ELUCIDATION OF ARPIN POLARISATION IN CELLS RESIDING ON ADHESIVE MICROPATTERNED SURFACES

MAGESWARY NAGESWARAN

ADVANCED MEDICAL AND DENTAL INSTITUTE

UNIVERSITI SAINS MALAYSIA

2015

ELUCIDATION OF ARPIN POLARISATION IN CELLS RESIDING ON ADHESIVE MICROPATTERNED SURFACES

by

MAGESWARY NAGESWARAN

DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE (TRANSFUSION SCIENCE)

ADVANCED MEDICAL AND DENTAL INSTITUTE

UNIVERSITI SAINS MALAYSIA

AUGUST 2015

ACKNOWLEDGEMENT

First and foremost, I wish to express my sincere gratitude and highest appreciation to my supervisors, Dr Siti Hawa Ngalim and Dr Ida Shazrina Ismail for the continuous encouragement, guidance and support from the initial to the final level of this project. Although there were many hurdles faced to complete this research work but yet I am glad that I have learnt lots of new skills.

Special thanks to all the lecturers, science officers and lab assistants of Cluster Regenerative Medicine Laboratory, who had given good cooperation and assistance throughout this project.

My heart warming thanks to all my family members especially my parents, Mr. Nageswaran and Mrs. Indrani for their love, support and encouragement. Not forgetting all my friends who has been helping me a lot along the way and being part of my wonderful journey.

Table of contents

List of figures

List of tables

List of abbreviations

Arp 2/3	Actin related protein 2/3
2D	2-dimensional
3D	3-dimensional
A.U.	Arbitrary unit
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
DAPI	4',6'-diaminido-20phenylindole,dihydrochloride
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
FA	Focal adhesion
F-actin	Filamentous actin
FBS	Fetal bovine serum
FN	Fibronectin
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RNA	Ribonucleic acid

ABSTRAK

Daya kelekatan dan penyebaran sel adalah penting untuk pergerakan sel. Sitoskeleton organisasi dan pembetukan lamelipodia yang stabil merupakan antara faktor yang penting untuk pembentukan lekatan tumpuan dan penyebaran sel. Sel HeLa telah digunakan sebagai model sel dalam kajian ini. Permukaan corak mikro dengan empat bentuk (I,Y,busaran silang,cakera) yang telah dikotkan dengan fibronektin telah digunakan untuk menyiasat kesan geometri dan topologi protein membran ekstrasellular pada kelekatan dan sel morfologi. Pertumbuhan sel atas permukaan kaca bertindak sebagai kawalan kajian. Permukaan geometri dan perubahan bentuk sel dipengaruhi oleh daya mekanikal daripada permukaan corak mikro. Pertumbuhan sel atas permukaan corak mikro mempunyai bentuk yang tetap manakala sel mempunyai corak yang tidak teratur atas permukaan kaca yang tidak mempunyai corak. Penyusunan filament aktin dan serat tekanan aktomyosin dipengaruhi oleh perbezaan bentul atas permukaan corak mikro. Penyebaran sel telah dikaji dengan menganalisis luas permukaan sel. Penyebaran sel atas permukaan geometri terhad adalah kurang berbanding dengan penyebaran sel atas permukaan berbentuk cakera. Sel-sel di permukaan berbentuk cakera pakai bentuk yang bulat dan secara rawak polarisasi. Lekatan fokus dan jorokan membran meliputi pinggir. Pertumbuhan sel atas permukaan berbentuk busaran silang mempunyai polarisasi yang kuat dan menerima bentuk kipas oriental. Pada permukaan berbentuk I, sel-sel berbentuk pseudo-persegi. Sel pada permukaan Y berbentuk segi tiga. Aktomyosin pembentukan berlaku di tiga tepi manakala filamen aktin terbentuk pada tepi pelekat. Tahap penyebaran sel menunjukkan penglibatan jumlah ruang diisi oleh protein membran ekstrasellular. Arpin merupakan protein yang mengehadkan penukleusan filamen aktin. Jadi, polarisasi dan penyetempatan Arpin dalam

sel telah dikaji. Hasil kajian menunjukkan bahawa pembentukan lamellipodia disekat oleh Arpin. Protein RhoA memainkan peranan yang penting untuk kontraktiliti akto myosin. Oleh itu, expresi gen RhoA telah dikaji di mana hasil kajian menunjukkan sel HeLa mempunyai expresi gen RhoA yang kurang atas permukaan corak mikro.

ABSTRACT

Cell adhesion and spreading are important components of cell migration. Multiple factors such as organisation of highly dynamic actin filaments and stable lamelipodium formation is crucial for focal adhesion and cell spreading. HeLa cell was used as a cell model. Micropatterns with four shapes (I,Y,crossbow,disk) coated with fibronectin were used to investigate the effects of geometric cues and topography of extracellular matrix protein on cell adhesion and morphology. Cells seeded on uncoated normal glass coverslip act as control. We hypothesised that the geometry of surfaces influences the internal organisation of the cells and cells can change their shape in response to the mechanical forces. The study depicts that micropatterns orchestrate regular cell shape as compared to an irregular shape adopted by the cells grown on uncoated glass cover slip. The assembly of actin filaments and actomyosin stress fibers differs with patterns. To support this data, cell area was obtained to examine the cell spreadiness. As expected, cells on confined geometry (I,Y,crossbow) spread less as compared to the disk-shaped pattern. Cells on disc shaped surface adopted a round shape and randomly polarised. Focal adhesion and membrane protrusion span the periphery. Cells were strongly polarised and adopted the shape of oriental fan on crossbow pattern. On surface with I shape, the cells were adopted pseudo-square shape. Cells on Y shape took up a triangular shape with actomyosin contraction at the three edges whereas actin filament formed at the adhesive edges. The extent of cell spreading is correlated with the total space covered with ECM-proteins. Arpin is an inhibitor of actin nucleation. Thus, the study investigates the polarisation and localisation of Arpin in cells residing on both patterned and non-patterned surfaces. The data obtained suggests that Arpin localises with stress fibers. The results presented illustrates that the

presence of Arpin influences actin filament formation thus restrics the protrusion of lamellipodium. RhoA protein plays a key role in actomyosin contractility. Thus, we studied the gene expression of RhoA gene in HeLa cell and found that the cells on patterned surface have a lower expression of RhoA gene compared to expression of RhoA gene expressed by cells grown on uncoated cover slip.

Chapter 1: Introduction

This chapter is a review of the literatures regarding cell adhesion and how the mechanical cues affects the cell behaviour. Firstly, it gives an overview of cells in which it highlights the important components in cell adhesion and migration. Secondly, it explains the internal framework of actin filaments that determines the cell shape and provides anchorage of cells attached to ECM. Then, the chapter describes the extracellular proteins that provide adhesion sites for cells hence enabling the cells to transmit signals between them. Detailed information about how adhesion proteins structures help the cell to attach and steers cell crawling is also explained. Next, the technique to engineer surfaces with micropatterns coated with adhesive glycoprotein was discussed. The influence of surface geometry and adhesion proteins on cell morphology is briefly explained. Finally, the chapter describes the techniques used to analyse fluorescent images to study cell adhesions.

1.1 Background

Physical interaction of a cell with surfaces in the extracellular matrix (ECM) is called cell adhesion. Cell adhesion is vital to facilitate other cell behaviours such as migration, growth and viability. Upon interaction between cell receptors namely integrins and the adhesive glycoproteins in the ECM, cells form nascent adhesions and later these structures mature into focal complexes and focal adhesions (Alexandrova et al., 2008). The size and turnover of these adhesion structures determines how fast or slow a cell can migrate (Kim et al., 2012). For example, during cancer cell invasion, cell that move faster play a dominant role by invading further and metastasising earlier. Therefore, cell adhesion proteins and their receptors act as a recognition system that provides the cells with anchorage and traction for migration. Meanwhile, the regenerative potential of transplanted cells is also dependent on speed of cell migration into the targeted area, the integration of the cells with native tissues and the quality of the cells to differentiate normally.

In general, biomolecules that are involved in cell adhesion structures include integrins, adaptor proteins (such as talin, α-actinin,paxillin and vinculin), signalling proteins (such as Rac1, RhoA and Cdc42) and cytoskeleton (actin and microtubules) (Parsons et al., 2010). Common examples of adhesive glycoproteins that provide cues for cell adhesions are fibronectin, laminin and osteopontin. In general, integrin which is a cell adhesion receptor binds specifically to Arginine-Glycine-Aspartic acid (RGD) regions within in these adhesive glycoproteins. Often, studies employ the full-length adhesive glycoproteins or short, RGD peptide for cell adhesion study.

Usually, these adhesive glycoproteins are presented in confined shapes. This spatial control help us to uncover the complexity of the tissue structures in vivo and simplify the role of each physical and chemical parameters, (Doyle et al., 2009), configuration (Jang and Nam, 2012) or spacing (Muth et al., 2013) on cell behaviour. These parameters influence cell morphology, of which hinting the dynamics of proteins underneath. Hence, we can uncover cellular processes and manipulate cell behaviours by regulating the presentation of these adhesive cues.

Cells migrate in random fashion without the guided signaling within the cells or from cues within the ECM. In order to dictate cell adhesion and cell migration, it is crucial to study the potential candidate protein that acts as a 'compass' within cells.

The networking and signalling between adhesion proteins and cytoskeleton enable cell adhesion to progress into cell migration. Actins polymerise into filaments, branch out to stabilise of cell shape and turnover for advancing cell movement.

In terms of directed cell migration, the focus for controlling the cell direction lies on actin-related proteins 2 and 3 (Arp 2/3)complex, which is responsible for initiating branched actin filaments (Delatour et al., 2008). Arpin, which acts as the inhibitor of Arp 2/3, has been suggested to direct cell migration (Dang et al., 2013). Therefore, further knowledge on Arpin polarisation will give us hindsight in developing applications for controlling cancer metastasis and a more robust tissue engineering system, both of which potentially useful in the field of transplantation.

1.2 Overview of the cell

Cells are the basic building blocks of all living organisms. The genetic information which encodes all the biological activities lies within the nucleus. The interior of a eukaryotic cell can be divided into two compartments; the cytoplasm and the nucleus. The plasma membrane of eukaryotic cell surrounds the cytoplasm.

Genetic material exists as chromatin and concentrated in the nucleus. The hereditary basis of every living organism is in the genome. Genome composed of a long sequence of deoxyribonucleic acid (DNA). The DNA is a double stranded, long polymer chains made up of nucleotides which are shaped like a twisted ladder run in opposite directions.

The information in the DNA is transcribed into ribonucleic acid (RNA). The RNA molecule is then translated to protein in cytoplasm (Sinden, 2012). Organelles in the cell cytoplasm are unable to synthesize proteins with except mitochondria.

Cells express collections of sensing proteins called receptors that recognise the extracellular stimuli. For example, haptotaxis, cells sense the chemical molecules bound on a surface. Most commonly used haptotactic surface is the ECM. The presence of bound ligands and the ability of cells to elicit a signal transduction pathway are responsible for transendothelial migration and angiogenesis (Bendas and Borsig, 2012).

The outermost surface of the cell is the plasma membrane which allows a cell to maintain homeostasis. Plasma membrane permits the aqueous condition within the cell to be different from those at the exterior of the cell.

Cytoskeleton is responsible to provide a more rigid internal structure that gives the shape of the cell and mediates movement by assisting the cell move in its environment. It is a non-membrane bound organelle that settled within a cell's cytoplasm. It is a dynamic structure made up of three main structural components known as microfilament, microtubule and intermediate filaments which are capable of assembly or disassembly.

1.3 Cytoskeleton system

Cytoskeleton is highly integrated network of fibers comprised of filamentous proteins that helps cell maintain their shape and internal organization. It also provides mechanical support that allows cells to carry out essential functions like cell division and cell migration. For instance, immune cells need to respond quickly when the body is infected. They need to be able to change their shape in order to perform their function. Immune cells such as lymphocytes need to move through the body to the infection site. So, lymphocytes change their shape to squeeze through the tightly packed tissue cells. Whereas, neutrophils need to change their shape to engulf the bacteria.

The cytoskeleton system is composed of three major types of structural proteins namely microtubules, microfilaments, and intermediate filaments. The machinery that powers cell movement is predominantly built from the microfilament which is made of the protein actin. Actin filaments are responsible for many cellular functions, such as cytokinesis, phagocytosis, and cell migration. Actin polymerises into long, fiber-like structures that are ~8nm in diameter.

The actin cytoskeleton generates force and cell movement by polymerisation of actin monomers into filaments and by the interaction of actin with myosin protein. Actin exists as a globular monomer called G-actin and as a filamentous polymer called F-actin (Dominguez and Holmes, 2011). In cells, actin polymerisation is regulated by actin binding proteins such as Arp2/3 complex (Ideses et al., 2008).

Arp2/3 complexes help to create the actin meshwork in lamellipodium. Remarkably, controlled polymerisation of actin filaments drives the growth and organisation of lamellipodia (Rottner and Stradal, 2011). The concentration of actin filaments in lamellipodium display a linear turnover from front to back.

Moreover, cells must be able to assemble and disassemble actin filaments rapidly in order to control the remodeling process. The regulatory mechanisms are able to inhibit spontaneous polymerisation of the monomeric actin, quickly nucleate new actin filaments, control the length and elongate preexisting actin filaments. These processes are crucial for the cell motility and changes in cell shape (Pollard and Cooper, 2009).

On the other hand, myosin is a molecular motor that converts chemical energy in the form of ATP to a mechanical energy generating force and movement. The crawling movement of cells is not only driven by actin polymerisation but also influenced by actin-myosin interactions. Myosin also enables the formation and dynamics of actin rich cell membrane such as filopodia (Yang and Svitkina, 2011).

In summary, dynamic regulation of F-actin filaments is crucial for many physical cellular processes, including division, cell adhesion and migration.

Each of these processes needs precise regulation of cell shape and mechanical force generation all of which mainly regulated by the dynamic in mechanical behaviours of F-actin bundles and networks.

1.4 Cell-matrix adhesion

Adhesion to extracellular matrix component is vital for cell survival, differentiation and migration. These processes can be exemplified in embryonic development, adult homeostasis and immune function. Cell matrix adhesion are composed of receptor (integrin), adaptor and signaling proteins such as (talin, vinculin, paxillin, kindlins, FAK) and stress fibers (myosin II) (Lock et al., 2008). Extracellular space is extremely important in governing the location and behavior of a cell. Cells can organise into different shapes through communication with one another, either directly via cell-cell contact or indirectly via signaling molecules that spans within the extracellular spaces (Doyle et al., 2009)

ECM is organised into fibers, layers, and sheetlike structures. ECM is a complex network composed of proteins, sugars, minerals, and fluids that simultaneously provide stable adhesion sites for cells. Hence, the cells are able to transmit signals between them. In a migrating cell, the ECM serves as a scaffold through which cells crawl. Cells reshape the ECM by controlling the assembly and degradation of ECM molecules around them. Moreover, cell surface receptors such as integrins connect cell to ECM (Larsen et al., 2006). ECM connected to the cytoskeleton on the cytosolic surface via integrin.

The proteins in gap junctions permit direct communications between adjacent cells by forming channels that allow exchange of cytoskeletons of adjacent cells so that a layer of cells can function as a single unit. Nonjunctional proteins such as integrins, cadherins, and selectins function as receptors that mediate adhesion without forming large complexes. Integrin is made up of two noncovalently associated transmembrane glycoproteins subunits; α and β chains. It binds to ECM proteins (fibronectin,laminin) which are responsible for holding tissues together. These receptors do not possess enzymatic activity of their own. Instead, they associate with adaptor proteins that connect them to signaling domain (Delon and Brown, 2007).

Cells use two mechanisms to control the degree of integrin binding: affinity modulation and avidity modulation. The strength in affinity modulation depends on the changes in receptor conformation which alters the affinity of receptor for its ligand. Whereas avidity modulation is the changes between the numbers of contacts formed between integrins and ECM proteins. Cells have their own signaling pathways that control both processes. Integrin conformation which occurs due to the changes at the cytoplasmic tails of the receptor subunits or in the concentration of extracellular cations is fundamental for both types of modulation.

Nascent adhesions form at the leading edge of migrating cell. Signalling proteins stimulate actin filament growth near the plasma membrane. These adhesions produce signals that activate Rac. This activation of Rac protein promotes actin polymerisation and prevents myosin II assembly in the lamelllipodium (Pollard and Cooper, 2009). However, over time nascent adhesions forms a stable link between the ECM and actin filaments which is

sufficient to integrate mechano sensitive proteins, paxillin and vinculin. This stable linkage permits nascent adhesions to increase in size and transition into focal complex.

Focal complex stays for several minutes after adhesion. This complex can mature into elongated structures called focal adhesions (Alexandrova et al., 2008). Focal adhesions are mature adhesions that evolve slowly over time. Focal adhesions normally connected to large actomyosin bundles. Focal adhesions are more stable and exhibit slow turnover. They are associated with the end of stress fibers and located at the cell periphery. These structures comprised of high level of vinculin, tain, paxillin, FAK and integrin (Lo, 2006). Focal adhesion further matures into fibrillar adhesion and is not usually seen in moving cells (Worth and Parsons, 2008).

In contrast to focal adhesion, fibrillar adhesions located more centrally in cells and are characterized by their binding to actin stress fibers (Le Clainche and Carlier, 2008). Fibrillar adhesions are the most stable integrin complex. These adhesions are associated with large actin bundles and fibrillar fibronectin (Worth and Parsons, 2008).

In short, cell undergoes spreading which regulated by the organization of actin cytoskeleton. Cell undergoes polarisation once the cell sense intracellular cues or signals from the binding between receptors and ligands. The adhesion matures from nascent adhesion to focal complexes to focal adhesions. Adhesion turnover occurs from the back to the front of the lamellipodium. This retraction process moves the cell forward.

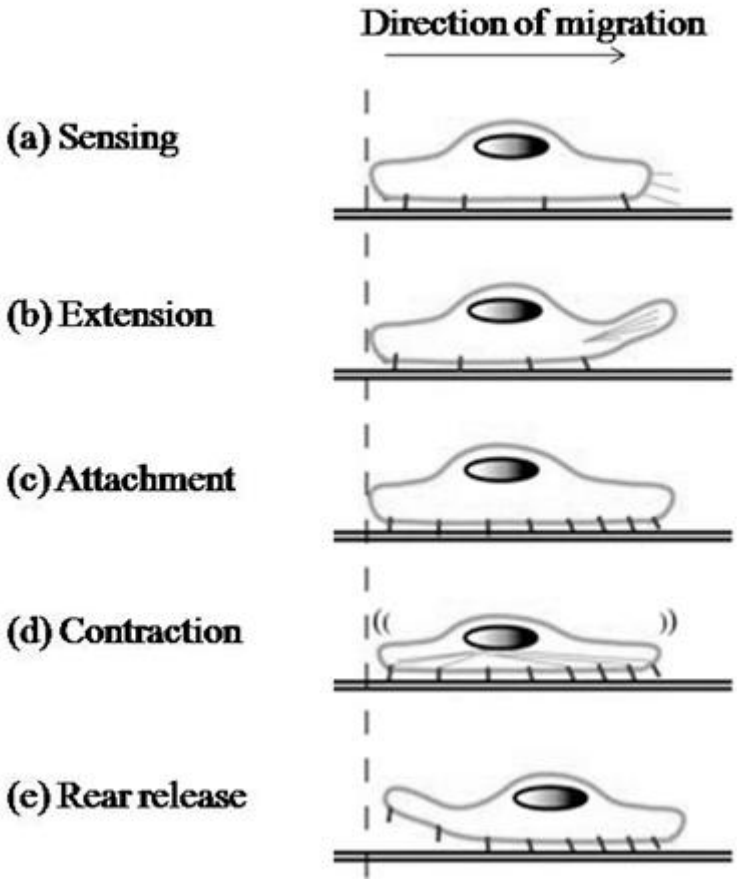

Figure 1.1 The overview of steps involve in cell migration. **a** A cell senses the motile stimuli. **b** Cell extension involving protrusion of lamellipodia. **c** Focal adhesion formed as a result of attachment of the lamellipodia to the extracellular matrix. **d** Stress fiber contraction causes cell traction. **e** Rear release mediates the cell to move forward. *Adapted from (Lamalice et al., 2007).*

1.5 Directed cell migration

Many physiological and pathological processes such as embryogenesis, immune surveillance and wound healing involve cell migration. Directed cell migration needs constant nucleation and coordinated turnover of actin filaments. In general, for a cell to move, it needs to change its morphological characteristic in response to the extracellular signals. Members of Rho family (Rac, Cdc42, RhoA) regulates actin polymerisation and dynamics (Pollard and Cooper, 2009). Activation of these proteins induces the formation of lamellpodia and filopodia. (Le Clainche and Carlier, 2008).

Rac proteins induce the formation of protrusive structures which is called lamellipodia. Cdc42 proteins stimulate the formation of filopodia. Meanwhile, bundles of actin filaments Rho proteins mediate the formation of contractile filaments that composed of actin and myosin II such as those in stress fibers (Hall, 2012).

These stress fibers maintain the mechanical stresses in cells and provide forces for adhesion and cell shape (Etienne-Manneville, 2008). Myosins make use of energy from ATP binding and hydrolysis to produce force and power motility along actin filaments (Pollard and Cooper, 2009).

Moreover, nucleating protein such as Arp2/3 allow the cell to control the time and place of de novo filament formation. Arp2/3 nucleation generates networks of branched filaments (Delatour et al., 2008). Interaction between regulatory proteins and sides of preexisting actin filaments activates the nucleation activity of Arp2/3 complex. Arp2/3 activation at the membrane is required for the formation of lamellipodium. Addition of actin monomers at

the leading edge and filament disassembly at the rear of the lamellipodium allow the cell to migrate forward (Rottner and Stradal, 2011).

Therefore, cell attachment to the ECM is crucial to direct cell migration. The ability of cells to make new adhesion at its leading edge and dissociates the old adhesion allow the cells to move forward. The role of adhesion in steering the cell migration is important to understand some of the pathophysiological processes within the body. For example, ligand binding induces integrin clustering. The affinity of integrin for its ligands is regulated by intracellular signaling that leads to integrin activation. Activation of integrin is critical for carcinoma cell migration.

.

1.6 Surface micropatterning

Surface engineering enable us to understand molecular mechanism of cell-surface interactions. It serves as a scaffold for different biological applications. Micropatterning technology provides structural guidance to cells cultured in vitro. The micropatterns allow a more cell signaling and various cell phenotype to be displayed. This technology can be a tool for understanding the relationship between structure and function of cellular components. Adhesive micropatterns, in which the size, nature of adhesive surface, and shape aid the research on controlled cellular physiology (Mandal et al., 2012).

Photolithography is a microfabrication process to produce micropatterns. This process involves a non-toxic cell repellent surface called cytophobic surface is degraded locally by ultraviolet (UV) light passing through a photomask (Pitaval et al., 2013). These micropatterns are coated with ECM protein called fibronectin to form adhesive substrates. This permits a controlled attachment and spreading of mammalian cells. Micropatterns provide micro environmental cues which control the shape, polarity, spreading, and internal organization of attached cells.

1.7 Cellular imaging

Fluorescence assay provides the analysis on synthetic biological molecules by the means of the fluorescent emission at a specific wavelength. When a fluorescent molecule absorbs high energy (short wavelength) light excites the system (shown by blue arrow), it promotes the electrons within the molecule to transit from ground state to excited state and relax to the lowest available energy state (shown by red arrow). Then, the electrons release their stored energy in an emitted photon. The emitted light is of a lower energy (longer wavelength) than the absorbed light (shown by green arrow). The principle of excitation and emission of light is illustrated in Figure 1.2.

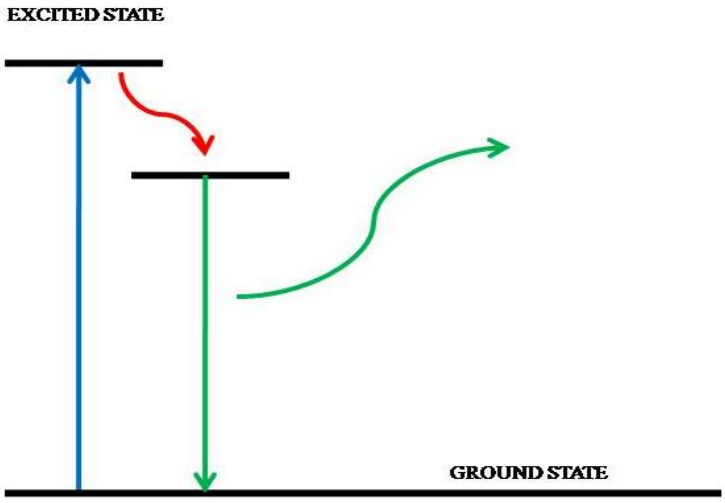

Figure 1.2 Diagram depicting the principle of excitation and emission of light

One of the imaging techniques that widely used to image fluorescently labeled specimens is confocal microscopy. Images of living cells, and three dimensional analysis with higher resolution are possible to obtain with confocal microscopy. Thus, confocal imaging is extensively used by researches from various field of study.

Table 1.1 Spectral properties for some of the fluorescent dyes and protein

Emission	Ex/Em (nm)	Dyes/protein
Blue	345/455	DAPI
Green	493/518	Alexa Flour 488, fluorescein, FITC
Red	555/580	Alexa Flour 555

1.8 Confocal microscopy

Laser scanning confocal microscope (LSCM) is a widely used to get high resolution optical images with depth selectivity. It is used for various analysis such as to study the spatial distribution of macromolecules in fixed or living cells, automated collection of three dimensional (3D) data, imaging multiple labeled specimens, measure physiological events in living cells and colocalisation between protein expressed by cells. The confocal principle is diagrammatically presented in Figure 1.3.

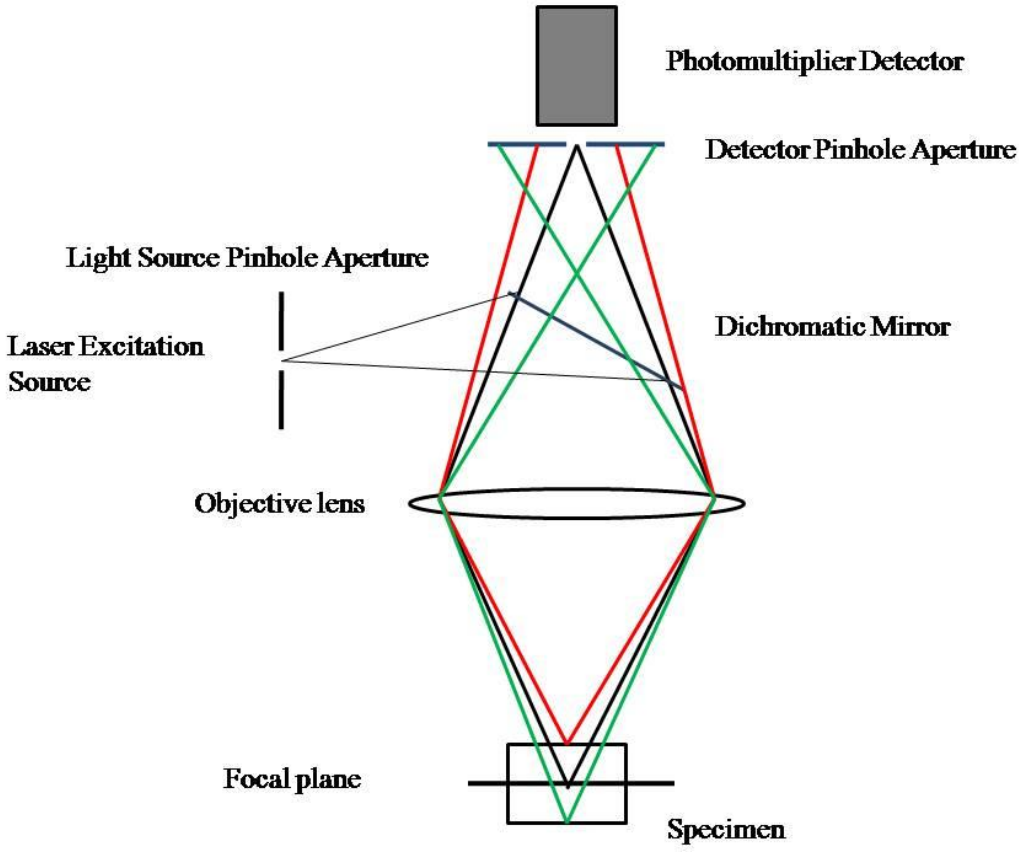

Figure 1.3 Principle of confocal microscope

Coherent light emitted by the laser system passes through a pinhole aperture located in a conjugate plane with a scanning point on the specimen. A second pinhole aperture located in front of the detector called a photomultiplier tube (PMT). The laser is reflected by a dichromatic mirror and scanned across the specimen in a defined focal plane focused as a confocal point at the detector pinhole aperture (Fujita et al., 2013). Zeiss 710 LSCM uses Plan-Apochromat 10x,20x,40x,63x objective lenses.

LSCM is able to collect bright field phase contrast, DIC, polarized light or dark field with the help of transmitted light detector. These signals are transferred to the PMTs via fiber optic. LSCM is used as a tool for quantitative imaging of biological events in a cell because of its ability to accurately record the brightness and the wavelength emitted by a fluorescent probe. Some of the measurements that use confocal microscopy are Fluorescence Resonance Energy Transfer (FRET), Fluorescence Recovery After Photobleaching (FRAP), Fluorescence Lifetime Imaging (FIL) and intensity.

Confocal microscopy techniques widely used to investigate the interaction of proteins with surfaces. This technique provides a better understanding about the process of cell adhesion in response to the protein studied.

In addition, quantifying live cell imaging by confocal microscopy normally carried out using time-lapse mode. Live cell imaging permits the study of molecular and cellular function of a cell.

The images are further processed and analysed using public domain such as ImageJ or FIJI software package (Degot et al., 2010).

1.9 Aim of this study

Arpin has been reported as a regulator for directed cell migration. To date, no studies have been looking at the polarisation of Arpin in response to micropattern surfaces. Thus, this experiment was looked at HeLa cell morphology on patterns with different shapes. HeLa cell is widely used and the properties are well known. However, the cell directionality is still unclear. So, HeLa cell was used as a cell model in this study for an understanding of cell adhesion and migration.

Cells were grown on micropatterned surfaces with has 4 different patterns. This allows the investigation on the distribution of Arpin proteins on the confined pattern surfaces. Cells express RhoA protein to regulate the cytoskeleton. Therefore, the gene expression of RhoA by HeLa cells on specific patterned surfaces was studied in this experiment.

1.10 Hypothesis

I. Micropatterned with different shapes affect filamentous actin (F-actin) branching

II. More Arpin expression on certain pattern reduces the actin filament branching

III. Less RhoA expression on micropattern surface

1.11 Objectives

1.11.1 General objective

To study cell morphology and polarization of Arpin and expression level of RhoA gene on adhesive patterned surfaces

1.11.2 Specific objectives

I. To quantify Arpin distribution on surfaces with different symmetry

II. To correlate Arpin and actin filament branching on the patterned surfaces

III. To investigate the level of RhoA expression

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Reagents and consumables

Reagents for this study were purchased from Sigma-Aldrich (USA), and Gibco (USA), unless stated otherwise. General consumables were obtained from the Central Research Laboratory (CRL), IPPT, USM.

For tissue culture, human cervical cancer cell, HeLa was used as the cell model throughout this study. Dulbecco's Modified Eagle Medium (DMEM, Gibco,11885-084) were used for cell culture. Fetal bovine serum (FBS, Gibco, 10270) was used in addition to the cell culture medium. Trypsin (Gibco,) was used to detach the adherent cells. Dulbecco's phosphate buffered saline (DPBS, Gibco, 14190-144) was used for rinsing cells in cell culture. HeLa cells were cultured on 22x22mm glass coverslips , CYTOOchips Starter FN and CYTOOchips Y-FN650 (CYTOO SA,France) to study the difference in cell morphology on normal surface versus patterned surface. The CYTOOchips are 20 mmx20 mm, 175 µm thick gridded coverslip with micropatterns.

For immunostaining, 4% paraformaldehyde (PFA) was used to fix the cells. Blocking buffer was prepared with 1% Bovine Serum Albumin (BSA, Sigma-Alrich A9647) in 0.2% Tween20 (Sigma-Aldrich,P2287) in PBS (BSA-PBT). Anti-Arpin antibody produced in rabbit (Sigma-Aldrich, SAB2107150) was used to stain arpin. Alexa Flour 555 donkey anti-rabbit IgG (Life Technologies,A31572) was used as the secondary antibody targeted against anti-Arpin antibody. DAPI (Gibco,USA) was used to stain cell nucleus in fixed cells. Vectashield anti fade mounting medium (Vector Laboratories,USA) for fluorescence

was used. Vectashield was used to hold the specimens in place between the microscope glass slide and coverslip. Alexa Flour 488 Phalloidin (Life Technologies, A12379) was used to stain the actin filaments.

2.1.2 Specific Instruments

Confocal laser scanning microscope (Zeiss LSM 710) was used to assess the distribution of Arpin on cells, to visualize the cell adhesion on patterned surfaces.

2.2 Experimental Background

The study was conducted to investigate HeLa cell morphology on different patterns with FN. The distribution of Arpin in cells adhered on micropatterned surface also studied. Arpin regulates actin polymerization by inhibiting Arp2/3 complex. Predominantly, Arpin inhibits actin branching at the lamellipodium tip. Thus, quantifying Arpin is crucial for a further understanding about the steering of cell migration by controlling its directional persistence. The regulation of cytockeleton and cell movement is controlled by RhoA protein. Hence, the PCR was performed to investigate the level of RhoA gene expression by HeLa cells. A flowchart illustrating the overall research process is provided in Figure 2.1

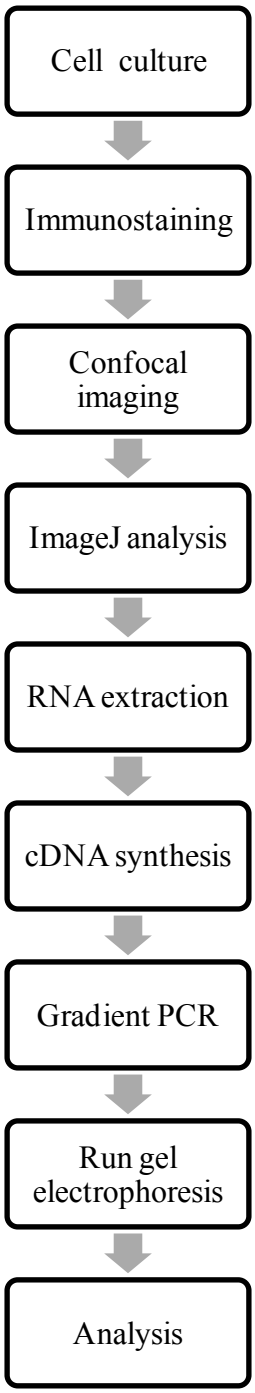

Figure 2.1 Summary of methodology

2.3 Cell culture

Cryovial containing HeLa cells were removed from -80'C freezer and thawed quickly in prewarmed water bath. Cells in the cryovial were added to a 15mL Falcon tube. DMEM with low glucose, L-glutamine, sodium pyruvate and 10% (v/v) FBS was added to the cells in the tube. The cells were centrifuged at 1300 rpm for 3 minutes. The pellet containing cells were mixed with complete DMEM medium by re-suspension and then subcultured into several culture flasks 75 cm^2 containing pre-warmed complete DMEM medium. The cells were grown in CO_2 incubator at 37ºC in a humidified atmosphere of 5% CO_2. Cell condition was continuously checked for stability and presence of contamination. The medium was change every three days until the cells reached 90% confluencey. Confluent cells were subcultured to new flasks on a regular basis.

2.4 Antibody staining

Confluent cells were collected by trypsinization. Prewarmed 0.25% trypsin was used to detach the cells from the culture flasks. Cells were centrifuged at 1300 rpm for 3 minutes. The supernatant was discarded and the cells were resuspended gently with DMEM complete medium. $1.5x10^5$ cells were dispensed into each well containing coverslips and CYTOOchips. The plate was placed in tissue culture incubator at 37ºC with 5% CO_2 for 5 hours to allow the cells to achieve full spreading. The cells were washed for 5 minutes with DPBS. Then, the cells were fixed with 4% PFA for 10 minutes and then washed three times with PBS. Once the cells were fixed, 0.1% Triton X-100 was used to permeabilize the cells for 10 minutes. The cells were then washed three times with PBS.

Cells were blocked for 1 hour in BSA-PBT. Cells were incubated with anti-Arpin antibody produced in rabbit (1:200) in blocking buffer. Cells were incubated with these primary antibodies overnight in a humidified chamber. Cells were washed three times with BSA-PBT for 5 minutes. Then, cells were incubated for 1 hour with Alexa Flour 555 donkey anti-rabbit IgG (1:200).

After, cells were washed three times with BSA-PBT for 5 minutes, the cells were stained with Alexa Flour 488 Phalloidin (1:40) for 20 minutes Lastly, the cells were incubated with 1µg/mL DAPI (1:1000) for 10 minutes. After 10 minutes, the cells were washed once with 0.2% Tween20 in PBS. The cells were washed for three times with PBS only for 5 minutes. One drop of Vectashield mounting medium (Vector Laboratories,USA) was placed on microscope slide. All the samples were loaded on microscope slide and left at 4ºC until further analysis. Samples were examined with a confocal microscope.

2.5 Imaging using confocal microscope

Cells were examined with a confocal laser scanning microscope (Zeiss LSM 710). The configuration used was EX405/488/543. The distribution and colocalisation of Arpin with branched actin filaments on patterned surfaces was determined by measuring the fluorescence intensity using Image J software.