CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

This chapter outlines the significance of cellular mechanotransduction, the fundamentals of cellular architecture and possible effects of microconfinement on cellular dynamics, succeeded by a concise review of pertinent literature. Based on the important conclusions drawn from literature review, the aim, scope and outline of the thesis is finally delineated at the end of this Chapter.

1.1. Importance of Cellular Dynamics under Mechanical Stimulus – Fundamentals of Mechanotransduction

Fundamental units of life are cells. Composed of myriad macromolecules whose activities range from providing structural integrity to modifying neighboring microenvironment, cells execute overwhelmingly wide spectra of physiological functions towards attaining an adaptive robustness of respective organ or tissue architecture. Essential dynamic cellular processes involve changes in cell shape, migration, modification of internal rheology or alteration physico-chemical nature of extracellular spaces. Bulk of these processes originates out of cells' inherent inclination towards responding to a stimulus of either chemical or physical in nature. The clan of chemical stimuli which include various nutrient compounds, growth factors, ions and other reactive organic specie, either permeate through cell boundary (i.e. plasma membrane) before directly interacting with intracellular components or binding to a specific receptor located onto the cell membrane and consequently triggering downstream signaling events. From a biochemical perspective, most of the major responsible chemical components and the related intracellular events have been well-characterized owing to decades of tedious research endeavors. In comparison, cells' response to mechanical stimuli has received only recent scientific attentions. Investigations related to this domain have attempted to address how cells sense a specific kind mechanical stimulus and transmit it into a cascade of entangled chemical signals eventually yielding a host of physiological responses (Ingber 2003, Ingber 2006, Kamm and Mofrad 2006). Another purview of intense

examination is the physical interface of the cell with its proximal microenvironment and how this interface triggers perturbations in cellular morphology and biochemical signaling events that consequently lead to effective adaptation or deleteriously mediate pathological circumstances (Griffith and Swartz 2006). Relevantly, *in vitro*, the natural conditions have roughly been reproduced by fluid shear stress (FSS), four-point bending, substrate stretch, gravity stress, gravity force, vibration, magnetic bead twisting cytometry, atomic force and shockwaves (Kamm and Mofrad 2006). In response, cells have been probed to elicit response either by transmitting the force directly to intracellular compartments with the aid of cytoskeletal elements or by activating an excitable molecular entity such as ion channel, membrane receptor or protein spontaneously linked to intracellular signal transduction (Orr *et al.* 2006). With the emerging importance of studying cellular dynamics under mechanical stresses in different physiological processes and pathological conditions, these have resulted into an altogether separate field called Mechanotransduction (Ingber 2006, Kamm and Mofrad 2006).

The basic ingredients of mechanotransduction and mechanosensitivity have been conspicuous in all cell types in both single and multicellular organisms. Here, the competent conversion of physical force to biochemical information is enormously essential in the sense that it guarantees constitutive stability of individual cells or the whole organism; organizes regulated morphogenic movements and facilitates the construction of precisely defined three-dimensional live-structures. Perceiving these irrefutable implications of the subject, in subsequent times, the development of mechanotransduction has been accelerated especially owing to the *in vitro* reconstitution of major skeletal architecture components such as actin filaments, microtubules, intermediate filaments and their cross-linking proteins and due to development of sophisticated techniques by which cells can be subjected to stresses of physiologically relevant magnitude, with unprecedented spatio-temporal precision, accuracy and repeatability (Kamm and Mofrad 2006). However, because of the complexity of tissue as well as of intracellular architecture, the fundamental routes of mechanotransduction are extremely diverse and often, case specific. In distinct physiological conditions and spatial locations, cells can be subjected to mechanical stresses of shear, torsional, compressive

and tensile types and in all of these scenarios, mechanotransduction has been perceived to determine the effectual physiology and ultimate fate of the tissue (Kamm and Mofrad 2006). For example, under the purview of vasculature and circulatory system, pressure and shear stress from pulsating blood flow dictate the morphology and pathology of the heart and vasculature. It is quite well-recognized that while pulsating shear of consistent directionality promotes normal vasculature formation, flows with spatio-temporally non-uniform direction or velocity profile assist rupture of regular architecture, inflammation and formation of atherosclerotic plaques (Hahn and Schwartz 2009). Within tissue matrices, the ultimate cell-phenotype is governed by the interstitial fluid circulation, compressive load of the neighboring cells and the traction forces exerted on the extracellular matrix (Griffith and Swartz 2006). Similarly, bone is structured by the mechanical stimuli from muscle contraction and gravity. This, in turn, regulates various osteoblastic physiological functions, including protein synthesis, extracellular calcification differentiation, migration and proliferation (Hughes-Fulford 2004). At another end of the spectrum, the process of hearing stems out of mechanosensitivity of inner-ear hair cells towards the vibration of inner-ear fluid, where sliding of bundled brushes of stereocilia conformationally open an ion channel, allowing a flux of positive ions (Hudspeth 2001). This, in result, triggers an electrical signal that upon arrival at brain, is conceived as sound. In addition, latest evidences emphasize upon the role of mechanotransduction in all phases of cancer progression (Griffith and Swartz 2006). Unlike other above-discussed instances, research of mechanotransduction in tumorigenesis is at its infancy and grossly elusive.

To elaborate, from a single or cluster of mutated cancer cells to large malign tumor formation at distant organs, tumor progression is segregated in different stages namely tissue dysplasia, intravasation, dissemination, extravasation, micro and macrometasasis and during each of these stages, cancer cells are subjected to mechanical homeostasis (Butcher *et al.* 2009, Kumar and Weaver 2009) with surrounding normal tissues and within itself. Evidently, even in primary stages, they display different mechanical phenotypes than normal tissues in terms of stiffer extracellular matrix, high traction forces and loss of inherent cell contractility. Moreover, during primary growth, tumor cells are also subjected to interstitial compressive and shear stresses. In fact, it is

presumed that compressive load on the cells lying at the core of a tumor forces the cells to migrate towards a new location (Butcher *et al.* 2009). Next, as the cluster of tumor cells separates from the primary tumor mass and intrudes into the surrounding parenchyma, the dynamic evolution of traction forces in cell body and protrusive forces at the leading end (known as invadopodia) governs the successful intravasation into blood capillaries. Interestingly, these impending mechanical forces are often coupled with coordinated biochemical behavior such as the specific release of matrix metalloproteases which degrade or soften the basal matrix obstruction ahead. Once into the circulation system, often known as "in transit", tumor cells are exposed lethal level of fluid shear forces which has been probed to activate several specific genes, elicit gross reorientation of cytoskeletal architecture and alter intracellular rheology (Miles *et al.* 2008). To ensure a successful metastatic cycle, tumor cells are required to strongly adhere with capillary walls (docking) and force subsequent intrusion (alternatively known as diapedesis) through adjacent endothelial cell lining. It is pertinent to mention here that till date, principles of mechanotransduction have been tested for endothelial and bone-related (osteoblast, osteocyte) cells while mechanotransduction in tumor progression has remained elusively uninvestigated. With the emergence of the startling irregularities regarding the accuracies of the prognostic indicators as diagnostic markers for probing the metastatic potential, strong needs do exist for mapping the distinctive features of the adaptive responses of cancer cells in contrast to the normal ones in micrometer (20-200 μm) sized confinements (hence, *microconfinements*) of tissue spaces and flow passages (Griffith and Swartz 2006), in an effort towards combating the mechanisms of successful dissemination, docking and metastatic colony or secondary nodule formation. However, standard cell-culture studies, routinely executed in macro-scale environments, have often turned out to be elusive in resolving the underlying physico-chemical events that may occur in response to the locally imposed perturbations over cellular length scales. Consequently, despite its vast scientific and medical relevance, the contrasting response of the cancer cells as compared to the other ones in a physiological microconfinement still remains to be poorly understood, especially within the purview of the experimentally tractable spatio-temporal scales. This deficit stems from the complexities in describing the underlying biophysical and biochemical interactions at physical scales that are

substantially smaller than those addressed in the standard cell culture experiments. In this context, microfluidic systems, owing to their conspicuous resemblance with the pores of tissue matrices and vasculature, offer a scope for *in vitro* elucidation of the aforementioned physiological event (Griffith and Swartz 2006).

1.2. Importance of Microfluidics in Studying Cell Dynamics

Microfluidics deals with liquid of sub-nanoliter volumes through purposefully designed channels having at least one of three dimensions of the order of micrometer (Whitesides 2006). Though, initially microfluidics has stemmed out of two distant fields namely analytical chemistry (deMello 2006) and microfabrication (Squires and Quake 2005), soon potentials of the subject were to be unleashed in the field of biology (Beebe *et al.* 2002). During next few years, its scope has stretched beyond exploring exotic mass transport phenomena and low Reynolds number fluid physics into the domains of biochemical analysis (Hong and Quake 2003, Melin and Quake 2007). The urgency of invoking microfluidic devices in solving relevant chemical and biochemical questions has been obviated because of two major beneficiaries that they have in promise. First, within microfluidic confinements, the assay volume requirement of liquid analytes has been discovered to be unprecedentedly low. Second, due to its intrinsic augmented surface area to volume ratio in reduced length scale, microfluidic devices can offer enhanced throughput, reduced reaction time and higher sensitivity (McDonald *et al.* 2000, Thorsen *et al.* 2002). Subsequently, adopting microfluidics in biology has revolutionized the paradigms of molecular biology, biochemistry and bioengineering in such a magnanimous extent that relevant fundamental science and applications are classified by the researchers under the tenet of a separate subject hailed Biomicrofluidics (Beebe *et al.* 2002). The initial flourish of Biomicrofluidics has been facilitated by the expanding necessity of achieving faster and high throughput Genomics during past decades (Wainright *et al.* 2003). However, in post genomic era, Biomicrofluidics applications seems to sprout everywhere in the vistas. Its spectrum encompassed vast bio-domains ranging from cell biology (El-Ali *et al.* 2006) to protein crystallization (Hansen *et al.* 2002), from nucleic acid isolation (Anderson *et al.* 2002) to lethal virus detection (Gu *et al.* 2004). Now, the subject has become progressive enough to miniaturize bulk of the

analytical experiments performed in laboratory scale within few square centimeter space of a monolithic platform and specific jargons such as Lab-on-a-Chip (LoC) and micro Total Analysis Systems (μTAS) have become cliché in the scientific world. Microfluidic systems have been demonstrated to possess potential in diverse spectra of biological applications, encompassing molecular separations, enzymatic assays (Mao *et al.* 2002), the polymerase chain reaction (Ottesen *et al.* 2006), and immunohybridization reactions (Verpoorte 2002). These are outstanding individual instances of down-scaled methods of laboratory techniques, but there also exist stand-alone functionalities, analogous to a single component integrated circuit. Given that the present day industrial approaches to address pertinent large-scale biological integration has emerged in the form of gigantic robotic based fluidic platforms which consume substantial space and expenses, Biomicrofluidics has a straightway objective of replacing them with powerful miniaturization (Melin and Quake 2007). This way, its projected functionalities are quite similar to the silicon based integrated circuit that replaced spacious valve-devices during VLSI (Very Large Scale Integration) chip revolutions of computation industry.

The very purpose of microfluidics devices is to deal with samples often dissolved in an aqueous phase and then, manipulate the system through characteristic analytical procedures such as heating, mixing and separation. Subsequently, processed solutions may be transported to some form of a detector or sensor and the data is acquired. Microfluidic channel networks (Mijatovic *et al.* 2005), generally and economically fabricated in a monolithic platform (Ng *et al.* 2002) made of a moldable silicon based polymer such as Polydimethylsiloxane (PDMS) or glass, include features such as separators, mixers, valves and injectors which are essentially microscale counterparts of existing macroscale analytical and bioreactor process components. Once the device has been designed and manufactured, what becomes indispensable here is to achieve apposite micro-macro interfacial connections to user accessible macroscale input-output components. The microfluidic technology in-complemented with macroscale interface, permits the consistent maneuvering of small sample volume with ensured reproducibility and accuracy.

Miniaturization and dimensional diminution in case of microchannel network has imparted most prolific effects on cell biology. Considering that biological cells

dimensionally scale in the order few tens of micrometers, microfluidic platform provides an exclusive way by which they can be handled individually (El-Ali *et al.* 2006). In some cases, on the basis of specific requirement, even different parts of a single cell can be physically or chemically manipulated though microfluidics (Andersson *et al.* 2002). These functionalities have implicated utterly unfathomed niches in fundamental cell biology and associated medical diagnosis (Helmke and Minerick 2006). While differential addressability of diverse parts of a single cell or several neighboring cells facilitates the study of intra and intercellular signal transduction (i.e. chemical communication between different cells or different parts of a single cell) (Lucchetta *et al.* 2005) and microfluidics based system biology (Taylor *et al.* 2009), the ability to isolate and study a single cell at a time bestows a distinctive approach to study infected cells *in vitro* (Maerkl and Quake 2007). Relevantly, on the basis of either attenuated or augmented deformability, microfluidics based detection systems pertaining to some of the lethal diseases such as cancer, malaria, AIDS and SARS have been proposed (Yager *et al.* 2006).

Again, with pertinence to the problem of distinctive survival advantage of cancer cells, microfluidic systems, if appropriately devised, providing the closest resemblance to the micron-sized physiological circulatory-renal flow systems and pores within tissue matrices, confers an unparalleled platform for *in vitro* simulation of *in vivo* cellular behavior (Lucchetta *et al.*. 2005, Griffith and Swartz 2006). The undeniable similitude between microchannel systems and blood vessels has encouraged researchers to an extent of adopting the technology of microfluidics in the domain of artificial tissue engineering (Andersson and van der Berg 2004). Moreover, since the illustration of mechanical influence on cell physiology, the fluid shear stress has been most emphatically utilized in the study of mechanotransduction, as the representative stress imparting mechanism. Within microconfinement, due to the dimensional commensurability, it is relatively easy to attain approximately accurate physiological conditions through precise regulation of incipient flow driving mechanisms such as pressure drop (Griffith and Swartz 2006).

Next, from the perspective of attaining a well-designed study, what remains unknown is the relevant cellular elements which demarcates the stress-adaptive response of a cancer cell from normal ones. Given the intrinsic complexity and tremendously large

number of intracellular elements, a pin-pointed study towards elucidating the distinctive stress survival of cancer cells should require a review of preceding literature on mechanotransduction with a view to isolate those elements which are maximally perturbed or influenced in response to fluid shear stress.

1.3. Brief Review of Important Cellular Architecture

In general, all mammalians cells are classified in two categories namely adherent and non-adherent. Adherent or anchorage dependent cells are relatively abundant in physiological construction and they constitute bulk of tissue architecture including skin, bone, different organs, nervous system and many other body components (Lodish *et al.* 2004). These cells require a significant adhesion to adjoining solid tissue matrices for the sustained survival and proliferation. In contrast, the non-adherent or anchorage-independent cells such blood leucocytes do not rely upon cell-matrix adhesion and thrive on the floating state within body fluids. Irrespective of this distinction, all of the mammalian cells share a generic self-consistent organization which consists of circumferential plasma membrane, the intracellular fluid i.e. cytosol, an intricate architecture, sub-cellular organelles such as mitochondria, ribosomes, lysosomes and glogi-complex and finally the nucleus (Fig. 1.1). In response to mechanical and chemical stimuli, various chemical signals are triggered which subsequently intrudes into the nucleus. In the nucleus, information stored in the sequence of DeoxyriboNucleic Acid (DNA) is transcribed into messenger RiboNucleic Acid (mRNA) molecules which comes out the nucleus and is consequently, acts as the template for protein synthesis within the ribosomal constriction (Fig. 1.2). The external stimulus perturbs the relative transcription rates of genes and in turn, propensity of intracellular protein molecules which are involved in multifarious cellular processes.

Among various components involved in transduction of chemical and mechanical stimuli, the roles of plasma membrane, cytoskeletal architecture (which shapes the internal structure of cell) and Cell-Substrate adhesion junctions are the most prolific. Following we outline the aforementioned elements in an abridged manner.

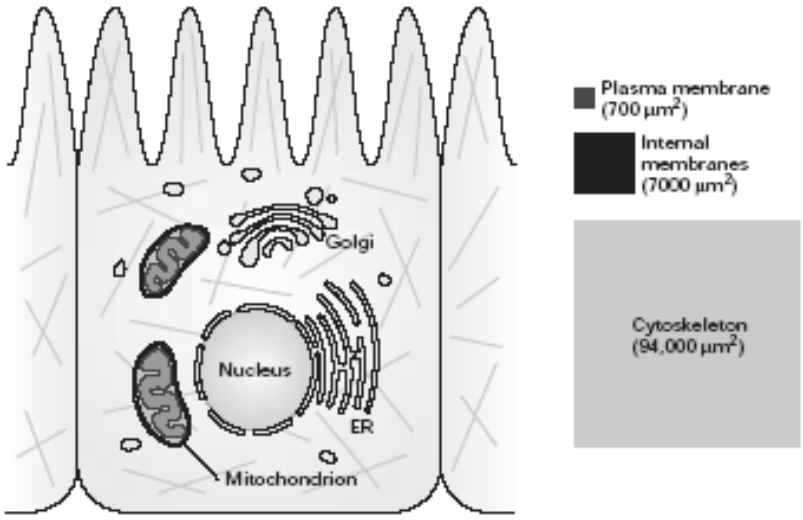

Figure 1.1. Schematic representation of cellular architecture as reproduced from Lodish *et al.* (2004).

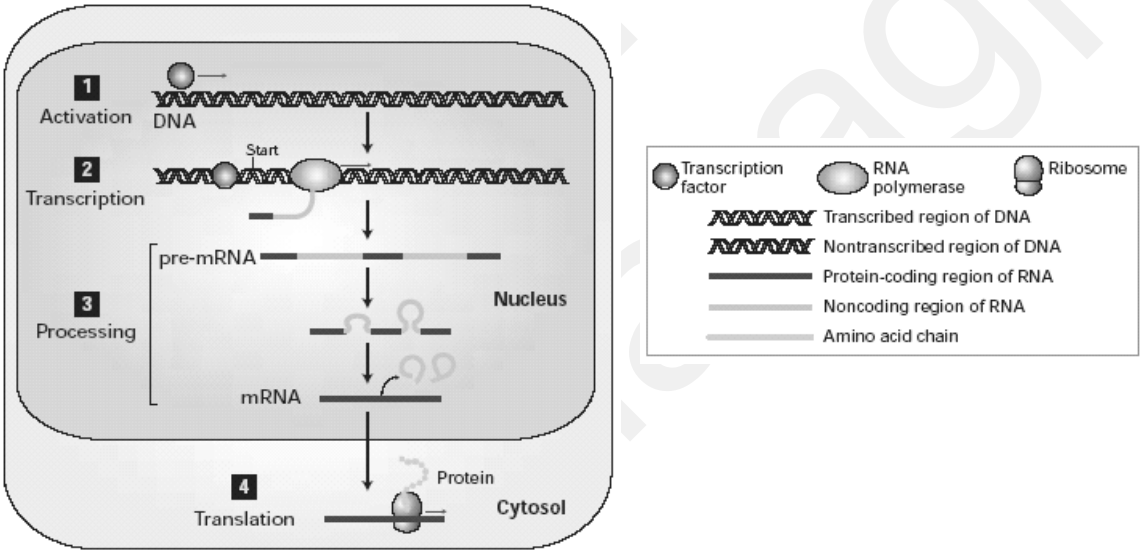

Figure 1.2. Schematic delineation of central dogma including transcription and translation culminating into protein synthesis. Reproduced from Lodish *et al.* (2004).

1.3.1. Plasma Membrane

The cell membrane or the plasma membrane is a predominantly double layer lipid structure which demarcates the intracellular components from the surrounding environment. Plasma membrane is essentially semi-permeable and plays imperative roles in governing the transport of water, ions and macromolecules in and out of the cell. From constitutive perspectives, it is composed of large number of lipids (about 25000, both

saturated and unsaturated kind), proteins (including receptors and ion channels), polysaccharides which enable to participate effectively in maintenance of structural integrity under stressful circumstances and to facilitate signal transduction (Lingwood and Simons 2010). Remarkably, the plasma membrane is penetrated by cellular structural components e.g. cytoskeleton. The cytoskeleton not only provides constructional robustness but also forms a coupling between cell's internal structure and the integrated adhesive elements which, in turn, are connected to the extracellular surface. Following the fluid mosaic model proposed by Singer and Nicolson (Lodish *et al.* 2004), the plasma membrane is represented as a high viscosity two-dimensional liquid where its constituents are freely mobile. However, through decades of tedious research endeavors, this concept has been reconsidered in the view of lipid-lipid, lipid-protein and protein-protein interactions which spontaneously yields to extremely heterogeneous compartments. This situation is further complicated by protruding portion of peripheral cytoskeleton and frequently distributed membrane cleavages.

1.3.1.1. Membrane Composition

Membrane Lipids: The bulk of the plasma membrane is composed of phospholipids which are amphipathic in nature owing to concomitant existence of hydrophobic tail and hydrophilic head groups. In cell membrane these molecules self-assemble themselves in such a way that exclusive hydrophilic head regions are exposed to polar environment, while alkyl tail is buried. Beyond this, it also contains glycolipids and steroids, especially cholesterol. Importantly, cell membrane composition differs cell to cell and even, between inner and outer layer of plasma membrane. Again, according to the characteristics of fatty acid segment of lipid molecules, lipids can be classified as saturated (e.g. sphingolipids) and unsaturated (e.g. phosphatidylserine). It is now well-recognized that the fluidity and the structure is exquisitely sensitive on the fatty acid chain length or the degree of unsaturation which augments membrane fluidity by virtue of their inherent loose packing. In contrast, saturated fatty acids are able to align themselves in relatively rigid configuration and often, forms robust nanodomains known as Lipid Rafts, in conjunction with membrane cholesterol. During cellular stress adaptation, alteration of membrane fluidity by desired modification of lipid composition

serves an indispensable part. In another situation, such as Apoptosis (Programmed Cell Death), elemental exchange may occur between inner and outer layer, again resulting into a change in the lipid composition.

Membrane Proteins: Broadly, membrane proteins are segregated as integral, peripheral and anchored classes. Integral proteins (also known as transmembrane proteins) such as membrane receptors and ion channels stretch across the cell membrane single or multiple times. The transmembrane region of an integral protein is composed of helix like structure where hydrophobic amino acids (building units of protein molecules) are placed outwardly placed towards the surrounding lipid tails. In contrast, lipid anchored proteins are covalently linked to single or multiple lipid molecules and are devoid of any transmembrane zone. Similarly, peripheral proteins are minimally attached to membrane or integral proteins. Functionalities of membrane protein include signal transduction, compartmentalization, engulfing of foreign materials (endo and pinocytosis) and structural regulation.

Beside lipids and proteins, plasma membrane holds numerous varieties of simple and complex carbohydrates which are presented as the surface modifications of lipid and protein molecules and are perceived to impart efficient regulation of signaling process.

Lipid Rafts: While much conventional Singer-Nicolson Fluid Mosaic Model presumes that the lipid bilayer exits basically as an impartial two-dimensional solvent, minimally perturbing the membrane protein function, research endeavors in biophysics have revealed the existence of several lipid phases even in the simplest of model lipid bilayers. Membrane lipid components, being expressed in several variants, impart the essential compartmentalized activities that a cell-boundary should possess in order to preserve the internal homeostasis and stability to external fluctuations. Here, differential interactions among components originating from the inherent mismatches between the hydrophobic thicknesses of molecular components, cohesive van der Waals interactions and lipid acyl chain packing entropy, evidently trigger the component segregation into distinctive domains (Simons and Toomre 2000, Lingwood and Simons 2010). Respectively, the lipid layer itself is entitled to uphold several spatially non-uniform structural elements. Of

these, a class of specialized cholesterol rich nanodomains (of size ~ 20-200 nm) known as lipid-rafts has been realized to participate in multifarious cellular sensing and signal transduction processes. With rest of the lipid-bilayer being composed of the unsaturated fatty acids in majority, the cholesterol within raft entities effectively cluster the saturated lipid-components such as sphingomyelin and most importantly, several types of membrane bound integral and anchored proteins. Even though the raft elements retain a small fraction of unsaturated phospholipids, the hydrophobic tails of the lipids clustered in raft domains are generally saturated and thus, constitute a rigidly aligned structure. Rigidity of this nanodomain is further enhanced by presence of cholesterol which, owing to its affinity towards saturated acyl chains, is preferentially partitioned and acts like gluing agent, reducing the fluidity. From the perspective of organizational behavior, the aforementioned physico-chemically distinctive portions of plasma membrane which are definitely noticeable in experimental time-scale, are described as lipid disordered (L_d) and ordered (L_o) domains respectively (Fig. 1.3). These raft elements are engulfed within a bulk phase of unsaturated lipids and perceived to be controlling several critical and specialized functionalities, including signals transductions, sensing, and sorting of proteins. While mobility within raft domain is minimal, these domains are themselves mobile within relatively more abundant unsaturated phospholipid layer. Although the size of raft domains prohibits direct optical visualization, the presence of these domains in the plasma membrane of living cells has been probed with detergent fractionation, particle tracking rheology, fluorescence correlation spectroscopy (FCS), fluorescence recovery after photobleaching (FRAP), fluorescence resonance energy transfer (FRET), proximity imaging techniques (Lingwood and Simons 2010). In recent times, a specific raft detecting fluorescence agent namely AF-CTxB (Alexa Fluor dye conjugated B-subunit of Cholera Toxin) has been introduced. Cholera Toxin B-subunit specifically binds to G_{M1} Ganglioside (Fig. 1.3b) which is favorably partitioned into raft domains (Balasubramanian *et al.* 2007). Importantly, lipid raft are recycled between plasma membrane and golgi complex (an intracellular organelle) with typical cycle time of 10-20 minutes. The inward and outward movements of lipid raft are known as endo and exocytosis respectively and consequently, the membrane presentation of raft elements are reliant on their rapidity.

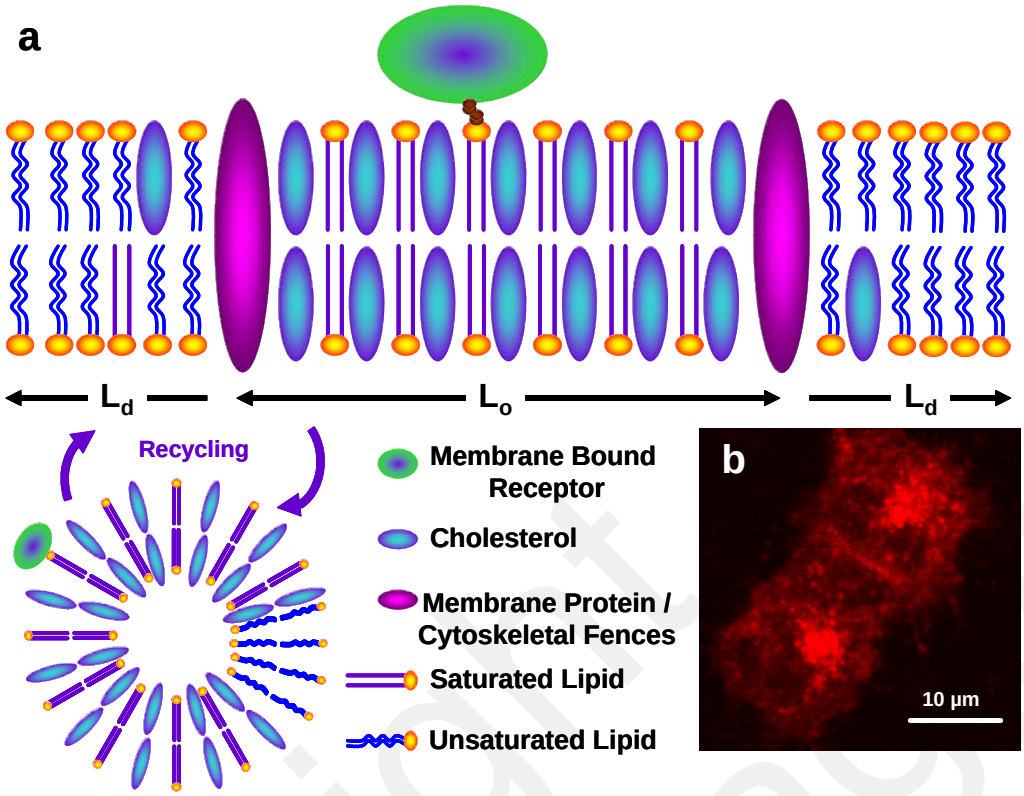

Figure 1.3. a. Schematic Representation of Lipid Ordered (Raft) and Disordered domains of cell membrane. The Ordered domain is stabilized by surfactant effect of membrane protein and continuous recycling. b. Confocal microscope image of lipid raft domains as obtained by staining HeLa cell membrane with AlexaFluor 594 conjugated Cholera Toxin B-subunit (CTxB).

1.3.2. Cytoskeletal Architecture

The cytoskeletal architecture, which provides the required constructional framework and solidarity to biological cell, is predominantly composed of actin microfilaments, intermediate filaments and microtubules. Cytoskeleton is fundamental scaffolding of cell and appreciably governs the cell shape, migration, division, adaptive response to external physico-chemical stimuli and even, the intracellular transport. It is being formed through a dynamic balance between polymerization and depolymerization of molecular subunits, several other cellular proteins (signaling and structural) have been implicated to influence the ultimate contour of the cytoskeleton and its environment responsive organizational rearrangement.

Actin Microfilaments: Actin microfilaments are formed by the directional polymerization of a specific protein called globular actin (G-actin) and of 6 nm in diameter (Lodish *et al.* 2004). In adherent cells, they stretch from one end of a cell to other, balance the cell-substrate traction force and preserves cell contractility. Within a single microfilament, two actin helices are intertwined. Actin molecules contain a barbed and a pointed end. During polymerization, the pointed end of subsequent monomeric unit binds to barbed end of the preceding unit. While the addition of G-actin is kinetically favored towards barbed end (or + end), the release preferably occurs in the point end (- end). Hence, in living cells, the growth or treadmilling of an actin filament is biased towards a specific direction and proceeds through coordinated influences Adenosine Triphosphate (ATP) hydrolysis and polymerization promoting molecules such as actoclampins. During, directed elongation, force exerted by growing end of microfilament is sufficient to deform the cell membrane and yield several extended structures such filopodia, lamellipodia and invadopodia (in case metastatic invasion) initiating cell migration. Actin cytoskeleton, especially the peripheral elements, are often used as the scaffolding domain for signaling molecules, which determines the microdomain formation within cytosolic and membrane signal transduction (Neves *et al.* 2008). In membrane, protruding fraction cytoskeleton acts like 'fence' or 'picket' that along with lipid raft governs the compartmentalization of membrane receptor proteins (Marguet *et al.* 2006). With the aid of myosin motor enzymes which hydrolyze ATP to bind and traverse along microfilaments, actin cytoskeleton actively participate in exerting contractile tension and transporting intracellular vesicles. Actin binding proteins such as Cofilin, Severin, Gelosin and Arp2/3 complex tights regulates action polymerization and microfilament branching processes.

Intermediate Filaments: These structural filamentous elements are 10 nm in diameters and generally, less dynamic then actin microfilaments. Only in recent times, numerous non-trivial roles of intermediate filaments have been identified with respect to generation of cell-substrate traction, structural integrity, intracellular distribution of organelles and cell-cell adhesion junction formation. Unlike actin microfilaments, basic constituents of intermediate filaments are multiple in types, including Lamins (A, B and C), Keratins,

Vimentin, Desmin, Peripherin etc. Though much remains to be elucidated, each of them possesses certain structure-functional significances. For example, during epidermal to mesenchymal transformation (EMT) stage of cancer progression, originally existing cytokeratin filaments are replaced by vimentin (Kumar and Weaver 2009).

Microtubules: Microtubules are formed by the polymerization α- and β-tubulin dimmers and are 25 nm in diameters. Uniquely, all microtubules in a cell emanate out of the Microtubule Organizing Center (MTOC) and extend up to the vicinity of plasma membrane. Similar to actin filaments, several motor proteins such kinesin and dynein bind to microtubules and utilize microtubular track for the transport of intracellular vesicles. In fact, the recycling of lipid raft elements between the plasma membrane and the golgi complex (an intracellular organelle) is facilitated by microtubule directed transportation. Microtubules have been probed to represent the cell polarity where the frontal-dorsal polarity is determined by the exact location of MTOC. During adaptive response to shear stress, MTOC has been observed to be displaced into a downstream position.

1.3.3. Focal Adhesions

For adherent cells, focal adhesions represent the dynamically formed protein complexes located at the cytosol-membrane interface (Mitra *et al.* 2005), which connects the internal cytoskeleton to extracellular matrix (ECM). Generally, focal adhesions are located at the basal region (i.e. bottom or substrate adhering) of the cell. They are constituted of main proteins of which Integrins and Focal Adhesion Kinase (FAK) are functionally the most pivotal ones. Integrins are heterodimeric proteins which span through the plasma membrane and binds to the specific motif (e.g. RGD peptide sequence) on ECM. The cytosolic end of integrin is indirectly connected to actin microfilaments with aid of mediators (such as FAK, talin, paxilin, α-actinin, filamin and vinculin) (Fig. 1.4). Due this connection, while at one end, any change in the matrix elasticity or chemical property can be physically sensed inside the cytosol, at the other end, in response to the requirement of the cell, the ECM can be effectively modified. Conspicuously, this integrin based coupling materializes the obligatory

mechanoreciprocity which an adherent biological cell must retain in order to elicit adaptive responses to imparted stresses.

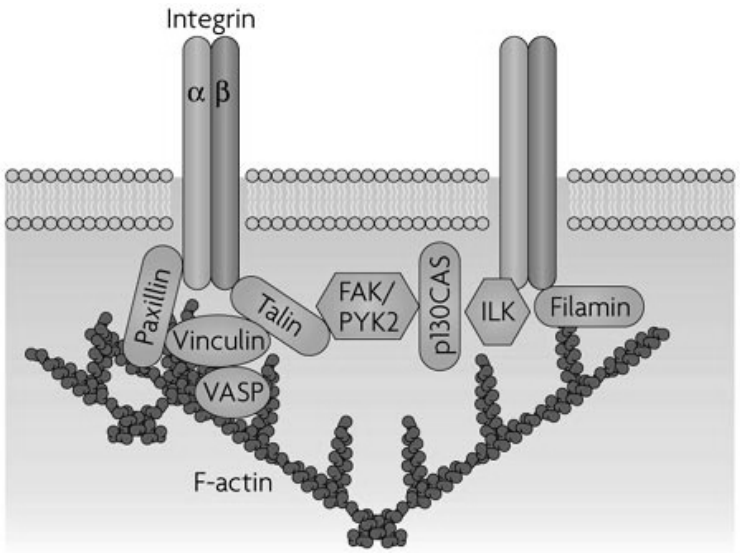

Figure 1.4. Schematic delineation of focal adhesion points with its components as reproduced from Billadeau *et al.* (2007).

Beyond this critical physical function, the member of focal adhesion complexes are capable of triggering explicit signaling events which culminate in the alteration in cytoskeletal architecture and cell physiology. In this regime, noticeably, the physical and chemical forces are extremely entangled in the sense that often localized confinement of physical stresses results in the conformational alteration of a protein component (such as FAK) and rest of the biochemical signaling just succeed from there. Pertinently, the FA induced signal transduction events are initiated during assembly or disassembly of focal adhesion complexes, implicating the process kinetics itself is profoundly important in stress adaptation. As the turnover of FA complexes dictates the cell migration, cell adhesion and the rheology of cytosol, this process is firmly regulated in every stage of cell's life cycle. It has been delineated that while large traction forces stabilizes FA, insufficient cell-substrate adhesion and activation of specific intracellular enzymes such as Calpain can lead to disassembly of FA (Wang and Lin 2007). To this relevance, extracellular mechanical stimuli, which propagate through FA, can stimulate the adhesive growth, stress fiber formation and stabilization by activating key signaling proteins Src Kinase and Extracellular signal Regulated Kinases (ERK1 and 2) (Hughes-Fulford 2004).

1.4. Description of the Transport Phenomena Around and Inside Biological Cells

Attributing to the complexity of biological architecture at every level (from cell to tissue to organ) and case specific regulation constraints for overwhelmingly large number of chemical entities whose dimensions may differ as much as by four orders of magnitude, the diversity of mechanisms underlying the intra and extracellular transport is exceptionally huge. Fortunately, most of them can be, at least grossly, summarized under few broad classes namely transport through blood and lymphatic circulation, interstitial flow, diffusion through membrane, transport across ion channels, intracellular diffusion, cytoplasmic streaming and directed vesicle transport. Following, we will attempt to portray each of these in an abridged manner, with specific emphasis on those (e.g. blood circulation, interstitial flow, intracellular diffusion and directed vesicle transport) which predominantly influences the chemo and mechanotransduction processes.

Blood Circulation: Guided by the coordinated cardiac pumping mechanism, the flow of blood though capillaries whose size varies over three orders of magnitude (mm to μm) is the most efficient and rapid method of transporting solid and gaseous elements to distant organ locations. Relevantly, the fractal like branching network architecture of vasculature systems enhances the transportation process. From fluid mechanics perspectives, due to the abundance of double discoid shaped red blood cells (RBC) that rotates and tumbles while flowing, blood is primarily conceived as a Non-Newtonian fluid with shear thinning properties. In spite of the small vorticies around the RBCs, the flow is emphatically laminar except for the diseased or hindered location. Consequently, the laminar property of blood has been perceived to impart enormous influences on the physiological of vascular endothelial cells which are, in aligned monolayer, responsible for constructing *in vivo* capillaries. The coupled biochemical and mechanotransductional studies (Li *et al.* 2005) have divulged that while a unidirectional time-independent laminar flow promotes normal vasculature and endothelial arrangement, disturbed flow near arterial bifurcations points are capable of eliciting inflammation like response and catalyze the formation of atherosclerotic plaques (Hahn and Schwartz 2009). In recent times, the effect of shear stresses for laminar and disturbed flows on the survival of

cancer cells, during dissemination stages of metastasis, is being progressively highlighted (Hahn and Schwartz 2009). The blood flow, in general, can exert shear stress as high as 20 dynes/cm^2 (2 Pa) which is adequate to persuade alterations in cellular architecture and to stimulate several intracellular signaling pathways (Hughes-Fulford 2004).

Interstitial Flow: Interstitial flow is defined as the fluid flow through the three dimesional porous matrix structure of physiological tissues (Griffith and Swartz 2006, Rutkowski and Swartz 2006). The effect of interstitial flow is most prominent over interstitial cells including fibroblasts, tissue immune cells and tumor cells and adipocytes. Owing to reduced porosity (and hence enhanced resistance) of tissue matrices, interstitial flows are generally slower than the blood circulation velocity. Nevertheless, importance of this type of flow has been recently unfurled in the domain of morphogen (chemicals that dictate the cell and tissue morphology) concentration gradient generation in an autologus (self generated and regulated) process, yielding guided cell migration and tissue development (Griffith and Swartz 2006). Interstitial flow is driven predominantly by plasma which is drained out of a blood capillary through endothelial wall into the immediate lymphatics. In healthy adult tissues, the interstitial flow can be augmented by tissue-specific pulsations, respiration, dynamic compression (in bone) and organ movement. To specific implication, it has been illustrated to govern the cancer progression by governing the spatio-temporal distribution of tumor promoting chemical factors (e.g. VEGF) and infusing synergistic physical perturbation.

Intracellular Diffusion: For Brownian motion in dilute solution, root mean displacement is directly proportional to the time interval (Δt) during which the displacement has been studied and Diffusion Coefficient *D*, yielding the following relationship:

$$\left\langle x^2 \right\rangle = 6D\Delta t \tag{1.1}$$

However, in pertinence to intracellular diffusion or diffusion of molecules over biomembrane, this does not hold true for most of the studied macromolecules. There are three specific reasons for this namely molecular crowding, facilitated transport and diffusive inhomogeneity of solvent. First, cytosol can be approximated as dilute solution. In fact it is molecularly "crowded" to such an extraordinary extent that the diffusion is

significantly damped and the molecules are consequently thought to undergo "subdiffusion" (Weiss *et al.* 2004, Banks and Fradin 2005). In contrasting situation, intracellular propagation of specific molecules can be facilitated by molecular motors or secondary messenger. Though this is intrinsically a reactive mechanism, on the ground of coupled reaction-diffusion mass transport theory, reactive terms can be absorbed into the diffusion part, yielding a resultant augmented diffusive transport, known as "superdiffusion" (Caspi *et al.* 2000, Brangwynne *et al.* 2008). There is another way which is commonly believed to be a prime source for anomalous diffusion. Cytosol or biomembranes are highly heterogeneous and the heterogeneity varies in both space and time. Though, in principle, diffusion in heterogeneous medium can be modeled in the following way:

$$\begin{Bmatrix} j_x \\ j_y \\ j_z \end{Bmatrix} = -\begin{bmatrix} D_{xx} & D_{xy} & D_{xz} \\ D_{yx} & D_{yy} & D_{yz} \\ D_{zx} & D_{zy} & D_{zz} \end{bmatrix} \nabla c \tag{1.2}$$

These equations are significantly cumbersome and expensive for obtain even a numerical solution let alone any possibility of achieving an analytical expression. In the wake of such imposed problem, the diffusion equation (1.1) is empirically modified as

$$\left\langle x^2 \right\rangle = 6D_{eff}\Delta t^{\alpha} \tag{1.3}$$

where D_{eff} is the effective diffusion coefficient and α is scaling exponent parameter. Sub and super diffusion is respectively defined for two regimes of α i.e. $0 \le \alpha < 1$ and $\alpha > 1$.

Most of the intracellular transport processes ferrying small molecules such as potassium, calcium ions or comparatively bigger macromolecules such as proteins, RNAs, take the advantage of molecular diffusive transport (also know as passive transport). Only when the transport in direction of increasing chemical concentration appears inevitable, diffusive mixing is replaced by the active processes involving consumption of intracellular energy in form of adenosine tri-phosphate molecules. In case of extracellular transport, active processes being inaccessible, the role of diffusion becomes even more vital. One medically relevant case is the diffusion of drug molecules through biological cell and tissues. There are three sets of governing parameters that influence drug adsorption namely its physicochemical properties, chemical formulation

and route of administration. There exist several dosage forms such as tablets, capsules and solutions which additionally include other ingredients. A specific dosage form is preferred for a particular drug which is then appropriately administered by various routes encompassing oral, sublingual, parenteral, buccal, rectal, topical and inhalational. Irrespective of the administration route, drugs should be in solution to be absorbed into the specific cell or tissue. Relevantly, drug molecules are required to cross numerous semi-permeable cell membrane barriers prior to reaching the systemic circulation. The process is executed through passive diffusion, facilitated passive diffusion, active transport, receptor mediated endocytosis or pinocytosis. Among these mechanisms, the passive diffusion is the most common and energy inexpensive manner by which the intracellular inclusion of drug molecules takes place. Drug diffusion predominantly occurring between high to low concentration, the diffusion rate is expected to be solely proportional to the concentration gradient. However, in physiological systems, effective diffusion coefficient of the drug molecules depends upon lipid solubility of drug, molecular density of extracellular space, molecular ionization and the area of absorption surface. Cell membrane being composed of lipid bilayer, diffusion of small un-ionized lipophilic drugs is favored to the highest extent. Given that the ionized molecules are weakly lipophilic, the administration route of a particular drug depends on pKa of the drug and pH of the relevant physiological fluid onto which the drug is predominantly absorbed. Hence, while for weak acids, administration through low pH medium such gastric fluid (pH 1.5) is preferred, weak bases are principally injected directly into the blood stream (pH 7.4).

Vesicle Trafficking: Many of the cellular components such as lipid rafts are packaged inside intracellular vesicles and are transported within cytosol (e.g. from Golgi apparatus to plasma membrane and vice versa) with the aid of motor proteins namely kinesin and dynein. Powered by the energy from ATP hydrolysis, these molecules along with the attached vesicles can move as fast as 3 μm/s, following the microtubular tracks. As stated earlier, during mechanotranduction, the trafficking rate can be efficiently regulated, yielding modifications in compositional distribution.

1.5. Signal Transduction in Cellular Domain

Signal transduction refers to the process by which cell converts one externally or internally generated stimulus to a sequence of biochemical reaction steps towards generating the ultimate response. A typical signal transduction pathway is consisted of receptor (that initiates the event upon conformationally activated by a specific stimulus), adapters (those facilitate the transmission), enzymes (those catalyses required conformational changes of adapter) and secondary messengers (those amplify the response reaction), most of which are essentially proteins. Owing to the overwhelming diversity of the relevant stimuli, the number of chemically response intracellular routes or pathways is exceptionally large. Often signal transduction components are shared between structure-functionally distinct pathways, rendering a cob-web like linkage diagram. Typical time scale of a signaling event can persist from milliseconds (for small ion e.g. K^+, Ca^{2+} currents) to minutes (for most of the protein dependent transduction events) even to days (which involves gene expression and large morphological as well as phenotypical alterations). Though according to upcoming understandings, signal can be of chemical or mechanical types, for sake of brevity, in this section, we limit our discussion to relatively conventional and well-studied chemical signal transduction only. Mechanotransduction has been discussed elsewhere (Section 1.1).

Universally, the chemical entities capable of eliciting the signaling response in a mammalian cell are either small sized molecules or polypeptides containing limited number of amino acid units. These molecules can either be synthesized by the cell itself (autocrine) or be released by the cells in the vicinity (paracrine) or be transported through bloodstream and interstitial flow from a distal origin (endocrine like hormones). From the functional perspectives, the stimulating molecules are classified as Growth Factors (e.g. Epidermal Growth Factor, EGF), Cytokines (e.g. Interferon-γ, IFNγ), Hormones (e.g. Insulin), Extracellular Matrix Components (e.g. Fibronectin), Neurotrophins (e.g. Nerve Growth Factor, NGF) and Neurotransmitters (e.g. Acetylcholine). In addition to these, signal transduction process may be triggered by alteration of ion concentration in neighboring environment or by the cell-cell binding. While the former is mediated by spontaneous structural modifications and opening of membrane located ion channels, the

later process involves direct inter-molecular coupling and is particularly customary in the immune system.

Upon approaching the cells, these molecules (also called ligands) bind to a specific receptor protein localized on cell membrane. Cell surface receptors are fundamentally integral membrane proteins, consisting of an extracellular ligand-binding binding part, an intermediate transmembrane helical segment (with hydrophilic core and hydrophobic surface interfacing lipid membrane molecules) and an intracellular fragment capable of initiating enzymatic activity. When a ligand binds to the extracellular antenna, the conformation of intracellular section is altered to unleash the enzymatic property. Consequently, the generic signal transduction proceeds through the phosphorylation (addition of phosphate groups from intracellular ATP molecules) of one or multiple Tyrosine, Serine or Threonine residues located on intracellular segment. This phosphorylation event then, in response, intrudes deep in the cytoplasm by the recruitment several transitional adapters which are also phosphorylated and thus, activated in sequence. In biological jargon, the ability to catalyze the phosphate transfer process is known as kinase activity which constitutes the central dogma of biochemical signal transduction. Although there exist several receptor classes according to their ways of triggering the downstream cascade or binding to a particular ligand type, from the perspective the current thesis, Growth Factor Receptors or Receptor Tyrosine Kinases (RTKs) such as EGFR and Integrins are enormously consequential.

Receptor tyrosine kinases (RTKs) generically contain an extracellular ligand-binding domain, a transmembrane domain and an intracellular kinase domain. For RTKs, ligand binding spontaneously prompts receptor homo-dimer formation and stimulation of tyrosine autophosphorylation within the cytoplasmic tyrosine kinase domains. The activation of kinase domain then initiates cascades of phosphorylation of downstream effector molecules such as Raf, Rac, Ras, Mitogen Activated Protein Kinase (MAPK), ERK, Grb2-SOS etc, few of which migrate to nucleus and regulate the gene expression (conversion of genetic information to working protein molecules) kinectics in order to control cell division, differentiation and migration.

Integrins mediate cellular adhesion and attachment to the extracellular matrix and inter-cell adhesion junctions. In this way, integrins uniquely act as the two-way signal

transducers. In one way, it converts the alterations of internal process kinetics into the perturbation to cell-surface traction forces while in other direction it facilitates the cell to sense the stiffness and the chemistry of extracellular matrix. Integrins bind to several extracellular matrix components such as fibronectin, collagen and laminin. The signal transduction through integrins engage multiple linker and adaptor molecules including focal-adhesion kinase (FAK), talin, paxillin, parvins, p130Cas, Src-family kinases, integrin-linked kinase (ILK) and Rho family small GTPases. As illustrated by previous research investigations, cell survival, proliferation, differentiation and apoptosis are actively controlled by cooperative interactions between integrin and receptor tyrosine kinase stimulated signaling events.

1.6. Confinement Effects: What to look for?

Fluid mechanics and interfacial phenomena over small scales are not only faceted with intriguing flow physics, but are also attributed to an intense interfacing with chemistry and biology over disparate spatio-temporal scales, often in a rather non-trivial manner. One of the pertinent open questions that remain to be comprehensively addressed in the literature concerns the two-way inter-connection between fluid dynamics and cellular dynamics in narrow confinements, having particular relevance to the fundamental understanding of cancer progression in biophysically relevant micro-environments (Tschumperlin *et al.* 2004, Jain 2005, Griffith and Swartz 2006, Anderson *et al.* 2006, Miles *et al.* 2008, Kumar and Weaver 2009). In reality, in the most prevailing circumstances, the biological cells, which serve as obstacles in the path of fluid flow and are of comparable size as that of the confining channel spaces, diminish the effective of flow passages significantly (Gaver and Kute 1998). As a result, the shear forces imparted by the incipient fluid flow become non-trivially augmented from their linearly approximated magnitudes. The situation may be further complicated by the dynamically adapting shapes of vivid cellular bodies and variable inhomogeneous composition and elastic properties of flow-exposed cell membranes. As a consequence, the non-trivial interactions between fluid mechanics and adaptive shear responses of cells in micro-confinement still remain to be poorly understood. This deficit stems from the difficulties in addressing the intricacies of fluid mechanics pertinent to dynamically evolving

confined biological cells and the perturbed biochemical and biophysical responses in cancer cells because of exclusive microfluidic interactions, which are bi-directionally coupled. With concurrent genetic and molecular studies failing to pin-point a consensus causal origin for favorable survival capabilities of cancer cells on tissues confinements and circulatory flow passages of micrometer dimensions, adopting a system approach towards quantifying the demarcating stress-responses of cancer cells in relevant microconfinement becomes an irrefutable necessity.

As we learn from the existing researches on shear stress exposed endothelial cells (Li *et al.* 2005) and metastasizing cancer cells (Kumar and Weaver 2009), the most important bio-physical responses that are believed to influence this dynamic adaptation of a cell to a stressed condition, as a part of its survival strategy, are the modification of the cell membrane fluidity (Butler *et al.* 2001) and rearrangement of focal adhesion pattern (Shyy and Chien 1997). Subjected to fluid shear stress, both increase in membrane fluidity and disassembly of focal adhesion points have been marked in the upstream region, which, in turn, are expected to influence the downstream intracellular bio-chemical responses. While these changes have been separately documented, one may anticipate a biochemical-connectivity between them from the clue that during the enzymatic detachment of mouse embryonic fibroblast, the disassembly of focal adhesion complex elicits lipid raft internalization in Rac1 (a small RhoGTPase) dependent manner (Balasubramanian *et al.* 2007). This biochemical process being consensus in both cancer and normal cells, a mere qualitative description of the process itself does not provide any hint towards the elucidation of distinctive stress responsive features of cancer cells within the confinement of tissue matrices and circulatory systems. Rather, a kinetic description of the process, parametrized by the characteristic times of relevant processes such as focal adhesion disassembly and change in membrane fluidity, should be appropriate to quantitatively delineate the underlying distinctive features in a conclusively pinpointed manner. Fortunately, there are biophysical methods namely Traction Force Microscopy (TFM), Fluorescence Recovery After Photobleaching (FRAP) which endows us with the capability of quantitatively monitoring the stress-adaptive cellular response in real-time.

Further, given that, in physiological construct, most of body cells live in the confinement of tissue matrices or circulatory vessels, the confinement and the

mechanotransduction of confinement borne physico-chemical stresses have been anticipated to impart non-trivial effects on morphogenesis, tissue functionalization and most importantly, malign tumor progression. Initial characterization of confinement effects on the pertinent physiological mechanotransduction points towards involvement of autocrine ('self-acting') epidermal growth factor receptor (EGFR) in transduction of both chemical and mechanical stimuli. As mentioned in the previous section, this is a membrane-bound Tyrosine Kinase type receptor which is activated by means of phosphorylation at specific Tyrosine residues when either the corresponding ligand (EGF) binds to it or it is conformationally altered by a physical stimulus (Lambert *et al.* 2006). It has been demonstrated in bronchial epithelium, where a compressive shrinkage in lateral intercellular space effectively augments soluble autocrine growth factor EGF concentration and stimulates associated downstream signaling cascade (Tschumperlin *et al.* 2004, Hughes-Fulford 2004, Griffith and Swartz 2006). It is then expected that similar phenomena can arise in microfluidic systems as the degree of confinement is purposefully increased (Paguirigan and Beebe 2009). It is extremely pertinent to mention that the involvement of EGFR in confinement effect may not be limited in direct ligand mediated manner. Conspicuously, biochemical studies concerning the mechanotransduction of FSS in endothelial and osteoblast cells have divulged a protein activation pattern akin to EGF activated signal transduction (Hughes-Fulford 2004). This information, in consummation, compel not only to presume a prevailing role of EGFR activation in impending confined spaces of microfluidic systems but also to investigate any plausibility of EGFR involvement in FSS mechanotransduction. The background reports that EGFR mediates cellular adaptive response in terms of re-orientation, migration and modification of tissue architecture definitely serves the preliminary motivation (Griffith and Swartz 2006) for the studying EGFR activation level in response to entangled physico-chemical stimuli within microconfined spaces.

In pertinence, experimenting in actual physiological domain being almost impractical, microfluidic cell culture systems, by their striking similarity with physiological confinements, provides an array of opportunities to sensibly examine the process dynamics *in vitro*.

1.7. Literature Review

1.7.1. Microchannel Based Cell Culture System and Confinement Effect

The intrinsic ability of microfluidics to deliver chemical stimuli with the spatial precision comparable to sub-cellular dimensions has opened a new paradigm of cell biology with excellent capacity to address cellular problems at single cell level. With microfluidic devices it is possible to expose cells to the gradient streams of chemicals such as hormones, cytokines, growth factors or chemoattractants and measure the corresponding responses individually (El-Ali *et al.* 2006). As the cell physiology, phenotype and functionalities are governed by the neighbouring chemical and physical conditions, microfluidics creates a whole new way of determining the fate of a cell in vitro. Pertinently, it is the motivation of integrating several time consuming assay steps into solitary monolithic micro-platform that has swept the initial impetus of fabricating microfluidic systems for cell culture related appliances (Dittrich and Manz 2006). Again, while majority of microfluidics-based cell cultures devices are simply miniaturized versions of the conventional lab scale processes, build on the basis of low sample volume requirement and compatible scaling of biophysical forces (Squires and Quake 2005), there are ample instances of microflow systems that have been designed to exploit hitherto unknown regimes of cell biology. It is therefore little undermining to consider microfluidic devices as small scale petri-plates, rather they should be appreciated as the latest weapons for biologists to unlock the inaccessible realms of cell biology. It is the inability of macro scale cell culture system to accurately quantitate the cell–cell, cell–analyte and cell-extracellular matrix (ECM) interactions which have prompted the development of microfluidic-based cell culture systems (Folch and Toner 2000). In recent years, the integration of cell culture with microfluidic systems has resulted in multifunctional platforms for obtaining fundamental biolophysical and biochemical insights on cells and tissues, as well as for the development of cell-based sensors with biochemical, biomedical and environmental applications (El-Ali *et al.* 2006). Although most of the microscale applications concentrate on the short term responses by the cells confined within the microdomain, several essential cellular processes occur over longer time scales. For instance, cell–cell interaction, cell differentiation, and proliferation necessitate the development of a microfluidics-controlled long-term cell culture platform

with a homeostasis microenvironment (El-Ali *et al.* 2006). With this view, long-term cell culture platforms (Balagadde *et al.* 2005) have been manufactured with functionality of exposing cells to the gradient of analyte concentrations (Huang *et al.* 2005). Typically, cells are restricted into the desired regions of microchannels by micropatterning the channel surface with ECM proteins (which promote cell adhesion), or by utilizing hydrodynamic trap arrays (Wheeler *et al.* 2003) or dielectrophoretic trap arrays (Voldman *et al.* 2002). Confined cells are then exposed to spatially (Jeon *et al.* 2002) and temporally (Lin *et al.* 2004) regulated chemical stimuli in order to perceive the nature of intra-cellular signal transduction that occurs in response. Another class of applications that microfluidic devices are targeted for is the implementation of micro-biomimetics and tissue-engineering where basic operations of organ or tissue are explored with the variation of neighbouring micro-environment (Andersson and van den Berg 2004). In this context, sincere and continuing research endeavours with respect to the fibroblast, vascular (Borenstein *et al.* 2002), bone (Holy *et al.* 2003), neuron (Taylor *et al.* 2003), cartilage (Peterson *et al.* 2002), liver (Powers *et al.* 2002) and stem (Kim *et al.* 2006) cell cultures are manifested by numerous research articles that have been published within a very short time duration. Although there has been an enormous progress of microfluidics in the field of cell biology, future researches should be directed to comprehend the adverse effects fluid shear on cells (Leclerc *et al.* 2006) and the packaging of long term cell culture units into the portable microfluidic framework (El-Ali *et al.* 2006). Instead of the aforementioned progresses, the distinct cellular behavior in microfluidic conduit has remained unaddressed. Though Beebe and colleagues (Yu *et al.* 2007, Paguirigan and Beebe 2009) have raised the issue of restricted growth factor and metabolite diffusion in microconfined spaces, the subject persists to be neglected, in order to avoid analytical complicacies while miniaturizing.

1.7.2. Development of Traction Force Measurement

The attempts to quantitate the traction force exerted by the adherent cells on the adjoining substrate have been initiated through measuring cell-induced deformations Cell-populated collagen gel (CPCG) and thin silicone membranes (Wang and Lin 2007).

However, in last decade, the technology has been progressively sophisticated towards force sensor arrays and traction force microscopy (TFM).

Cell-Populated Collagen Gel (CPCG): Originally introduced by Bell *et al.* (1979), in this methodology, cells are embedded within a gel disk of collagen, an important component of extracellular matrix. As the cells start spreading, cellular traction force (CTF) triggers a contraction of gel-disk as well as an increase in diameter (Ehrlich 1988; Moon and Tranquillo 1993). Subsequently, CTFs can be determined from the alteration in gel diameter. In further developments, this protocol has been improvised by including a free-floating collagen gel which essentially acts as a cell force monitor (Campbell *et al.*. 2003; Delvoye *et al.*. 1991). The major limitation of seemingly straightforward process is its inability to estimate traction force of a single cell. Rather, by CPCG, the resultant traction force of a cell population is determined with little spatial resolution. The protocol is additionally complicated by dynamic cellular processes including incessant remodeling of the extracellular matrix gel itself.

Thin Silicone Membrane: In this method, the wrinkles formed by the adhering cells on a thin silicon membrane are treated as the markers for estimating the CTF. Use of this technology has been apparent in pioneering measurement forces due to fibroblast adhesion (Harris *et al.*. 1981), cytokinesis of individual cells (Burton and Taylor 1997) and migration of fish keratocytes. However, development of the system is predominantly inhibited owing to lack of accurate wrinkle characterization method. The generation of wrinkles due to CTFs being effectively an intricately anisotropic process, prediction of the governing dynamics is computationally infeasible.

Force sensor array: With the advent of photolithographic and micropatterning technologies, it has becomes pragmatic to fabricate an array of cantilever beams whose deformation in reaction to CTF enables refined determination of traction forces (Galbraith and Sheetz 1997). Though through tedious calibrations, accurate measurements of CTF can be achieved, the process is unfortunately unidirectional and does not encompass the entire cell-adhesion region. To eliminate this bottleneck, in relatively recent times, cellular micropost arrays have been proposed (Tan *et al.* 2003). While the underlying principle remains unaltered, micropost evidently covers entire basal membrane surface area, providing enhanced spatial resolution. Yet, the system

inadequately mimics the cell adhesion over a flat substrate and traction magnitudes can be evaluated only in discrete post locations (Wang and Lin 2007).

Traction Force Microscopy: TFM relies on measuring the resultant displacement of substrate-embedded fluorescent beads or any other marker due the deformation of the substrate onto which cell applies the traction force. Bead displacements are measured in effect to the cell attachment relative to the undisturbed substrate (cell being removed from it). Subsequently, from the displacement data, the causal traction force field is computed. After the introductory efforts by Dembo and Wang (1999), TFM has been utilized to explore several cellular scale biophysical processes (Butler *et al.* 2002) such as cell migration (Munevar *et al.* 2001, du Roure *et al.* 2005), response to DC electric field (Curtze *et al.* 2004), nature of adhesion on variable substrate characteristics (Lo *et al.* 2000). Algorithm to calculate TFM has been recently improved utilizing finite element method (Yang *et al.* 2006). Till date, surface modified polyacrylamide gels have been used as TFM substrate, which suffer from mild cytotoxicity and importantly (Wang and Lin 2007), from the inherent incompatibility towards the existing microfabrication technology.

1.7.3. Mechanotransduction and the Effect of Fluid Shear Stress

1.7.3.1. Generic Principles of Mechanotransduction – Current State of the Art

For all organisms, physical forces govern a diverse spectrum of physiological functions, in coordination with prevailing biochemical reactions. Frequently, it is the mal-regulation of mechanotransduction that harbors the undesired pathological consequences. In this respect, bulk of the original thrust in the field of mechanotransduction must be accredited to the discoveries that aberrant stresses in physiological system can confer diseased conditions such arterial blockage or atherosclerosis (Lehoux and Tedgui 2003) and bone degradation or osteoporosis (Favus 2003). Since then, unprecedented research endeavors (Hughes-Fulford 2004) have been directed towards elucidating the principle signaling cascades which are eligible to force induced activation (Fig. 1.5). First, it has been proposed that stretch-activated ion-channels in the cell membrane may be instrumental in converting mechanical stimulus to chemical signal (Martinac 2004). The hypothesis is necessarily based on the presumption that stress induced alteration in

membrane tension may mediate the conformational changes in the ion-channels, forcing them to open even in absence of chemical excitation. Investigators have delineated brain sodium channel-1 (BNC-1) as pivotal for touch sensation in mice (Price *et al.* 2000). Simple this may be, however the veracity of proposition remains dubious as results from molecular dynamics simulations continue to be far from conclusive (Orr *et al.* 2006). Nevertheless, there has been a mounting consensus (Orr *et al.* 2006) that in the molecular level, the transduction of physical stimuli can be accomplished through modification in protein conformation; much akin to chemical alteration (e.g. protein phosphorylation or other posttranslational modifications) mediating signal propagation (Kamm and Mofrad 2004). For large number proteins which are faceted to the structural integrity of biological cell, it has been demonstrated that the required strength of the physical stimuli to elicit a mechanoresponse is much higher than the thermal noise and sufficient enough to mediate conformational change towards influencing the reaction affinity (Geiger *et al.* 2009). Additionally, the polymerization and structure formation kinetics of cytoskeletal elements have been revealed to depend on the precise spatiotemporal nature of mechanical loading (Alenghat *et al.* 2004, Orr *et al.* 2006). It is important to note that cell stretching can infuse changes in the binding affinities of proteins linked to the focal adhesion complex, yielding either stabilization or disassembly of focal adhesion points (Orr *et al.* 2006). The fate of focal adhesion, in turn, dictates the dynamics of cytoskeleton and stress fiber formation, which primarily adjusts to balance the imposed physical stimulus. In spite of the molecular complexity of the cytosol, the process of mechanotransduction, here, is well-synchronized and exceptionally fast (Na *et al.* 2008). On the basis of this observations, researchers (Ingber 2006, Na *et al.* 2008) have conceived that efficient propagation of forces to the mechanosensitive elements (such as focal junctions, cytoskeleton) augments the sensitivity by amplifying small forces to large structural deformation. One emerged conception here is that forces (e.g. shear stress, substrate stretch) can be transduced through the intracellular structures to distant locations within the cell including cell-cell junctions, focal adhesions, nuclear membrane and intracellular organelles, without exhibiting significant dissipation (Hu *et al.* 2003, Na *et al.* 2008). While the process is certainly incredible, according to Ingber and his colleagues (Wang *et al.* 2001, Ingber 2006), this becomes possible owing to the tensional

integrity alias tensegrity of the discrete cytoskeletal elements (Wang *et al.* 2001, Ingber 2006). To date, most of the investigations have emphasized upon Focal Adhesion Complexes as the key signal transducer in mechanotransduction process (Geiger *et al.* 2009). Given that, in addition to cytoskeleton, FA is coupled to many proteins involved in a variety of biochemical signaling pathways including tyrosine kinases, inositol lipid kinases, ion channels, heterotrimeric guanine nucleotide-binding proteins (G proteins) and growth factor receptors, mechanical forces, either imparted to the cell-matrix junction or propagated outward from the cytoskeleton, congregate with biochemical culmination at the focal adhesion (Kamm and Mofrad 2004).

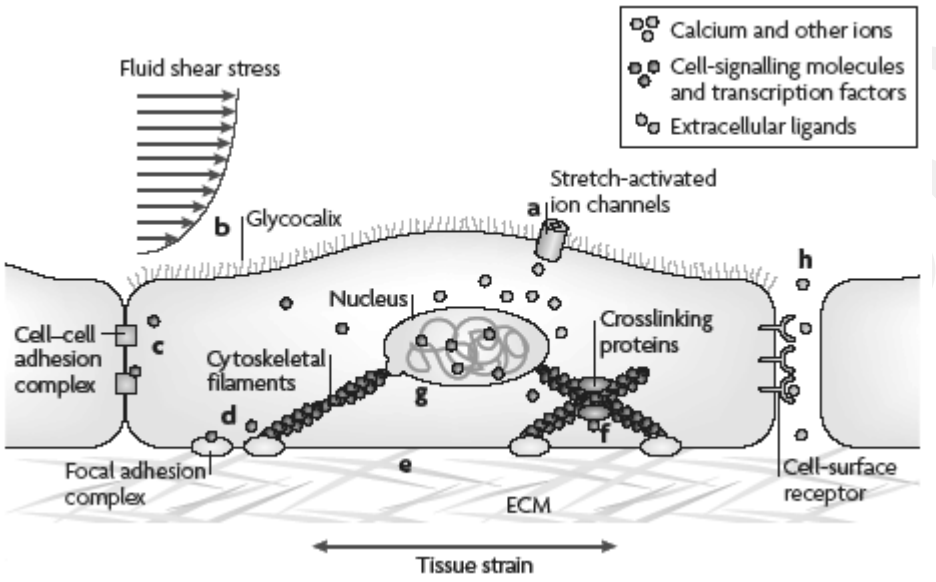

Figure 1.5. Schematic representation of major routes of cellular mechanotransduction, as reproduced from Geiger *et al.* (2009).

1.7.3.2. Effect of Fluid Shear Stress

Fluid shear stress is the most effective means of applying controlled stress over adherent cells. Most of the investigations in this field have involved laminar shear stress between 10-20 dynes/cm^2 (1-2 Pa) for endothelial cells and 7-24 dynes/cm^2 (0.7-2.4 Pa) for osteoblasts (Hughes-Fulford 2004, Li *et al.* 2005). Laminar shear stress of the aforementioned magnitude occurs within straight parts of aorta and bone matrix. Considering the implications of shear stress (Chien 2007), researchers have identified several mechanosensitive elements such as Plasma membrane, Integrins, Receptor Tyrosine Kinases, ion channels and G-protein coupled receptors (GPCRs) (Li *et al.*

2005). The effects of shear stress on the fluidity of endothelial plasma membrane have been investigated in a flow channel by utilizing the fluorescence recovery after photobleaching technique with lipid-binding carbocyanine dye (DiI) as the fluorescence probe (Butler *et al.* 2001). In comprehensive spatiotemporal elucidation, it has been observed that membrane fluidity in terms of lipid diffusion coefficient (D) increases in upstream location while decreases in downstream. Later, similar incident has been observed while the membrane fluidity is monitored with a molecular rotor fluorescent dye namely DCVJ (Haidekker *et al.* 2000). Interestingly, it has been noted while increase in membrane fluidity facilitates shear activation of ERK and JNK, application of cholesterol which decimates the fluidity, can inhibit the above-mentioned stress induced activation (Butler *et al.* 2002b, Li *et al.* 2005). However, a detailed mechanism dictating these phenomena persists to be obscure. One of the indirect consequences of shear induced alteration in membrane fluidity is the activation of K^+ ion channel Kir2.1, culminating in an inward-rectifying ion flux (Hoger *et al.* 2002). Shear has been observed to increase the influx of Ca^{2+} ions too (Yamamoto *et al.* 2000). The observed transient increase of increase Ca^{2+} level, then, has been anticipated to trigger several signal transduction pathways. Role of integrins in shear stress mechanoresponse is evident from the fact that inhibiting integrin binding to extracellular matrix down regulates the shear stress-induced changes in cellular function (Wang *et al.* 2002). For endothelial cells, shear stress directly activates (ligand independent route) vascular endothelial growth factor (VEGF) receptor Flk-1 which further promotes ERK and JNK activation through an adapter protein called Shc (Chen *et al.* 1999). Once, the signaling proteins such ERK, JNK are phosphorylated, these are translocated to cell nucleus and stimulate expression or attenuation of a number of functionally important genes (Hughes-Fulford 2004). In homologous experimentations on osteoblasts, increased levels of morphogenesis determining proteins cox-2 (cyclooxygenase-2) (Hughes-Fulford 2004) and c-fos (Bao *et al.* 2000) have been probed. It is conceived that cox-2 and c-fos expression is mediated by the components of MAPK pathway which may be inflamed by the activation of membrane receptors (e.g. EGFR, VEGFR) or focal adhesion components. Yet, the process by which such membrane receptor can be activated by shear stress has not yet been elaborated. Importantly, the shear stress of 12 dynes/cm^2 (1.2 Pa) has been found to

enhance the stiffness and the viscosity of cytosol matrix of Swiss 3T3 fibroblast cells in a Rho-kinase (ROCK) dependent way (Lee *et al.* 2006). Although this phenomenological change perceptibly influences the shear adaptation and mechano-response of biological cells in a profound manner, the original mechanism, the universality and the nature of stress induced hardening remains unknown (Lee *et al.* 2006, Butcher *et al.* 2009).

1.7.3.3. Mechanical Influence in Tumor Progression

While the role of mechanical stimuli in guiding the morphology of vasculature and bone matrix is indubitable, only recently scientific interests have been focused to emphasize on the involvement of physical forces in tumor progression and metastasis (Kumar and Weaver 2009). In the sense that tumor growth and metastatic development proceeds through intrinsic genetic changes and integrated response of the tissue establishment, the precise physical nature and the dominating forces of neighboring microenvironment have appeared to be eloquently pivotal (Miles *et al.* 2008). With the startling ambiguity over biochemical marker for tumor invasiveness, researchers have begun to appreciate the role of mechanical phenotype encompassing the elasticity, geometry and topology of the extracellular matrix that synergistically orchestrate with the biochemical signaling pathways (Anderson *et al.* 2006, Kumar and Weaver 2009). For example, in mammary epithelial cells (MECs), normal acinar structure is disrupted towards developing tumor phenotypes when the stiffness of the *in vitro* extracellular matrix is changed from the normal physiological value to comparatively higher magnitude of *in vivo* tumor stroma (Butcher *et al.* 2009). Meticulously analyzing the fate of such cell population over a gradient of matrix rigidity, adjoining biochemical events such as integrin clustering, FA stabilization, ERK activation, ROCK dependent stress fiber formation and contractility have been unraveled (Paszek *et al.* 2005).

Further, during the invasive stages of tumor, the force generating mechanism in the protrusive end (known as invadopodia) has been demonstrated to play an important role (Yamaguchi *et al.* 2005a, Kumar and Weaver 2009). Formation of well-organized invadopodia entails extremely localized force generation by actin polymerization and harmonious activation of several microfilament-binding proteins such as cofillin, Arp2/3, and N-WASP (Yamaguchi *et al.* 2005b). However, the protrusive forces could have gone

ineffective, if coordinated degradation of basement by matrix metalloproteases (MMPs) were absent (Kumar and Weaver 2009). Pertinently, how these diverse processes remain so well-synchronized even at multicellular level remains elusive. Relevant to the prevalence of mechanical forces during tumor progression, stresses from the interstitial (Griffith and Swartz 2006) and blood flow (Miles *et al.* 2008) seem to impart prolific impact on the success frequency of distant colony formation. At the morphologic level, interstitial stress can assist in blood vessel formation (angiogenesis) by promoting VEGF expression predominantly through hypoxia (oxygen depletion in extracellular space) (Harris 2002, Roose *et al.* 2003). Again, exposure to shear stress can stimulate myriad signaling pathways in cancer cells, culminating into drastic rearrangement of the cytoskeletal network and focal adhesions (Roose *et al.* 2003, Kumar and Weaver 2009). This, in turn, ultimately promotes structural reinforcement and enhanced adhesion to the vascular wall. When subjected to shear stress, tumor cells have been observed to initiate strong adhesion formation and remodel the extracellular matrix (von Sengbusch *et al.* 2005, Thamilselvan *et al.* 2007).

1.8. Aim of the Present Thesis

1.8.1. Important inferences drawn from the literature review

Despite the fact that microfluidics based cell culture system has extended its applicability in vast arena of diagnostic devices and biomedical assay systems, the fundamental of cell-system interaction within microcofined spaces remains uninvestigated. With the startling non-triviality and immense physiological relevance, such interactions hold key towards comprehending the distinct survival advantage of cancer cells in the microconfinement of tissue matrices and vasculature network.

The bottleneck against the elucidation of cellular dynamics within microconfinement arises due to the intricate nature of cell fluid interaction and the absence of compatible quantitative technology which will enable the precise determination of the biophysical state of the cell. Owing to the deficiency of ample air-water interface within microfluidic conduits, a juxtaposed advective-diffusive transport mechanism in form of fluid flow dictates the supply of nutrients, soluble gases and necessary growth factors. In contrast, it is well-recognized that microfluidic systems

intrinsically foster lethally large shear rates because of the reduced characteristic length scales and larger relative obstacle dimension. Hence, the fluid flow in such domain should be regulated to optimal magnitude depending upon the biophysical state of adhered cells and their response to the imparted shear stimulus, in order to eliminate the detrimental consequences of fluid flow and simultaneously, to develop a homeostatic cell-culture microenvironment. However, this feat cannot be accomplished without the elaborated consideration of biophysical states of the cell. The cell-surface traction force symbolizing the physiological state of an adherent cell, integration of Traction Force Microscopy with cell-culture platforms immediately appears to be a viable option. Yet, till now, no such endeavor has been undertaken owing to the incompatibility between conventional polyacrylamide based traction force measurement protocol and material characteristics of microfluidic devices.

Again, while it is known that cellular adaptation to shear stress is predominantly mediated through the spatio-temporal alternation of plasma membrane fluidity and the arrangement of focal adhesion points throughout the basal surface, the dynamic quantification of the aforementioned biophysical processes with inception of fluid shear stress in the milieu of microfluidic conduits remain unaddressed. Further, the specific molecular mechanism underlying reorientation of membrane fluidity is not well-characterized. Through a non-trivial alteration in the fraction of ordered (less flexible solid-like raft complexes) and disordered (liquid-like phospholipids layer) phases in a dynamically evolving manner, one may explicate the stress-responsive regulation of plasma membrane fluidity, which has remained far from being clarified. Most importantly, in order to gain a deeper understanding of remarkable stress adaptive character of cancer cells, the kinetics of these processes must be compared for normal as well as malignant specimens. Dynamical natures of these events incidentally impose one of the most challenging problems of biomicrofluidics, whose consequence is easily envisioned in the prognosis of myriad pathogenic incidents, including a very special one called metastasis, a truly lethal phase in cancer progression.

From the previous research reports pertaining to fluid shear stress mediated mechanoresponse, two consensus trends become obviously apparent. First, the activation pattern of intracellular signaling proteins in response to FSS resembles that of Epidermal

Growth Factor Receptor stimulated downstream signaling. However, it remains uncertain how EGFR activation is elicited in response to incipient shear stress. In the scale of microconfinement, EGFR phosphorylation due to mechanical stimuli can be overtly entangled with the contribution from ligand mediated activation, upholding essentially a scenario where linkage between chemo and mechanotransduction becomes remarkably consequential in determining the eventual landscape of molecular activity. Second, the molecular processes involved in mechanotransduction often culminate into the alternation of intracellular rheology, which in turn, endorses a variety of biological progressions including flow-adaptation, migration and phenotypic morphogenesis. Consummating the existing evidences, seemingly disagreeing rheological behaviors may prevail during the continuation of a single event such as metastasis. Yet, it is not adequately enlightened what governs the tight regulation of intracellular rheology under stressful circumstances.

1.8.2. Problem Definitions

1.8.2.1. Introduction of Microfabrication Compatible Traction Force Microscopy for Monitoring Cellular State within Microconfinement

Considering the incompatibility of exiting polyacryalmide based traction force microscopy technology, we develop a microfabrication compatible force measurement technique based on low-elasticity elastomers termed as Ultrasoft-Polydimethylsiloxane based Traction Force Microscopy (UPTFM). This technique is devised for mapping the cellular traction forces imparted on the adhering substrate, so as to depict the physiological state of the cells surviving in the micro-confinement. We subsequently integrate the technique with a microfluidic platform for evaluating different states of stress in adherent mouse skin fibroblast L929 and cervical carcinoma HeLa cells. Utilizing this technique, we monitor the spatio-temporal evolution of cellular traction forces for static incubation periods with no media replenishment as well as for dynamic flow conditions that inherently induce cell deformation and detachment. While the studies conducted on a quiescent fluid medium should enable us in obtaining an optimal static cell incubation period, those executed under dynamic flow conditions can provide us with the minuscule details of the cellular response, deformation and detachment processes.

1.8.2.2. Probing the Existence of Confinement Effect

Metastasis is segregated into several stages, of which shear stress adaptation of cancer cells during the "life in transit" has been the least illuminated phase. Concerned by the inconclusive culmination of prior genetic studies which have grossly neglected the effects of extracellular environment, specifically the fluidic confinement onto which cells live and adapt, we have attempted here to quantify the effect of confinement in cancer progression. Our primary objective has been essentially focused to answer the following challenging question: 'does the size/extent of the surrounding fluidic confinement play a controlling/decisive role in dictating the effective survival capability of a cancer cell?' Standard cell-culture studies have often turned out to be elusive in characterizing the underlying details, primarily attributable to their apparent limitations in resolving these events on substantially small physical scales consistent with realistic bio-physical confinements. Towards this, we first establish a strategy for pinpointing the stress-responsive spatio-temporal events leading to the activation and regulation of the intracellular signaling, which are executed primarily through lipid rafts of apical membrane surfaces and focal adhesion (FA) points at cell-surface contact. From the perspective of combined reaction-diffusion transport, we have endeavored to probe the hitherto elusive existence of amplified spatiotemporal extents of intracellular signaling within microconfinement.

1.8.2.3. Molecular Origin of Confinement Effect and Its Implications on Rheology of Cell Cytoplasm

Deriving from the clues given in the preceding studies partially related to the effect of physiological confinement on tissue morphogenesis, we enquire whether the aforementioned confinement effect in terms of amplified response kinetics of cancer cells can be linked with the pattern of EGFR activation. Given that this membrane receptor can be activated by both ligand-mediated and direct shear stress induced ways, we investigate the entanglement of these processes in defining the ultimate cellular kinetics. Again, we examine the original basis of stress activation of EGFR through fluid flow induced perturbation to membrane raft elements, which has remained hitherto unexplored. As we attempt to delineate the landscape of downstream molecular stimulation, succeeding EGFR activation, its implication on cytoplasmic viscosity has also been questioned. En

route, we have introduced a novel molecular rotor based measurement technique of intracellular rheology.

1.9. Outline of the Thesis

The remaining part of the thesis is organized as follows.

- In Chapter 2, the fabrication process of microfluidic cell culture platform has been given, along with basic fluid flow measurements and temporal characterization of cell compatibility.
- In Chapter 3, the novel microfabrication compatible cell-substrate traction force measurement technique namely Ultrasoft-Polydimethylsiloxane based Traction Force Microscopy (UPTFM) has been introduced. With the help of UPTFM, the evolution of cellular state is delineated for quiescent culture condition as well as during inception of fluid shear stress.
- In Chapter 4, using coupled UPTFM and FRAP platform, the kinetics of cellular adaptation to imparted shear stress has been elucidated for normal and cancer cells, both in microconfinement and non-confined conditions. The drastic reduction in response time in microconfinement (termed as "the confinement effect"), exclusive for cancer cells has been revealed in this chapter.
- In Chapter 5, the molecular origin of confinement effect has been identified as the Synergistic overlay of ligand-dependent and stress mediated activations of EGFR through disruption of lipid rafts. In this chapter, downstream consequence of EGFR activation is mapped and its impact on intracellular rheology has been deciphered.
- Finally, in Chapter 6, important inferences drawn from the studies executed as a part of the present thesis and the scope of relevant future works are presented.