not an all or nothing affair. Topography can alter the probability of formation of successful attachments, therefore guiding the cells in their direction of migration based on where these successful attachments form [4, 5].

Despite the higher persistence of movement of cells on pillars, cells showed a reduced distance of translocation on pillars compared to smooth controls. The reduced distance is because cells on pillar substrata spent significantly less time on the run compared to smooth controls.

B. Morphology:

Depending on the location of cells initial contact with the pillar substratum, cells assumed different spreading patterns; resulting in more branched and elongated morphologies on pillars compared to smooth controls. Cells generally spread bi-directionally on two adjacent pillars and gradually covered them with the number of covered pillars increasing with time. Similar results were found by Vasiliev *et al.*, who showed that epitheliocytes spread on metallic grids, gradually covering the openings of the grids. They argued that the mechanical bending of the cell is related to the presence of linear stress fibers within the cell [31]. They argued that the presence of circular peripheral actin bundles and focal adhesions distributed along the cell periphery were responsible for the epitheliocyte spreading pattern observed on their substrata [25, 35, and 36]. In our study epithelial cells showed numerous actin stress fibers throughout the cell, nevertheless, the cells were capable of climbing down from the pillar top onto the square floor. Thus the linear stress fiber bundles didn't impose any absolute restriction in sliding off the pillar tops. As the pillar top surface makes an angle of approximately 135° with the sloping walls, the actin stress fibers need to bend some 135° in order for the cell to climb down the walls of the pillars or alternately be reassembled at the lower level so that individual fibers need not bend.

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C. Cell number and cell projected area:

Cell number was significantly lower on pillared surfaces compared to that of smooth surfaces during the course of 7 days. In a review article, Walker et al. [38] have mentioned that ERK activity is required for cyclin D1 expression and S phase entry in hepatocytes plated on collagen films. Integrin engagement leading to ERK dependent cyclin D1 expression is also needed for S phase entry in fibroblast cells. Taken together, it was concluded that spreading/tension requirements for ERK dependent cyclin D1 expression and subsequent S phase entry were associated with cell spreading. Walker et al. also mentioned that cell spreading induced by the actin organization is required for G1 phase cell cycle progression [38]. Generally anchorage dependent cells need to be well spread to enter the S-phase of their cell cycle; the significantly lower cell projected area on the pillar substrata compared to the smooth surface may be instrumental in observed differences between the surfaces in cell proliferation.

D. Cytoskeletal organization:

MTs and focal contacts (FC) are generally found to be the first elements to align within the gaps at 1 hour post seeding.

Focal complexes form at the front of the cell behind the advancing leading lamellipodium. These transient structures mature into larger central focal adhesions as the cell body advances over them. Focal complexes have been associated with higher motility

while more mature adhesion plaques are characteristics of more well spread stationary cells [1, 8, 20, 22, 23, 26, 39 and 40]. Our data showed the alignment of a few mature focal adhesions within the gaps in the first hour. Hamilton *et al.* showed that focal contacts were the first cytoskeletal elements to align within the gaps of DES, suggesting that the stabilization of the cellular extensions by focal contacts determines cell orientation and directional migration on this substratum [17].

Cells formed adhesions on all surfaces of the pillar substratum. The adhesions on the top surface of the pillar, however, were larger than the adhesions formed within the gaps. Smaller adhesions predominated on the walls and in the gaps of the pillars (figure 7C, D). The presence of the larger plaques on the top surface of the pillar may indicate that the cells exert greater tension on this part of the surface compared to the gaps [8, 20, 22, 23, 39 and 40].

Failure of dissociation of adhesions at cell rear will lead to cell immobilization. It has been shown that the disruption of microtubules leads to activation of Rho which consequently induces enlargement of focal adhesions and formation of stress fibers. It is also believed that microtubules are responsible elements for delivering relaxing factors to the adhesion sites, resulting in their dissociation [2, 16 and 28]. Therefore the relatively limited microtubule formation on the pillar tops may explain the presence of large adhesion plaques on the pillar tops, while the presence of the MTs in the gaps could account for the absence of the large super mature adhesion plaques in this region.

As noted earlier, time-lapse observations showed that lamellipodia and filopodia formed in the gaps. MTs are thought to be important for cell migration as they maintain cell polarity by stabilization of cell's leading edge and dissociate selective focal adhesions at the cell rear to allow for the cellular translocation [2, 16 and 28]. Asymmetrical microtubule arrays maintain cell polarity. Aligned MTs in the gaps would thus be expected to stabilize the lamellipodia of the cells in a preferred direction and guiding cell migration through the gaps. Microtubules showed great flexibility around the walls of the pillars where they can bend around the corners of the walls of the pillar, a phenomenon that is termed "wall hugging" by *Oakley et al.* (submitted) [23]. One possibility is that MT's can suffer a large strain from a small stress compared to microfilaments (MF) and intermediate filaments (IFs). Due to the anisotropic properties of the MTs, it is easier to bend a filament than to exert a shearing force to it. Therefore the MT's align in a shear-free plane [18, 33]. Another possible explanation is that alterations in tubulin concentration in the gaps and around the walls of the substratum may account for the preferential formation of MTs in the gaps compared to the pillar tops [18]. The few microtubules formed on pillar tops showed a random arrangement similar to smooth surfaces as opposed to the diagonal alignment formed in the gaps. Flat surface of the pillar tops lack any orienting feature and microtubules are correspondingly randomly oriented.

The presence of actin condensations at 1 hour at the edges of pillars is consistent with the suggestion of Curtis and Clark on grooves that actin condensations are associated with substratum discontinuities. Actin filaments were the last cytoskeletal component to align within the gaps 2 hour post seeding, consistent with the findings of Oakley *et al.* who

studied fibroblasts on grooves [24]. Actin filaments showed a preference to form on the top surface of the pillars. The presence of actin stress fibers oriented in multiple directions on pillar tops in one cell suggests that cells exert tension in multiple directions at this location.

Conclusion:

In summary, this study indicates that a complex substratum of 10 um deep six-sided pillars with feature spacing of 83.76 um and walls that slope of the pillar tops with a 135° angle, slowed epithelial cell migration substantially. Diverse morphologies were assumed by the epithelial cells on pillar substrata; however, epithelial cells generally covered the pillars with time, reflection of a behavior innate to many epithelial populations in vivo. Although stress fibers formed readily on the pillar substrata, an increased surface area provided by this substratum allowed the formation of an increased number of mature focal adhesions which resulted in an imbalance between the adhesiveness of the cell to the substratum and cell contractility leading to limited cellular translocation on pillar substrata. Despite the limited cell motility, the gaps between the pillars were capable of guiding epithelial cell migration and cytoskeletal distribution therefore resulting in a higher directional persistence in cells on the pillar substrata.

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Figure 1
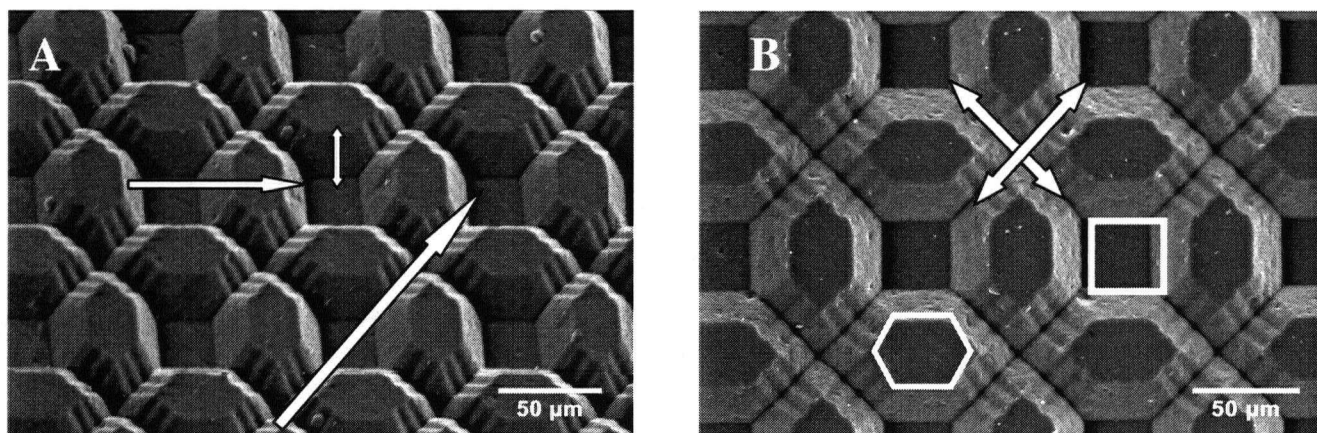
Figure 1. Topography of pillar substrate. SEM image of the pillar structure employed in the study. (A) is an inclined view of the pillars, where the height and substrate feature spacing are indicated by the vertical and horizontal arrows respectively. The diagonal arrow shows the gaps. (B) is the top view of the pillars, and the square box, top surface of the pillar and the depression created by the presence of 4 neighboring pillars are outlined by the white square, hexagon and arrow respectively.

Table I

Height of pillar	Area of pillar top	Area of square well	Distance of adj. pillars	Distance of opposite pillars	Angle of top to the slope of walls
10 Um	130.1 ± 3.9 um^2	114.85 ± 5.7 um^2	84.31 ± 2.9 um	83.76 ± 3.04 um	$132.2° \pm 3.16°$

Table II

	Pillar	Smooth
Distance (μm)	51.3 ± 21	1795.7 ± 727
Distance (a-z)	15.1 ± 12	288 ± 405
Time on run	75.8 ± 25	278.8 ± 6
Persistence	0.31 ± 0.2	0.14 ± 0.11
N of stops	11.5 ± 4	3.0 ± 1
Average run (μm)	6.9 ± 1	111.5 ± 51
Average velocity (μm/hr)	10.8 ± 4	371.5 ± 150

Table I. Description of the dimensions of pillar features.
Table II. Description of measured variables from the analyzed time-lapse videos. A designated spreadsheet created by Dr Mathis Riehle at the Centre for Cell Engineering (freely available for download at http://www.gla.ac.uk/centres/cellengineering/mathis).
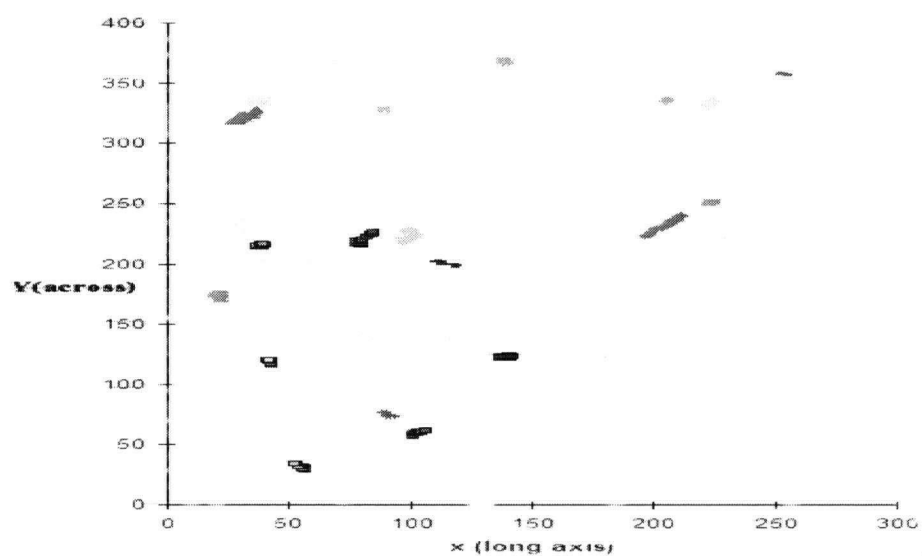
Figure 2

A
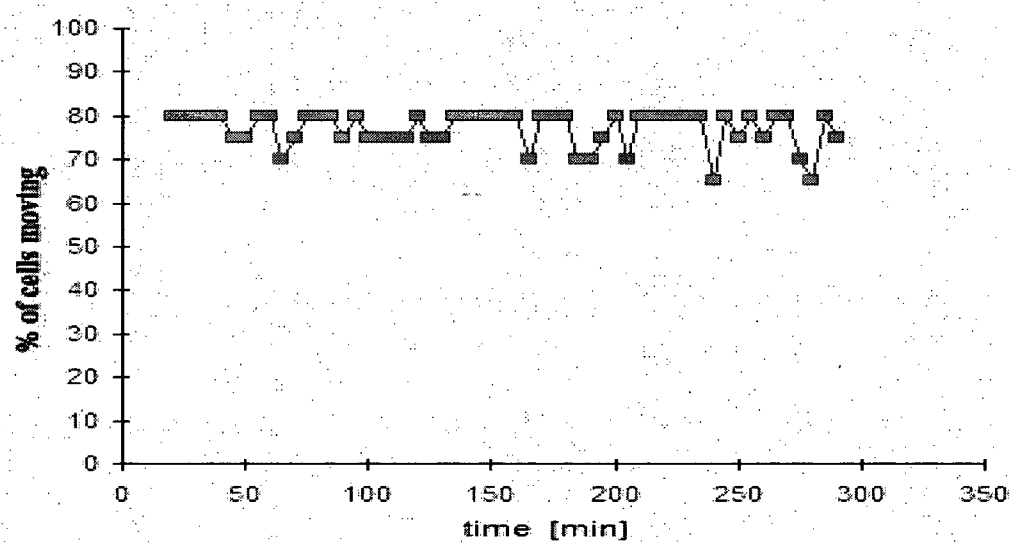
B
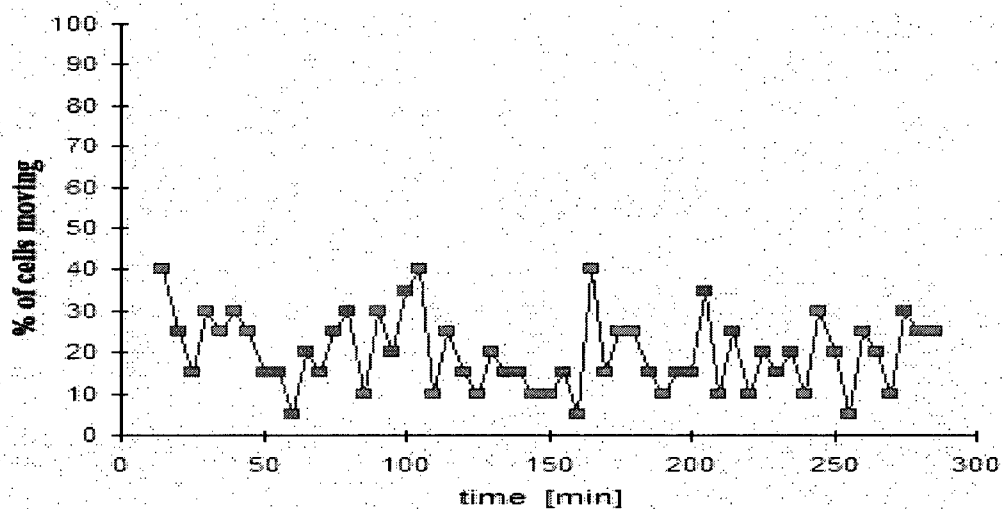
Graphs of percentage of moving cells plotted against time. A)Smooth , and B)Pillar
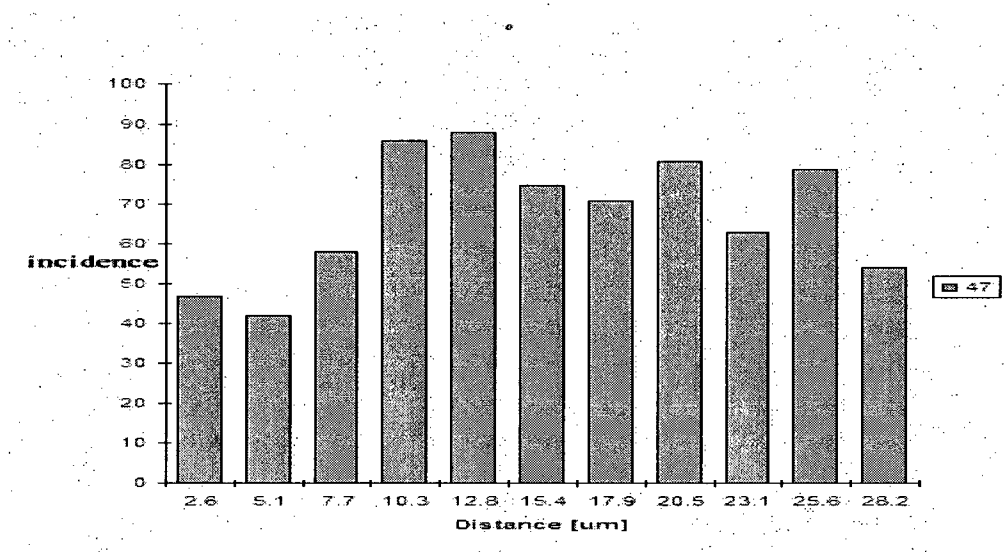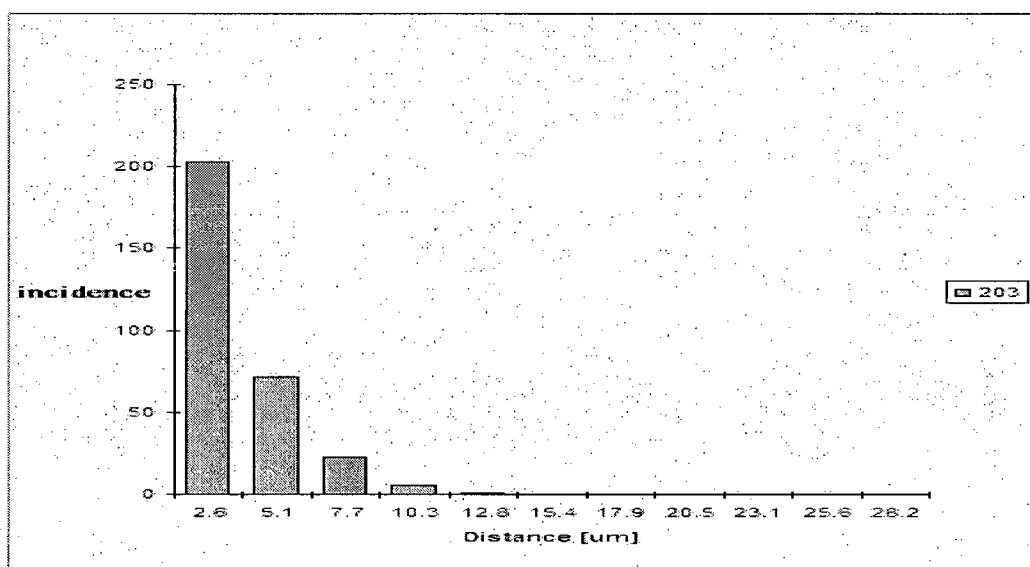
Figure 3

A
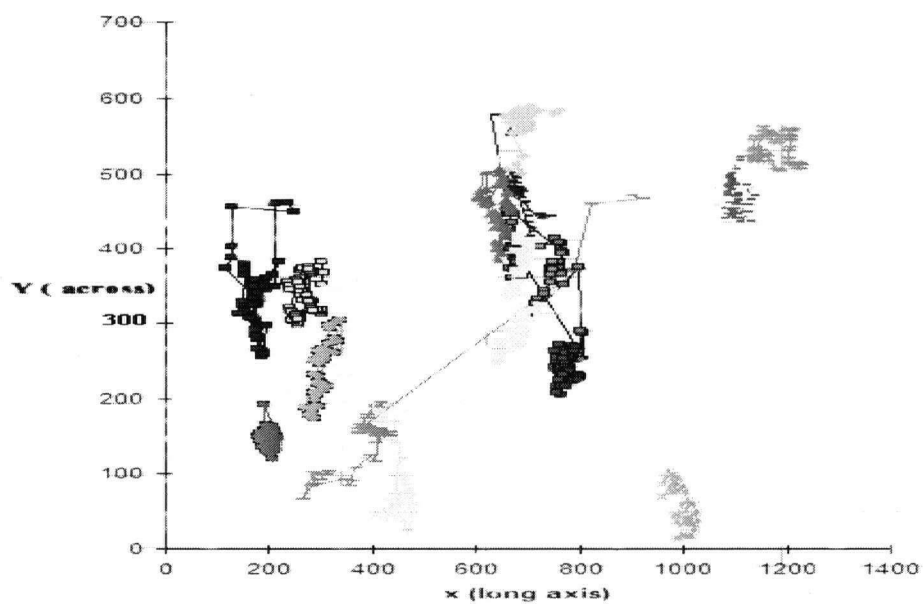
B

Path of cell migration on x and y axis . A) Smooth and B) Pillars
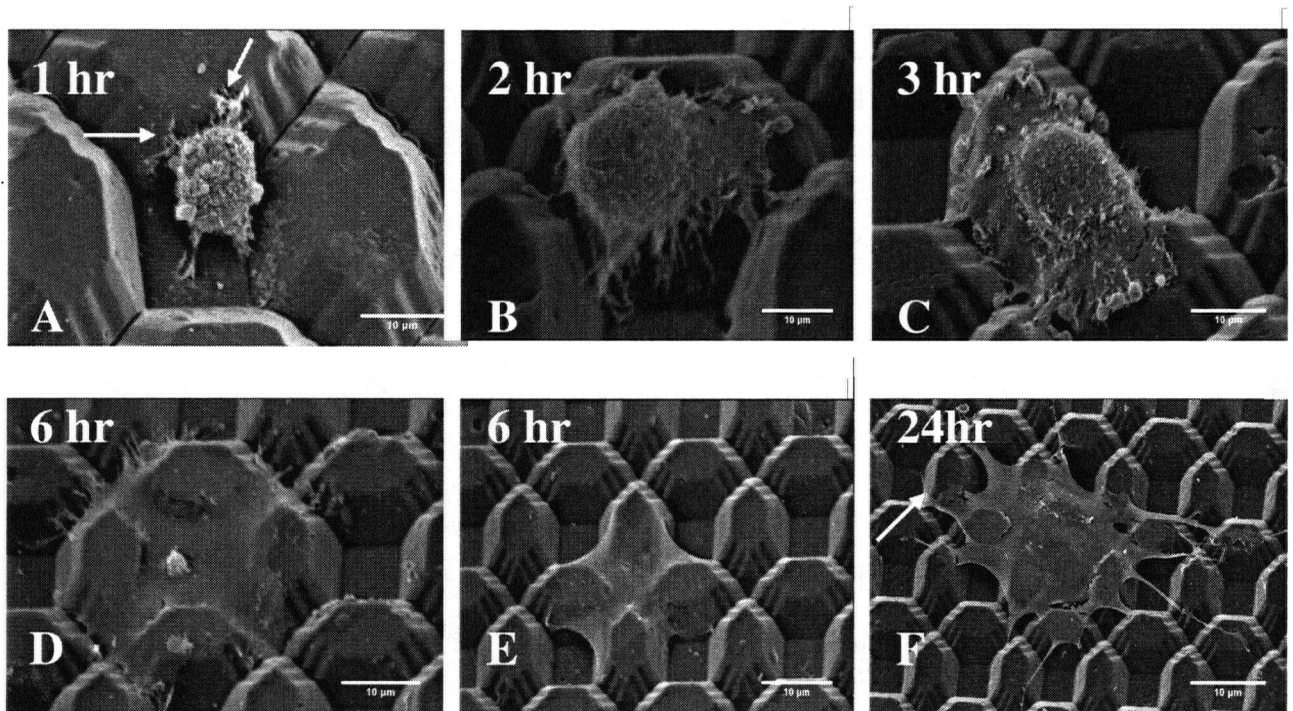
Figure 4

A

B

Graph plotting incidence of cell migration distances on A) Smooth and B) Pillars

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Figure 5

Morphology of the PLE cells on pillars SEM images of cell grown on pillars. (A) is the epithelial cell 1 hour post seeding . Arrows show formed attachments on the wall and the top edge of the pillar. (B) 2 hours post seeding where cell is extending a lamellipodium on the top of the pillar. (C) Cell covering 2 adjacent pillars 3 hours post seeding. Cell covering 4 pillars 6 hour post seeding, presenting hexagonal (D) and cross morphology (E). (F) Cell covering approximately 8 pillars with filopodia elongating through the gaps as indicated by the arrow.
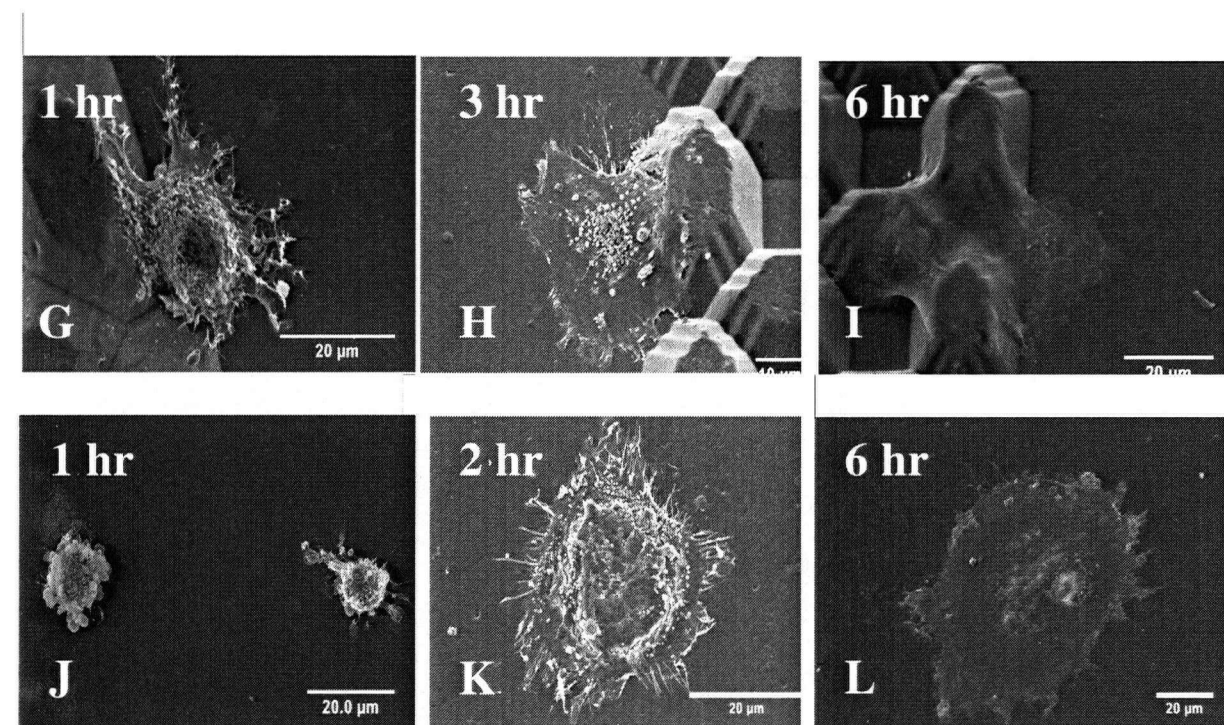
Morphology of PLE cells on border of pillar/ smooth and on smooth surfaces. Cells at the border of the structure, 1 hours (G), 3 hours (H) and 6 hours (I) post seeding. Cells do not show any restriction on climbing onto the pillars from the adjacent smooth structure. (J) Spherical cell on smooth control 1 hour post seeding, (K) Radial spreading at 2 hours post seeding and (L) Spread cell with flattened nucleus 6 hours post seeding.

Figure 6

1 hr	2 hr	3 hr	6 hr
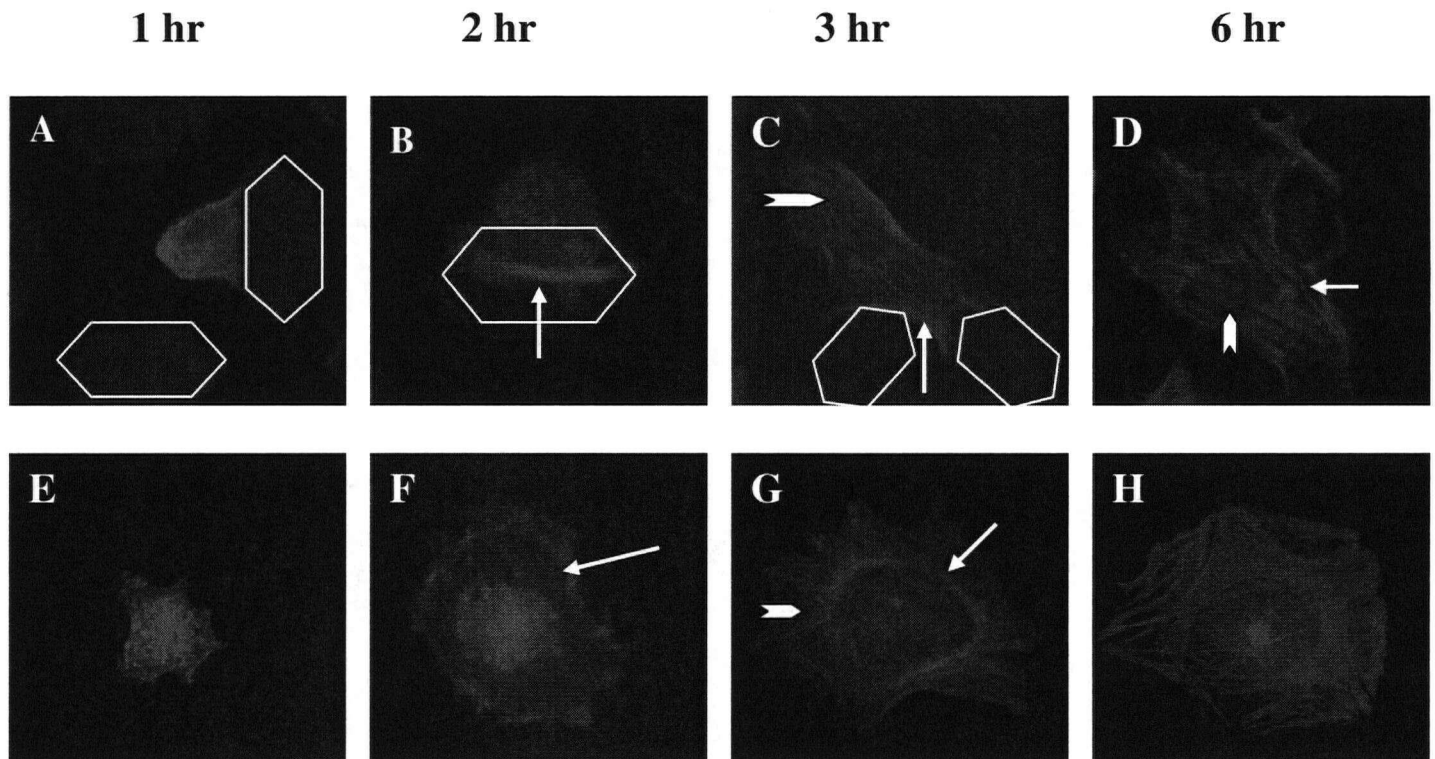
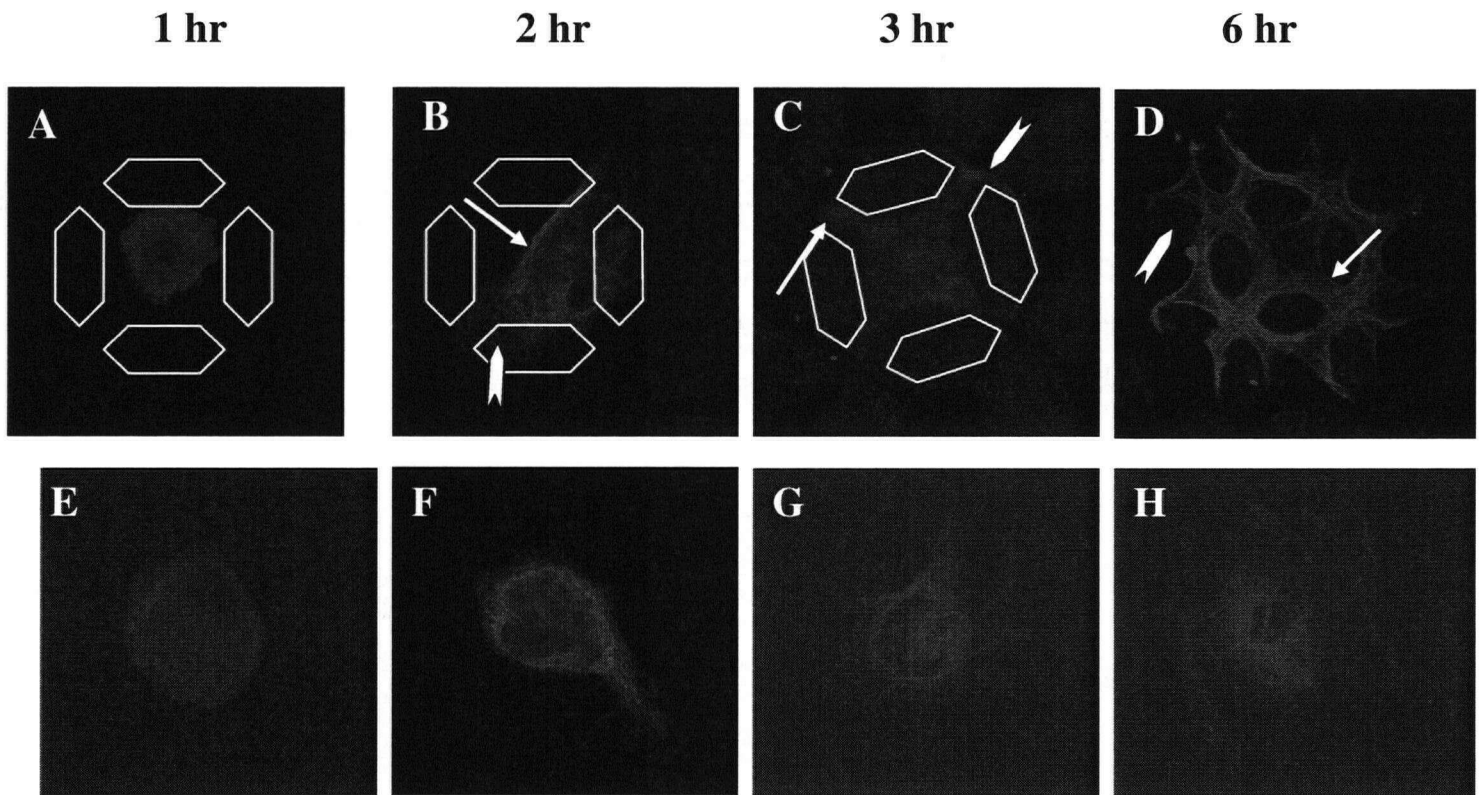**CLSM digital image of PLE cells stained for F-actin on pillars and smooth controls.** (A) Phalloidin positive staining of a cell attached to the walls of the pillars with no distinct actin filaments 1 hr post seeding (B) Cortical actin present at the periphery of the cell's lamellipodium (indicated by the arrow) 2 hours post seeding on pillar top . The cell body was located on the wall of the pillar. (C) Simultaneous presence of diagonal actin stress fibers (arrow head) and cortical actin at the pillar edge (arrow) 3 hours post seeding. (D) Diagonally aligned actin stress fibers in the gaps and the top surface of the pillar 6 hour post seeding shown by the arrow and the arrow head respectively. (E) Phalloidin positive staining cell on smooth control 1 hour post seeding. (F) Appearance of cortical actin at cell periphery 2 hours post seeding. (G) Simultaneous presence of cortical actin ring (arrow) and actin microfilaments with no preferred orientation (arrow head) 3 hour post seeding. (H) Fully spread cell with numerous actin stress fibers traversing the width of the cell with no specific orientation at 6 hours.

Figure 7
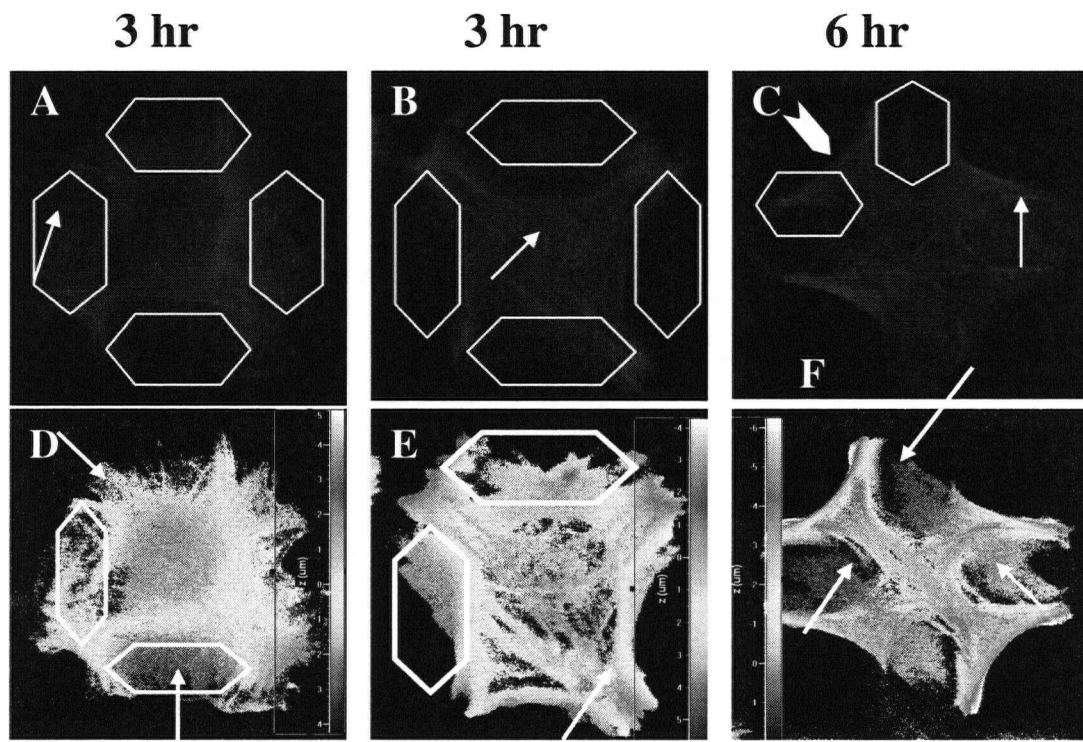
CLSM digital image of actin stained PLE cells on pillars on different z-axis levels. (A) Presence of radial array of actin microfilament on pillar surface (arrow) 2 um below the cell's dorsal surface at 3 hours. (B) Diagonally aligned actin stress fibers (arrow) 3 um below the cell's dorsal surface at 3 hours. (C) Diagonally aligned stress fibers throughout the height of the cell body (arrow points to stress fibers in cells dorsal surface and the arrowhead points to diagonal stress fibers 4-5μm below cell's dorsal surface) . (D) Height measurement of cell in (A), arrows showing the random arrangement of actin microfilaments. (E) Aligned microfilaments at different cell heights. (F) Diagonal aligned stress fibers at different cell heights at 6 hours

Figure 8

1 hr	2 hr	3 hr	6 hr

CLSM digital image of PLE cells stained for microtubules (MT).(A) Round cell 1 hour post seeding with no evident microtubule structure. (B) Cell spread onto two pillars with radial array MTs on pillar tops (arrow) and diagonal MT through the gap (arrow head) 2 hours post seeding. (C) Cell spread in the square box with diagonal MTs in the gap and in the square box (arrow) and close adaptation of the MTs to the walls of the pillar (arrow head) at 3 hours. (D) Aligned MTs in the gaps and radial array on MTs on pillar tops (arrow head) at 6 hours. (E-H) Radial MT arrangement of epithelial cells on smooth controls at 1-6 hours.

Figure 9
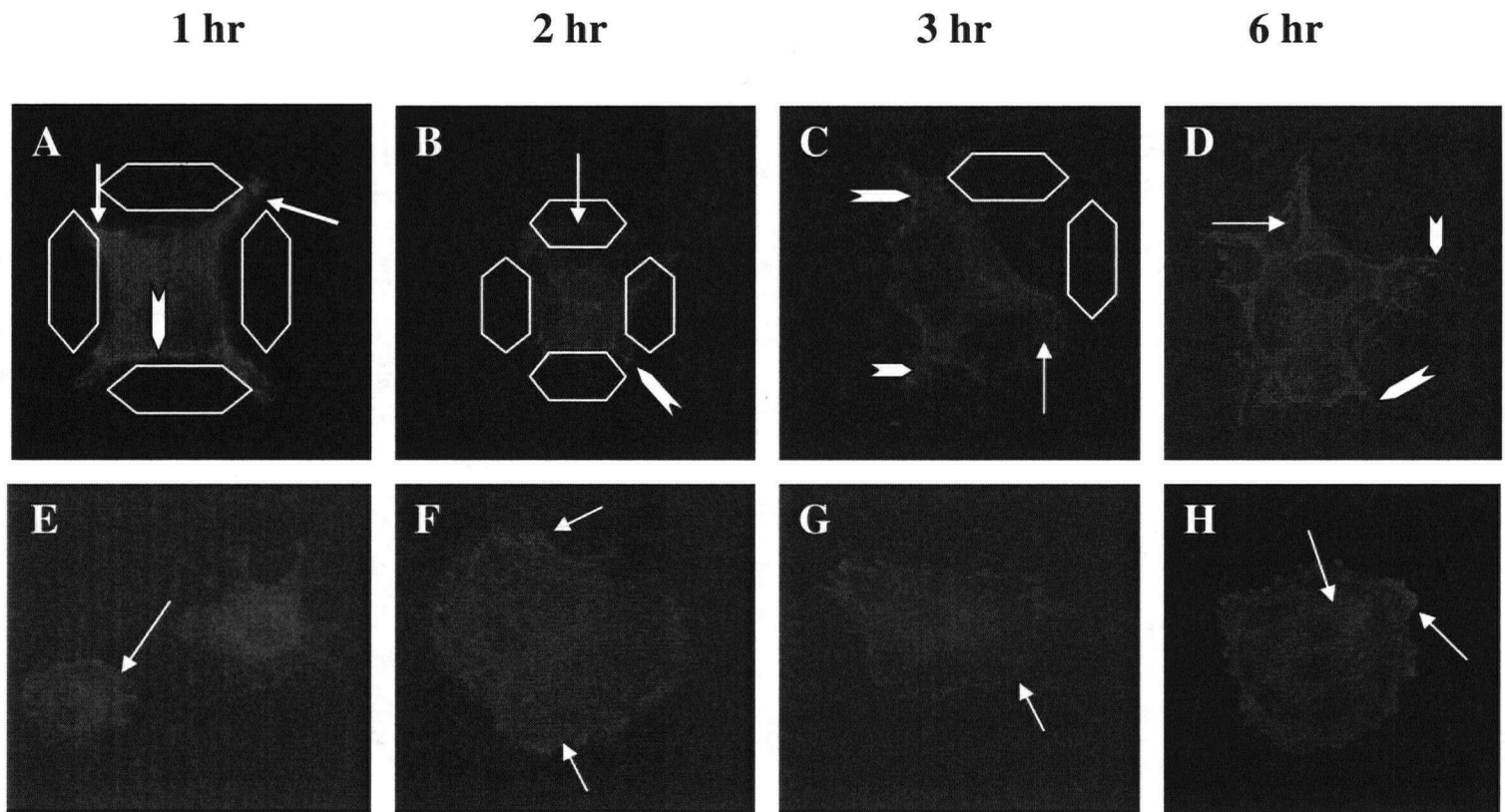
CLSM digital image of PLE cells stained for vinculin. (A) Presence of punctuate vinculin staining in the gaps (arrows) and on the pillar edge (arrow head) at 1 hour. (B) Punctuate vinculin staining on the pillar top (arrow) and in the gap (arrow head) 2 hr post seeding. (C) Presence of super mature focal contacts indicated by the presence of large adhesion plaques on pillar top(arrow) and smaller mature focal adhesions in the gaps (arrow head) at 6 hours. (D) Vinculin plaques some diagonally and some with no preferred orientation on pillar top (arrows), diagonally aligned smaller focal adhesions in the gaps and on pillar edges (arrow head). (E-H) Vinculin staining smooth controls at 1-6 hours. By 6 hours mature vinculin plaques have formed in the central areas of the cell (H).
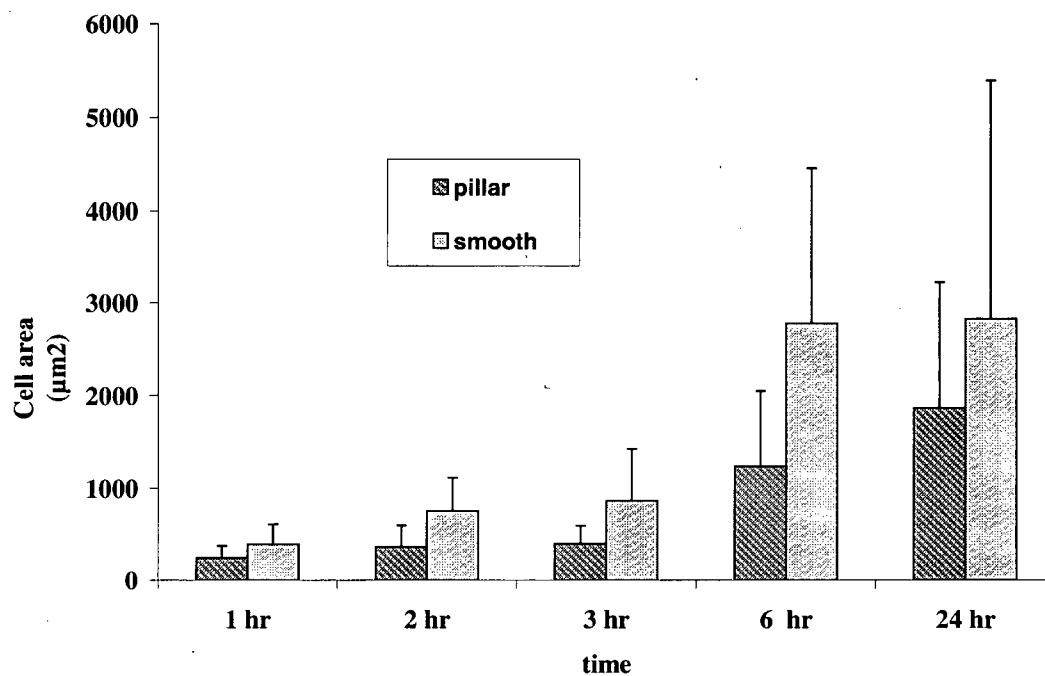
Figure 10

A
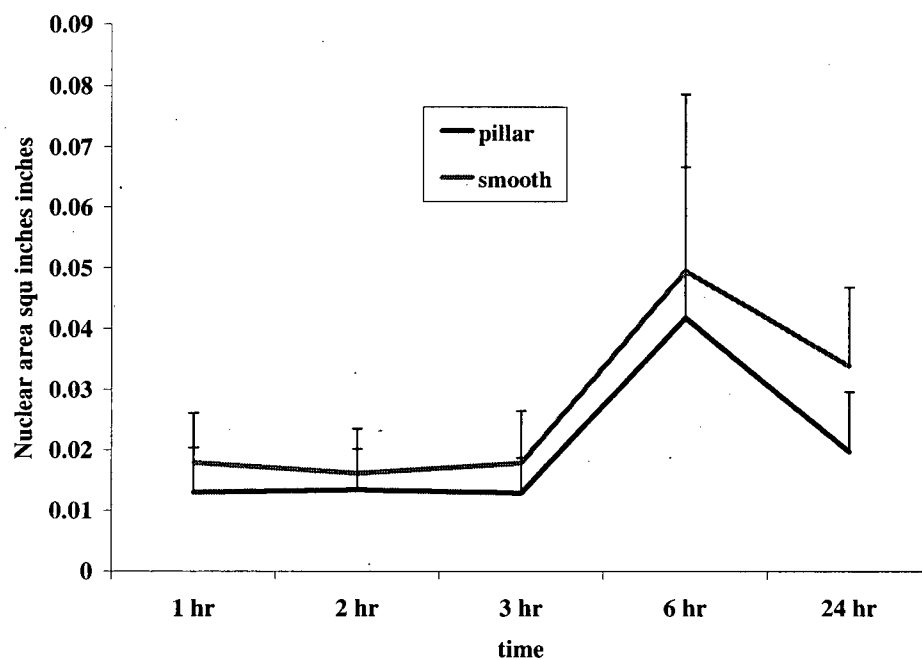
B
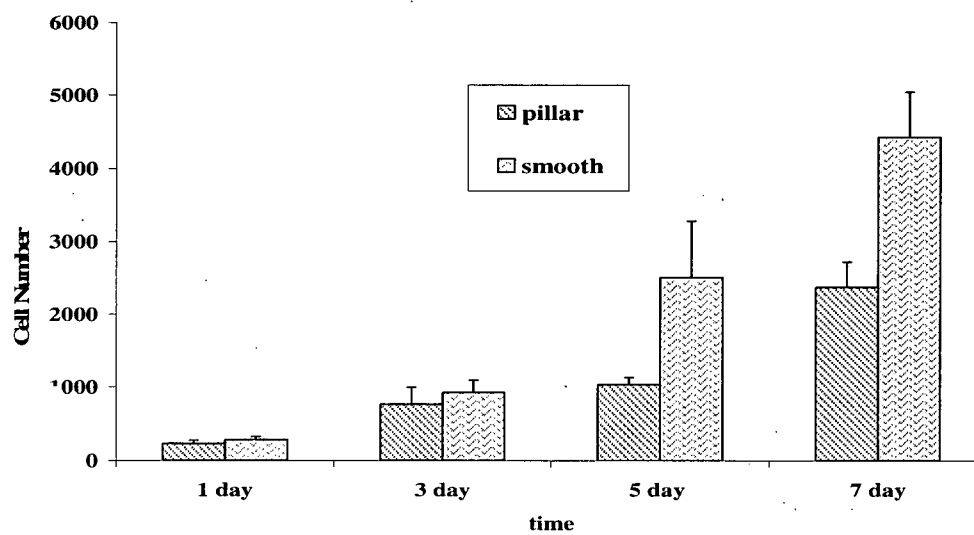
C

Nuclear projected area and cell number analysis of PLE cells on smooth and pillar substrata. (A) Nuclear projected area on pillar and smooth controls. Bars represent mean ± stdev of 40 cells from 3 repeats of experiments. (B) Cell number on pillars and smooth controls. Bar represent mean±stdev from 2 replicates of 3 repeat experiments. C). Cell projected area on smooth and pillars.

Chapter III

Conclusion and Future Directions

In case of endosseous implants, the key biological consideration is the integration of the implant with the surrounding bone (osseointegration). However, transmucosal (transgingival) portion of the implants, penetrate the soft tissue comprising of epithelial and connective tissue [1 and 2]. Therefore it is also mandatory to achieve proper soft tissue integration with the implant to ensure a stable biological seal, especially around the transmucosal portion of the device. Because of the innate tendency of the epithelial tissue to cover a denuded area, epithelial tissue can migrate and proliferate down the interface of the implant compromising the survival rate of the device. Several failure modes are associated with the proliferatory and migratory behavior of epithelial tissue. *In vivo* studies by Chehroudi *et al.*, demonstrated that placement of two stage implants with a subcutaneous and a percutaneous component, allows for significantly less epithelial migration on the surface of the implant resulting in a better survival rate over a 24 week period [1]. However, several studies have shown that placement of one stage implants (non submerged approach), allows for formation of biological width around the dental implant from the time of the implant placement [2]. Therefore, designing substrates that impede epithelial migration and proliferation will potentially eliminate failures associated with this behavior of epithelial tissue.

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Conclusion:

Impeded migration and proliferation of individual cells on our pillared substrata potentially allow for the formation of a tight seal in the transmucosal portion of the oral implants where the soft tissue integration has proven to be crucial for the survival of the implant. Although epithelial cells are gap guided by the gaps of the pillar structure, they have a tendency to gradually cover the pillar tops. Since epithelial tissue is considered to act as a lining tissue within the body, epithelial cells tend to cover discontinuities on a surface. It seems that their response to surface topography takes effect from this innate phenotypic property that the cells exhibit in tissues *in vivo.*

The hexagonal pillars have plausible implications in guided tissue regeneration in periodontology, in transmucosal portion of the implant surface and they can serve as the biomaterial for the *in vitro* tissue expansion for engineering skin/ oral mucosa constructs.

Future direction:

We studied the effect of hexagonal pillars on individual epithelial cells *in vitro*, however it has been known that epithelial cells move as a sheet in vivo. The mechanism of movement of epithelial cell sheet differs from that of individual cells [5, 11].

Epithelial cell-cell contacts make the epithelial cell sheet stiffer compared to individual. It would be interesting to see the effect of pillar geometry on the migration of epithelial cell sheet to closely investigate the possible behavior of these cells *in vivo* [8] .

1-Time lapse observations were recorded for a period of 28 hours. The limitation with staining the cells with Cell Tracker Orange or Cell Tracker Blue, is that eventually after four divisions the dye precipitates out of the cell and the stain fades away. Transfecting the epithelial cells with GFP will provide an interesting alternative to record the cells for longer periods of time.

Ultimately it is the goal of every biomaterial to find clinical applications *in vivo*. A recent study by Hamilton *et al.* on 120um pits *in vitro* and *in vivo* showed that pit surfaces did not inhibit epithelial tissue migration. In fact epithelium bypassed the adhered connective tissue and migrated down the surface of the implant [5]. It is interesting to implant these substrata *in vivo* to see whether they would be able to discourage epithelial cell migration and proliferation in longer time points while permitting connective tissue insertion into the pillar gaps.

2-FAK plays a pivotal role in the ability of cells to break away from the substratum. It has been proposed that FAK drives the turnover of focal adhesions [6]. Since the epithelial cells were relatively immobile on the pillars, it would be interesting to find out whether FAK gets phosphorylated on these structures. If so, when does the phosphorylation initiate and how long does the phosphorylation last for. Failure to observe FAK phosphorylation on pillars compared to smooth controls infers that the focal

adhesions on pillar substrata are more stable compared to that of smooth controls and this stability of attachments in turn results in the immobility of the epithelial cells on pillar topography.

3-Small Rho GTPases are known to be involved in actin and microtubule dynamics within a cell. Rho, Rac, Cdc42, ARP 2/3 complex are all involved in actin dynamics. Profiling these molecules will disclose information about the effect of geometry on the expression of these molecules and as a result their effect on actin dynamics [7, 9 and 10]. Polarity is an essential feature of eukaryotic cells and phosphotidylinositol 3,4,5 triphosphate plays a central role in polarization of some cell populations. In epithelial cells, PI3 is localized at the basolateral plasma membrane is known to be absent from the apical membrane [3].

4-Microtubules are detyrosinated when they are stabilized within a cell. The presence of the detyrosinated microtubules stabilizes the leading lamellipodia and tracing the detyrosinated microtubules will show whether pillars encourage the stabilization of microtubules in any preferred direction and whether the directional persistence of cells through the gaps may be explained by the formation of the stable lamellipodia that are due to the presence of detyrosinated microtubule populations. Related to this matter, it would be of interest to stain for intermediate filaments to observe their interactions with microtubules. It has been postulated that kinesin, a microtubule motor protein facilitates the interactions between microtubules and intermediate filaments [4].

5-Once an implant is placed within body, macrophages are attracted to the centre of the wound. Once stimulated, macrophages release reactive oxygen species such as H_2O_2 and

NO. It is interesting to co culture the epithelial and macrophage cells to see if the geometry of pillars will result in the activation of macrophages resulting in the production of these reactive oxygen species. These reactive oxygen species will result in the breakage of the epithelial barrier. Therefore, if the surface geometry of the pillars results in the stimulation of macrophages that lead to the production of these reactive oxygen species, formation of a continuous sheet of epithelial cells alone will not last, if the barrier is broken as a result of inflammatory response within the body.

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