DEVELOPMENT OF A NOVEL CELL TRACTION FORCE TRANSDUCER BASED ON CHOLESTERYL ESTER LIQUID CRYSTALS

Characterisation, quantification and evaluation of a cholesteryl ester liquid crystal based single cell force transducer system

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ABSTRACT

Development of a novel cell traction force transducer based on cholesteryl ester liquid crystals

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Keywords: cholesteryl ester liquid crystals, cell force transducer, cell traction force mapping, lyotropic liquid crystals.

Abstract:

In biomechano-transducing, cellular generated tension can be measured by soft substrates based on polymers but these techniques are limited either by spatial resolution or ability to detect localised cell traction forces (CTF) due to their non-linear viscous behaviour under shear rates. A newly developed cell traction force transducer system based on cholesteryl ester lyotropic liquid crystals (LCTFT) was developed to sense localised traction forces of human keratinocyte cell lines (HaCaTs), in which the length of the deformation line induced represents the intensity of the CTF exerted. The physical properties of the cholesteryl ester based lyotropic liquid crystals (LLC) were characterised by using polarising microscopy, rheology, atomic force microscopy (AFM) based nano-indentation, spherical indentation, and micro-tensile tests. The interactions of LLC with cells were studied by using cell viability studies, cytochemical treatments, widefield surface plasmon resonance (WSPR) microscopy and various immuno-staining techniques. The results show that LLC is thermally stable (0 - 50 °C) and linearly viscoelastic below 10 % shear strain at shear rates of < 1 s^{-1}. AFM nano and spherical indentations show a good agreement on the Young's modulus of both determined at ~110 kPa which is close to the elastic modulus of the epidermis. The Poisson's ratio of LLC was determined at ~0.58 by using micro tensile tests. The biophysical interaction studies indicated that LLC is biocompatible and allowed cell attachment. Cell relaxation technique by cytochalasin-B treatment suggested that the attachment and contraction of cells on LLC was due to the contractile activity of actin cytoskeletons that are mediated by focal adhesions. The staining experiments showed that cells consistently expressed the same suites of integrins (α2, α3, α5 and β1) and ECM proteins (collagen type IV, laminin and fibronectin) on both glass and LLC coated substrates. Interfacial interaction of cells with LLC observed via the staining of actin and vinculin, and WSPR imaging suggest the association of marginal actin filaments and focal adhesions in attaching HaCaT cells to the LLC. Linear static analysis applied in the Finite Element model of focal adhesion-LC confirmed the compressive force patterns induced by cells. By applying cell relaxation techniques and Hooke's theorem, the force-deformation relationships of the LLC were derived and used for direct quantification of CTF in culture. The sensitivity of the LCTFT was implied by a wide range of CTF (10 - 140 nN) measured at high resolutions (~2 μm). Nonetheless, a custom-built cell traction force measurement and mapping software (CTFM) was developed to map CTF of single cells. Reliability of the LCTFT was evaluated by using a known pharmacological active cytokine, TGF-β1, in inducing contraction of human keratinocytes. This study inferred internal consistency and repeatability of the LCTFT in sensing contraction responses of HaCaT cells in a concentration dependent manner of TGF-β1. The overall LCTFT and CTFM software had shown good potential for use in the study of contraction and migration of keratinocytes.

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CHAPTER 1

INTRODUCTION

"This journey of a thousand miles begins with a single step"-Lao Tzu

1.1 Research Background

Pharmacology is an experimental science which involves studying the effects of chemically active molecules on physiological activity. As one of the cornerstones in new drug development, pharmacology investigates the effects of new drugs through screening for desired activity, determining the mode of actions and defining the drug therapeutic function. Pharmacological studies involving animals in biological assays started as early as the 19th century (Fastier and Reid, 1949). Mammals such as rats, guinea pigs, rabbits and dogs are the most commonly used experimental subjects. In pre-clinical testing, organs or tissues are excised surgically and prepared for pharmacological study in oxygenated physiological solution within an organ bath. The organ bath technique was first applied by Henrick Magnus in 1904 on a strip of small intestine (Fastier and Reid, 1949). Today, the organ bath is still being used extensively to investigate the physiology and pharmacology of various tissues such as muscle, arterial rings, uterine tissue, ileum, colon arterial and diaphragm. In an organ bath, a section of tissue is suspended between a fixed point and an isotonic or isometric force transducer in a pre-warmed Krebs solution (Bennett and Pettigrew, 1974). Rhythmic contraction and relaxation of the tissue creates variable load forces which the force transducer converts into electrical signals (Bennett and Pettigrew, 1974). In modern systems, the electrical signals are digitised and displayed on a computer monitor as traces of contractility. Stimulation of the tissue can be induced by chemical, electrical or physical means. The range of sensitivity in terms of force transduction of commercial systems is from 50 μN to 250 mN (ADINSTRUMENT Incorporation and DMT Incorporation).

New developments in the agricultural and pharmaceutical industries require stringent evaluation and assessment of compounds before clinical trials. Prudent pre-

clinical trials usually involve large numbers of rodent and non-rodent mammalian species (Ganter et al., 2005, Jacobson-Kram and Mills, 2008). Recently, the United States Food and Drug Administration (FDA) issued a guide on Exploratory Investigational New Drug (IND) to reduce animal exploitation and simultaneously to accelerate the development of new pharmaceutical agents (Jacobson-Kram and Mills, 2008). Similarly, a new directive concerning cosmetic products (2000/0077(COD)) aims at reducing animal testing by promoting the design of in-vitro toxicological assays. Thus, cell line based culture assays are attracting interest as an alternative for drug and cosmetic testing. However, to enable this highly sensitive and high throughput screening, accurate analytical techniques for sensing cellular activities are required. During pre-clinical testing, application of an agonist and an antagonist to a tissue in an organ bath induces contraction and relaxation of a tissue, which is measured in terms of force. The question is how did the drug trigger the action of the tissue? This analysis in a tissue constructed from many different cells types is difficult (for example, the same section of tissue may contain neurons, smooth muscle cells and fibroblasts), thus, the characterisation of cell physiological responses may provide more insights if determined by examining the contractile properties of the individual cells (Bitar and Makhlouf, 1985, Li et al., 2009, Ma et al., 2002).

Motivated by the need to assess single cell contraction induced by chemical stimuli, contractility assays measuring the change in average cell length of a large population of smooth muscle cells were developed to study the responses of smooth muscle to agonists such as endothelin-1, interleukin, C-terminal octapeptide of cholecystokinin, acetylcholine and methoionine-enkephalin (Bitar and Makhlouf, 1985, Moummi and Woodford, 1992, Dallot et al., 2003, Akiho et al., 2002). These studies identified a number of advantages of single cell based biological assays for the

pharmaceutical industry such as: (a) the easy characterisation of surface receptors as a consequent of drug action, and (b) highly repeatable screening (Dallot et al., 2003, Akiho et al., 2002).

Thereafter, many single cell based biosensors have been developed for use in pharmacological screening (Stenger et al., 2001, Park and Shuler, 2003). In a cell based biosensor system, the cell is the primary detector which converts the molecular signals into electrophysiological or mechanical signals; these signals are then transduced by a secondary transducer in the form of electrode, magnetic or optical detector. Overall, the techniques in measuring physiological responses of single living cells to analytes can be classified into two broad categories: electrophysiology and mechanobiological measurement systems. Each category has its own advantages and disadvantages.

Electrophysiological techniques originated from neuroscience, in which, systems were developed to study the electrical activity of nerves cells (Hodgkin and Keynes, 1955, Hodgkin, 1937, Gross et al., 1972). These systems have since been employed to study other electrogenic cells such as smooth muscle and cardiac myocytes (Thomas, 1972, Hara et al., 1986, Cranefield and Hoffman, 1958). These techniques involve the use of electrodes to sense the depolarisation or hyperpolarisation of action potential of cells attaching to the electrodes (Gross et al., 1972). Patch clamp (Kraft and Patt, 2006, Betz. W. J. and Sakmann, 1971), potassium chloride filled glass electrode (Dole, 1941) and planar patch clamp (Behrends and Fertig, 2007) technique are among the classical electrophysiology techniques developed to monitor the activities of individual ionic channels in the cell membrane. In these systems, the recording can be intracellular or extracellular in a cell culture. Unfortunately, these techniques are time consuming and because of their invasive nature, they often result in cell death. One of the alternatives is monitoring cell electrophysiology using micro-fabricated

extracellular electrode recording or microelectrode array systems (Kovacs, 2003, Fromherz, 2003). These systems rely on culturing electrogenic cells over miniaturised recording sites made of gold or indium-tin-oxide, or arrays of field effect transistor electrodes to monitor signals from those cells directly position over the recording sites (Fromherz, 2003, Kovacs, 2003, Thomas et al., 1972, Gross et al., 1977, Offenhausser et al., 1997). These systems have been used to generate dose response curves for known pharmacological agents (Yu et al., 2006) but are subject to limitations associated with sensitivity and reliability (Muthuswamy et al., 2005, Shoham et al., 2006)

In the measurement systems using microelectrode, the electrodes allow recording of electrical activity at single point of the cell membrane or tissue but this does not provide information about the spatial distribution of bioelectric activity over the cell membrane (Loew, 1996). To circumvent this issue, an optical electrophysiology technique has been developed which involves the use of potential-sensitive dyes or fluorescing proteins that are capable of changing the fluorescence intensity when these probes detected sub-millisecond membrane potential changes or intrinsic birefringence (Obaid et al., 2004, Cohen and Salzeberg, 1978, Loew, 1996). The potential-dependent characteristic is able to shift the excitation spectra, and thus allowing the quantification of membrane potential (Waggnoner, 1979, Cohen and Salzeberg, 1978). After the tissue or cell is perfused or injected with the potential sensitive dye, two-dimensional (2D) distribution of colour intensity along the cell membrane corresponding to the field potential can be visualised and recorded. Some dyes developed earlier such as cyanine dyes are believed to inhibit the metabolic activity or inducing photodynamic damage due to the strong illumination of these dyes (Waggnoner, 1979, Obaid et al., 2004). Therefore, the potentiometric dyes developed later (Potential-sensitive ANEP dyes, Invitrogen) aim to overcome these problems.

These dyes improved the signal to noise ratio and reducing dye internalisation (absorption into the nucleus). However, these dyes still needed to be solubilised in dimethyl sulfoxide (DMSO) and are limited by moderate phototoxicity (Obaid et al., 2004). Phototoxicity increases oxygen radicals in cell cultures and can be cytotoxic in the long term (Obaid et al., 2004).

Electrophysiological measurements are more suitable for electrogenic cells that fire large action potentials. Monitoring the physiological changes of epithelial cells is more related to the measurement of cell mechanical activity in terms of forces (for example, contraction or traction forces). Traction forces are defined as forces that a cell exerts tangentially on a substrate (Oliver et al., 1998). They are the indications of the contractile forces generated within the cell cytoskeletons. Studies on how the contraction forces are generated and transmitted by a single cell as traction forces to the adjacent cell and extracellular matrix (ECM) set the foundation for the development of cell traction force (CTF) measurement technique.

1.2 Cellular Adhesion, Contraction and the Measurements of Cell Traction Force

1.2.1 Epidermal Tissue

The skin is made up of three major layers, the epidermis, dermis and hypodermis (Gawkrodger, 2003). The epidermis forms the outermost layer of skin and is constructed from four layers known as the basal, spinous, granular and cornified layer (Figure 1.1). The epidermis protects the underlying tissues of skin from abrasion and contains four major cell types (keratinocytes, melanocytes, langerhans cells and

merkels cells) (Gawkrodger, 2003). Of these cells, the keratinocytes make up about 90 % of the epidermis (Rocchad and Barrodon, 2009).

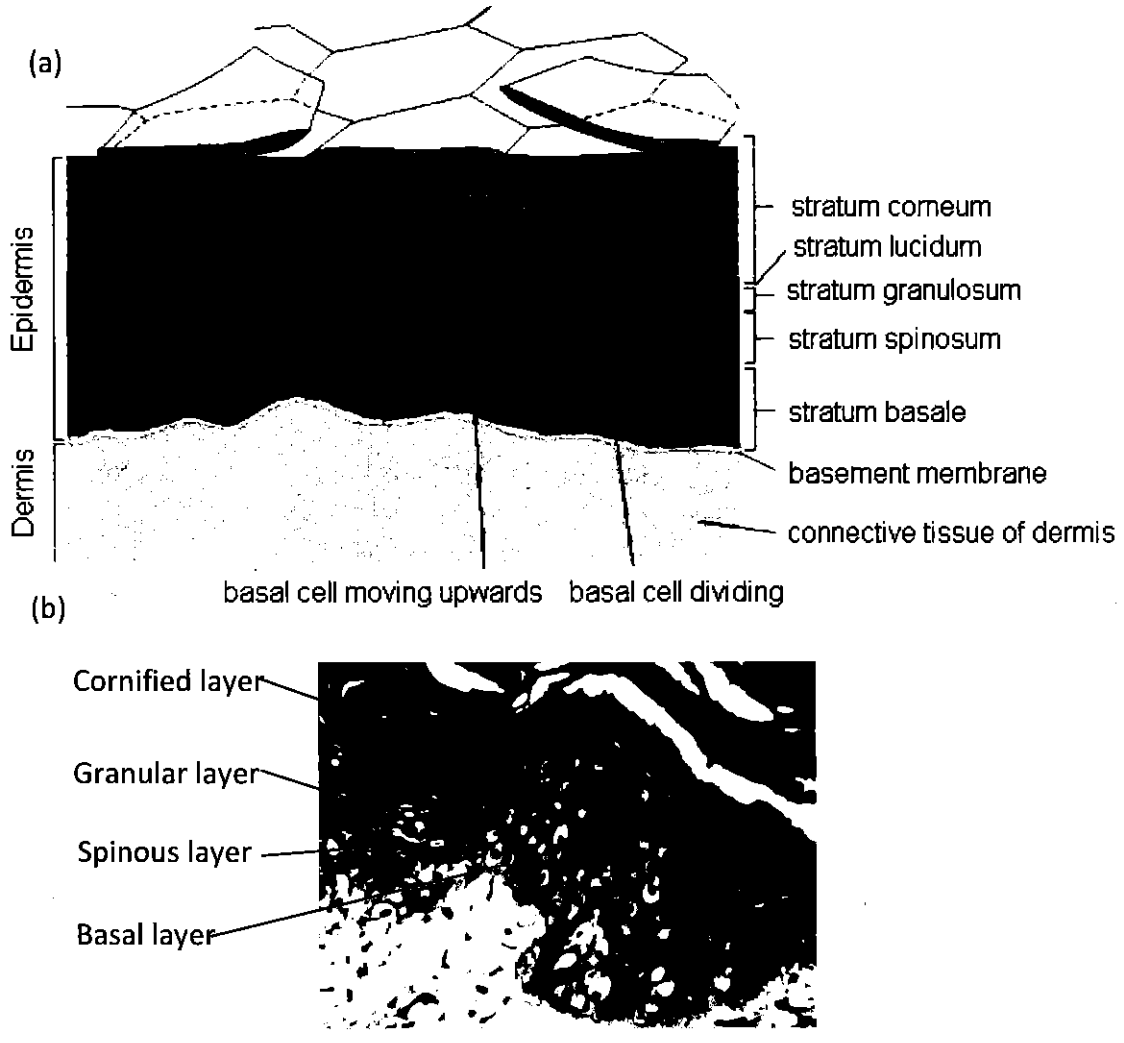

Figure 1.1. (a) A schematic diagram of epidermis and dermis (Alberts et al., 2002). (b) A photomicrograph of histological section for an epidermis. (Source: courtesy of School of Life Sciences, University of Bradford).

By division of keratinocyte stem cells at the basal layer, new epidermal cells (keratinocytes) are formed at the spinous layer (stratum spinosum) and further division continues to form the granular layer (stratum granulosum) (Hendriks, 2005). The presence of lipids in the stratum granulosum creates a hydrophobic barrier between the stratum granulosum and the stratum lucidum that prevents dehydration (Feingold, 2007). As the keratinocytes translocate superficially, the cells increase their

keratin content to protect the skin and underlying tissue from environmental damage such as ultra violet light and dehydration. Newly produced keratinocytes gradually differentiate and move upwards to replenish the overlying cells of the stratum lucidum (Gawkrodger, 2003). This process of terminal differentiation and migration continues until the cells die and become enucleated cells in the protective stratum corneum or cornified layer (Feingold, 2007).

Even though protected by the stratum corneum, the skin is delicate and can be wounded when being exposed to abrasion of sharp objects. However, under small levels of compression or shear on the skin surface, the tissue is elastic and can return to its original state upon release of applied stress. The elasticity and integrity of the skin is due to the structural organisation and strong traction forces between cells, and also, between cells and the extracellular matrix (Silver et al., 2003).

1.2.2 Cellular Adhesion

The contact which leads to the binding of a cell to a surface, another cell or the extracellular matrix is known as cellular adhesion (Dzamba et al., 2001). Cell adhesion is mediated by cell junctions (Vasioukhin et al., 2000). Cell junctions are divided into two broad classes, the intercellular junctions and the extracellular junctions. Intercellular junctions (Figure 1.2) are those that link cells to other cells such as the tight, gap, adherens and desmosomal junctions (Vasioukhin et al., 2000, Farquhar and Palade, 1963). Multiple adhesion molecules (for example, hemidesmosome and focal adhesions) are found in extracellular junctions and they function to attach cells to the extracellular matrix (ECM) (Farquhar and Palade, 1963).

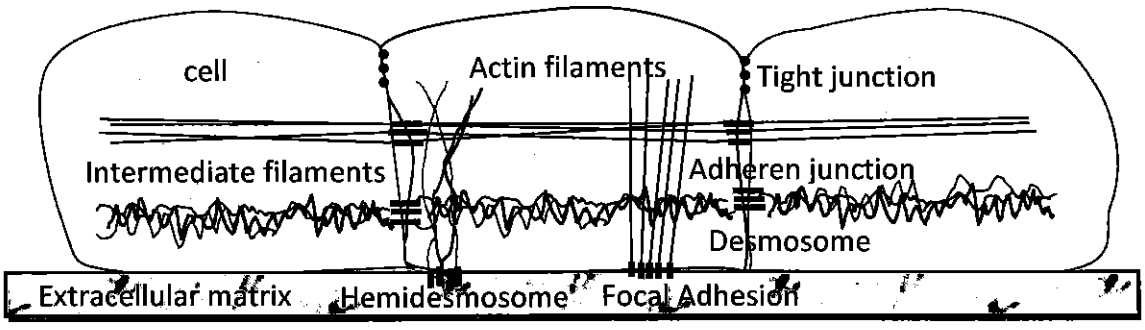

Figure 1.2. A schematic diagram of cellular adhesion.

Tight junctions (Figure 1.2) are formed from membrane proteins such as the claudins, occludins and e-cadherin that strongly couple the adjacent cell membranes (Schneeberger and Lynch, 1992, Furuse et al., 1998, Farquhar and Palade, 1963). As their names implies, molecules cannot pass through the sealed junctions, thus, allowing them to form a selective barrier. For example, it is this selective barrier in the endothelial cells that gives rise to the blood brain barrier (Wolburg et al., 1994).

Gap junctions are formed from membrane spanning proteins called the connexions. Gap junctions are continuous channels between two plasma membranes. These channels allow intercellular cytoplasmic communication and provide for cell-to-cell diffusion of small molecules, including ions, amino acids, nucleotides, and second messengers (Lampea and Laub, 2004). Gap junctions allow the propagations of action potential between cells (Rohr, 2004). Each gap junctions consists of 6 connexins subunits. These subunits are associated with the connexins of a neighbouring cell membrane, thus, forming channels that passes through the membrane of both cells (Lampea and Laub, 2004). Gap junctions are like valves and can be controlled locally. They are found between cardiac myocytes and smooth muscle cells that enable a coordinated contraction mediated by direct cell-cell impulse transmission (Lampea and Laub, 2004, Rohr, 2004).

Desmosome and hemidesmosome (Figure 1.2) are directly connected to the intermediate filaments at the extracellular junctions (Vasioukhin et al., 2000). Desmosomes link the intermediate filaments of a cell to the adjacent cell via calcium-dependent adhesion molecules or specialised cadherins called desmogleins and desmocollins (Gumbiner, 1996, Farquhar and Palade, 1963, Vasioukhin et al., 2000). Desmosomes are patch like junctions that are randomly distributed and fill the 30 nm gap between the attachment membranes (Green and Jones, 1996). Hemidesmosomes have a different function which is to connect the intermediate filaments of a cell to the ECM (Dzamba et al., 2001). They can be found in muscle cells or in stratified epithelial cells.

As shown in Figure 1.2, two extracellular junctions that are closely associated with the actin cytoskeleton are the adherens junctions and focal adhesions (Farquhar and Palade, 1963). Adherens junctions or Zonula adherens are complex proteins found at the sites of cell-cell adhesion (Farquhar and Palade, 1963). At the adherens junctions, the calcium dependent transmembrane proteins (E-cadherin) mediate cell to cell attachment (Vasioukhin et al., 2000). E-cadherins binds β-catenin which in turn binds α-catenin that link the actin cytoskeleton to the adherens junctions (Krendel et al., 1999). Focal adhesions are large macromolecules, comprise of a sub-membrane plaque which is made up of at least 50 different proteins and they mechanically bind the cell membrane to the ECM via specific transmembrane receptors (Matthews et al., 2006). Both the adheren junctions and focal adhesions connect to the actin filaments of the cytoskeleton and it is these actin filaments that exert traction forces on neighbouring cells and ECM (Burridge et al., 1986, Geiger and Ginsber, 1991).

In the epidermal layer, the ECM forms a complex meshwork which is produced and secreted by cells into their surrounding medium (filling between cells) (Buck and

Horwitz, 1987). In the connective tissue, fibroblasts mainly produce macromolecules in the ECM matrix. These macromolecules are formed from heavily glycosylated proteins giving rise to hydrogel like materials (such as, glycosaminoglycan (GAG) and proteolycans) which contain various fibrous proteins (O'Toole, 2001). Fibrous proteins embedded in the polysaccharides gel (Hay, 1981) are made up of two functional groups, the structural (collagen and elastin) and adhesive proteins (fibronectin and laminin) (O'Toole, 2001, Gray et al., 2000). Structural proteins help to organise the matrix and provide resilience (Hay, 1981, Hook et al., 1984). For example, the GAG and proteolycan gels withstand compressive forces or tension applied to the ECM while allowing the diffusion of nutrients and hormones between the blood and the cells (Hook et al., 1984). The adhesive proteins including collagen facilitate cell attachment to the ECM (Geiger et al., 2001). For example, laminin is associated with the attachment of epithelial cells to basal lamina, and fibronectin enables the attachment of fibroblasts and mast cells in the connective tissue (Kirfel and Herzog, 2004, O'Toole, 2001).

In the connective tissue of the dermis (Figure 1.3), collagen is the most abundant fibrous protein. This protein is characterised by long, stiff and triple-stranded helical structure of three single-polypeptide chains (or α chain) (Rich and Crick, 1955, Rest and Garrone, 1991). The single α-chain is constructed from many proline and glycine molecules (amino acids) (Rich and Crick, 1955, Rest and Garrone, 1991). About 28 types of α-chain molecules have been identified, including type I, II, III, V and XI collagen (Rest and Garrone, 1991, Heino, 2007). Type I is the principal collagen molecules that constituted the collagen fibres in tissue (Rest and Garrone, 1991). Type IV collagen molecules are network-forming collagen fibres that made up the meshwork

of the basal lamina, while type VII collagen binds the basal lamina to the underlying connective tissue (Heino, 2007).

Elastin is another structural protein and like collagen fibres are embedded in the ECM of skin, blood vessels and lungs (Hay, 1981). The network of elastin fibres exhibit elastic behaviour and they give tissues their elastic properties (Gotte and Serafini-Fracassini, 1963). The collagen fibrils are interwoven with the elastin fibres in order to prevent the tissue from over-stretching and tearing (Cleary and Gibson, 1983). The collagen fibres provide tensile strength while elastin fibres support tissue elasticity (Rosenbloom et al., 1993, Hay, 1981).

Fibronectin is an adhesive fibrous protein found in the connective tissue. This protein is a dimer composed of two very large subunits connected by disulfide bonds near the carboxyl terminal (Hynes and Yamada, 1982). Each subunit in the dimer has repeated sequences of what are referred to as type III fibronectin repeats (Hynes and Yamada, 1982). Along the repeating unit, there are binding sites for the heparin, collagen and specific transmembrane receptors found on the surface of the cells membrane (Hynes and Yamada, 1982). Some studies suggest that isoforms of fibronectin produced during wound healing specifically help in guiding cell migration and proliferations for new tissue development (Singer et al., 1999, Bartkova et al., 2003). Therefore, the adhesive function of fibronectin in attaching cells needs to be balanced with the needs of cell migration during wound healing (Larjava et al., 1993).

The basal lamina or basement membrane is formed from ECM proteins and it is especially rich in adhesion proteins (collagen, fibronectin and laminin) that are organised as a thin sheet underlying the epithelial cells. Hence, the basement membrane separates the cells from the connective tissue (Lillie et al., 1988, Larjava et al., 1993). In the epidermis, the basal lamina produced by the epithelial cells is divided

into two layers: lamina lucida overlying the lamina densa (Figure 1.3) (Laurie et al., 1982, Stanley et al., 1982b). The lamina lucida forms a boundary with the epithelial cells overlying the lamina densa (Laurie et al., 1982), whilst the lamina densa is connected to the underlying connective tissue by collagen fibrils (mainly made of type VII collagen molecules) (Laurie et al., 1982, Stanley et al., 1982b). The basal lamina containing the adhesion proteins can influence cells polarity, regulate cell metabolism and induce cells differentiation (Stanley et al., 1982b).

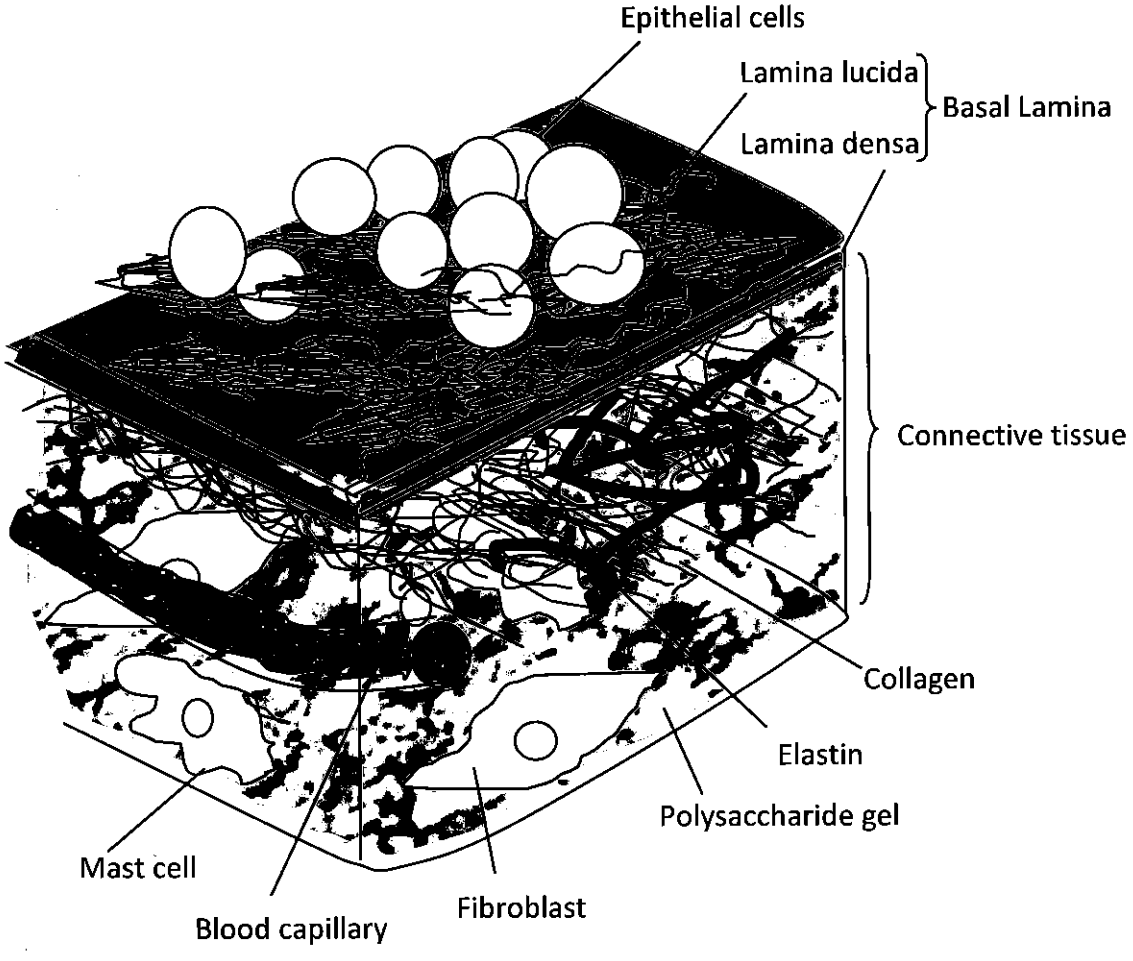

Figure 1.3. Structure of basal lamina and connective tissue

The exact composition of basal lamina varies from tissues to tissue (Pruniéras et al., 1983). A majority of the mature basal lamina consisted of type IV collage, laminin, perlecan, and entactin (Stanley et al., 1982b). During the early synthesis of basal lamina in the development of embryo, the basal lamina does not consist of collagen

type IV molecules but instead consists of a large laminin network (Turkesen et al., 1985, Pöschl et al., 2004). In the absence of collagen, laminin molecules are capable of self-assembling into a sheet in an in-vitro culture (Kirfel and Herzog, 2004, Pruniéras et al., 1983, O'Toole, 2001). Laminin has three polypeptide network that are disulfide bonded and this networks contains binding domains for collagen type IV molecules, entactin, perlecan and cell surface receptors (Beck et al., 1990). In large networks of basal lamina, laminin can directly bridge to the type IV collagen molecules which in turn, associated with type IV collagen, perlecan and entactin (Beck et al., 1990). In terms of the binding domains of laminin to the cells, fragment 1 and 8 of laminin are exposed as the adhesion sites for cell surface receptors (Aumailley et al., 1987).

1.2.3 Integrin Receptors and Focal Adhesion

The function of various types of receptors found on the surface of the cell membrane is to provide adhesion to the ECM proteins (Dzamba et al., 2001). These receptors are transmembrane proteins of a large family known as integrins. Integrins are classified as the heterodimers which are composed of two distinct chains, the α (alpha) and β (beta) subunits (Burridge et al., 1997). In cell-ECM attachment, the specific ligands in the ECM determine the type of α and β subunits of the integrins being expressed by a cell (Geiger and Bershadsky, 2001). Some examples of integrins are as given in Table 1.1. Multiple integrins exist on the cell membrane and they work co-operatively with the cell adhesion proteins in cell-cell and cell-ECM interaction (Hynes, 2002).

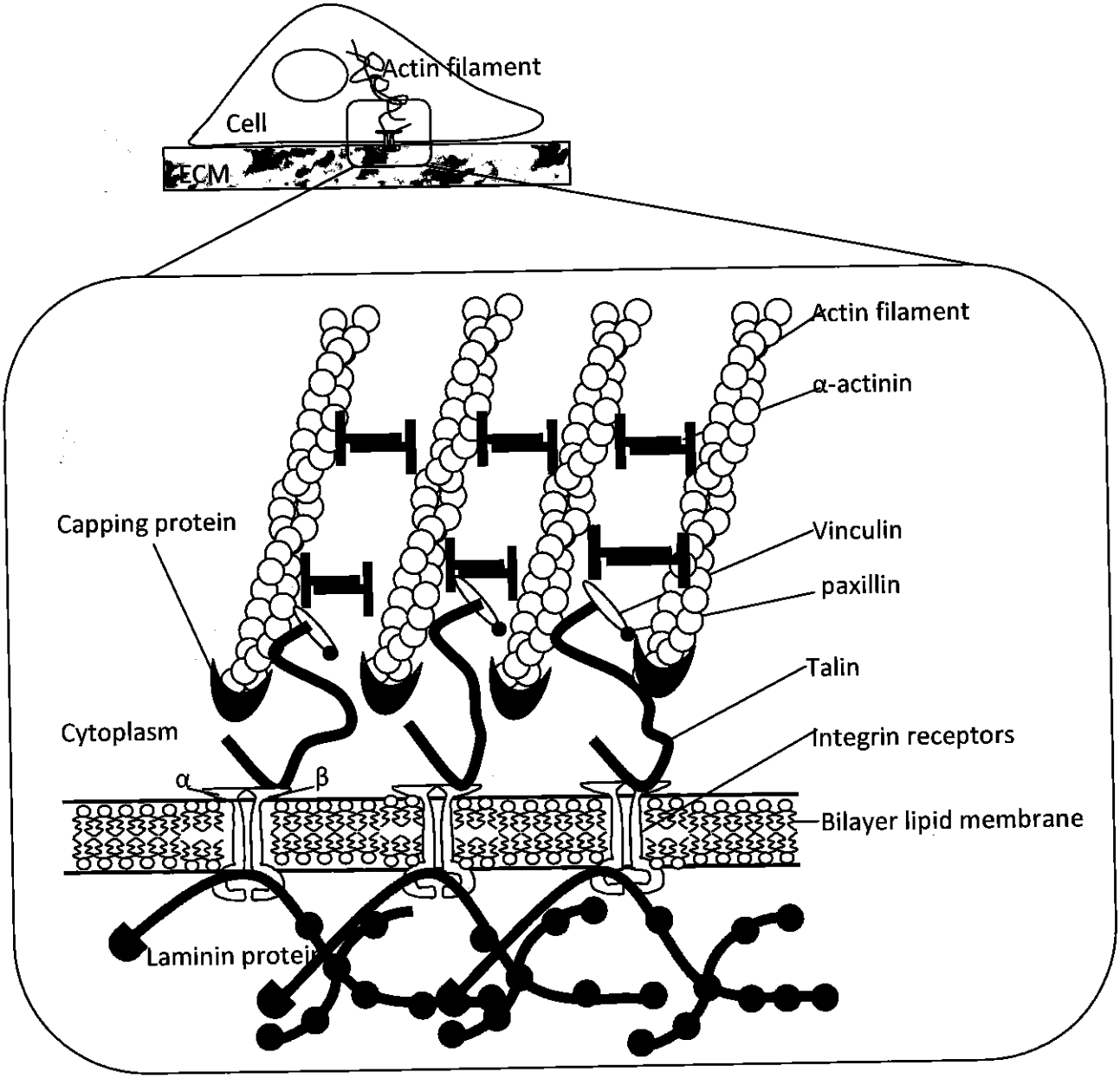

Figure 1.4. The structures of actin cytoskeleton, focal adhesion complexes, integrin receptors, and adhesion proteins.

Integrins have a dual function, that of transducing signals (either biochemical or physical) from the ECM to the cell and also the function of mediating the transmission of forces generated within the cell to the ECM (Geiger and Bershadsky, 2001, Hynes, 2002). Hence, they work as mechanosensors in sensing the stiffness of the ECM (Bershadsky et al., 2003, Rivelinea et al., 2001) whilst functioning as transmembrane messenger which trigger signals in regulating cellular attachment, migration and differentiation (Dzamba et al., 2001, Geiger et al., 2001).

Table 1.1. Associated ligands receptor identified for human keratinocytes migration.

Integrins	Ligands	Reference
α2β1	Type I Collagen	(O'Toole, 2001, Kim et al., 1992)
α2β1, α3β1	Laminin 5	(Decline and Rousselle, 2001, Kainulainen et al., 1998)
α5β1, α3β1	Fibronectin	(O'Toole, 2001, Kirfel and Herzog, 2004)

Focal adhesions (FA) or focal contacts (FC) located within the cell (Figure 1.2) are adhesion plaques which assist the actin cytoskeleton to anchor to the ECM *via* the integrin receptors (Figure 1.4). Focal adhesions consist of a number of proteins such as α-actinin, talin, paxillin and vinculin (Burridge et al., 1997, Geiger and Bershadsky, 2001, Wong, 1999) as seen in Figure 1.4. Among these protein molecules, vinculin is the major protein molecule at the sub-membrane plaque (Schwartz and DeSimone, 2008).

1.2.4 Polymerisation of Actin Filaments and Generation of Traction Force

Actin filaments of the actin cytoskeleton are the main machinery in regulating the contractility of a cell. Actin is a globular protein (Boron and Boulpaep, 2004), however, they can assemble and polymerise in a helical fashion to form filamentous actin double helix (Figure 1.5).

Figure 1.5. Assembly of globular proteins into long chain of actin filaments in helical structure.

The contractility of the non-muscle cells is dependent on the translational interactions between myosin-II and the filamentous actin (F-actin or sub unit of actin filament) (Langanger et al., 1986). Upon stimulation of cell contraction, the

phosphorylation of the F-actin allows the myosin II head to bind and sliding along the adjacent F-actin fibres in opposite directions (Peterson et al., 2004) as shown in Figure 1.6 (a-b). The mechanical actuation and phosphorylation of myosin II heads are driven by harnessing the energy released from adenosine triphosphate (ATP) when it is hydrolysed to ADP, this is regulated by myosin light chain kinase (Tan et al., 1992). This mechanism regulates the stretching and contraction of actin filaments in the longitudinal direction. To gain movements in transverse direction, α-actinin reduces the spacing between the two filaments by drawing bundles of actin filaments closer to each other (Figure 1.6c) (Pellegrin and Mellor, 2007). The collective actions of both myosin II and α-actinin in the actin filaments trigger the formation of stress fibres and cell contractility. With the binding of the actin filaments, FA and integrins receptor to the ECM, this creates a shear force exerted on the ECM, consequently, inducing cell surface traction (Lazarides and Weber, 1974, Pellegrin and Mellor, 2007).

Under 100x magnification of immunofluorescence microscopy, the stress fibres appear in a periodic "sarcomeric" organisation (Figure 1.7) are similar to the myofibrils of muscle (Peterson et al., 2004), in which the sarcomeres observed in non-muscle cells are the repeatable patterns of α-actinin (Pellegrin and Mellor, 2007).

The contractility of actin filaments bundles allows cells to actively re-structure their cell body to accommodate to the stiffness of the surrounding environment. When cells are grown on a glass cover slip, a broadly spread morphology is acquired by cells to sustain their bodies on the stiff substrate. For cells attached to a hard surface, isometric contraction and the parallel arrangement of aligned stress fibres are the dominant features of the cytoskeleton as shown in Figure 1.7 (Peterson et al., 2004).

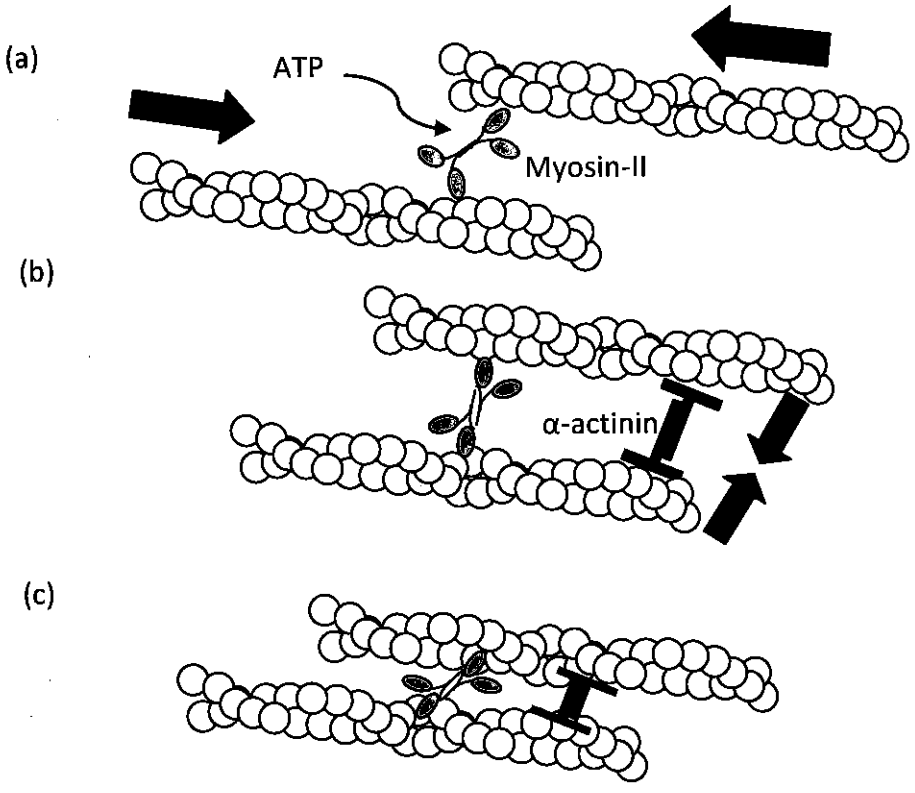

Figure 1.6. The mechanism of actin filaments contraction in bi-directions.

Figure 1.7. Sarcomeric appearance of the actin filament (Peterson et al., 2004). (Scale bar: 2 μm)

Some studies (Sagvolden et al., 1999, Rivelinea et al., 2001) show that cells are very sensitive to their interface tension and this can be seen when tension is applied to a cell membrane (for example, indenting the cell membrane with a microneedle). In this example, the cell rapidly generates stress fibres at that localised region (Rivelinea et al., 2001). In resisting tension, the cell reorganises the network of filaments to adapt to the stress. Consequently, focal adhesions are stimulated and assemble into clusters

in response to the onset of the contractility (Peterson et al., 2004). A higher recruitment of stress fibres increases the size of the focal adhesions, and hence induces higher contractile forces (Goffin et al., 2006). Reversible disassembly of the focal adhesions results in a disruption in the contractility of cells (Burridge and Chrzanowska-Wodnicka, 1996).

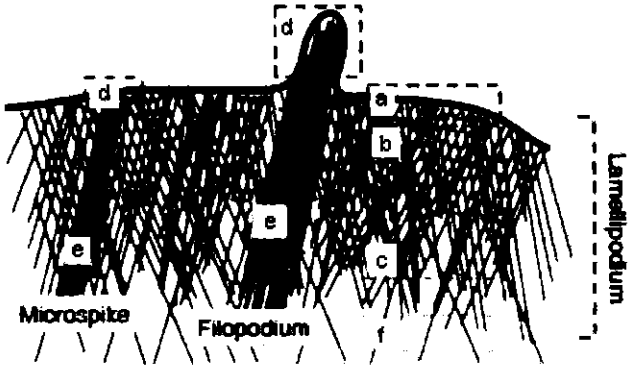

Figure 1.8. A schematic showing the sub-components of the lamellipodia and filopodia: (a) tip of lamellipodium, (b) actin meshwork, (c) region of major disassembly, (d) tip of filopodium, (e) bundle, and (f) undegraded filament which contributes to the cytoplasmic network (Small et al., 2002).

In addition to the functions of contraction, these filament networks are able to protrude and pressurise intracellularly against the cell membrane in the form of lamellipodia and filopodia. Lamellipodia contains quasi two-dimensional actin meshes that extends across and to the edge of the cell membrane (Figure 1.8a-c, f) to make contact with a surface (Small et al., 2002). Beyond the frontier of the lamellipodia, further microscopic protrusion of the membrane exists as microspikes, otherwise known as the filopodia (Figure 1.8d-e).

The assembly and disassembly of the actin filaments and associated integrin receptors are regulated by the GTPase family members (Figure 1.9) such as Rho, Rac, and cdc42 (Hall, 2005, Mackay and Hall, 1998). GTPase is an enzyme which hydrolyses guanosine triphosphate (GTP) that consists of monomeric GTP-binding proteins. These

are specialised molecular proteins that control the transduction pathways within the cell (Hall, 2005, Burridge et al., 1997). Rho triggers the bundling of actin filaments into stress fibres (Figure 1.9) (Mackay and Hall, 1998, Hall, 2005). Activation of Rac leads to the assembly of actin filaments in a meshwork at the periphery of a cell to form lamellipodia and membrane ruffles (Mackay and Hall, 1998). The third member, Cdc42 activates the actin filaments to form filopodia (Figure 1.9) (Mackay and Hall, 1998). There is also cross-talk between the monomeric G-proteins, for example, Rac can activate Rho in fibroblasts (Hall, 1998, Mackay and Hall, 1998). Hence, these signaling pathways control the structural changes that in turn are responsible for cell attachment and migration on the underlying substrate.

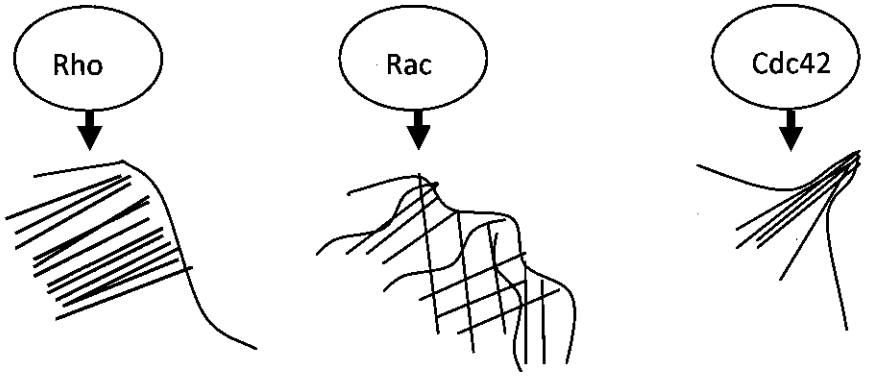

Figure 1.9. Activation of actin filaments by Rho, Rac and Cdc42

During cell migration, G-protein activates the down-stream signaling in order to elicit the formation of stress fibres, which then leads to the regulation of cell contractility. In triggering epithelial cells to change from a static to a migratory phenotype, the messaging could be regulated by cytokines such as Transforming Growth Factor Beta (TGFβ). TGFβ is a multifunctional cytokine which regulates many cellular process in keratinocytes such as the cell differentiation, proliferation and migration (Cross and Mustoe, 2003, Massague and Wotton, 2000). Over the cell membrane surface, TGFβ binds to type II receptor (TGFβRII) which then

phosphorylates type I receptor (TGFβRI) as shown in Figure 1.10. TGFβRI and TGFβRII are both obligate heteromeric transmembrane receptors (Wrana, 1998). The phosphorylation of TGFβRI leads to phosphorylation of Receptor activated-Drosophila gene Mothers against dpp (R-Smads) (as seen in Figure 1.10) with members termed Smad2 and Smad3 that continue to form complexes with Smad4 (Massague and Wotton, 2000). The complex of R-Smad and co-Smads translocates to the nucleus where these transcription factors regulate the target gene and activations of subsequent cellular processes (Massague and Wotton, 2000, O'kane and Ferguson, 1996).

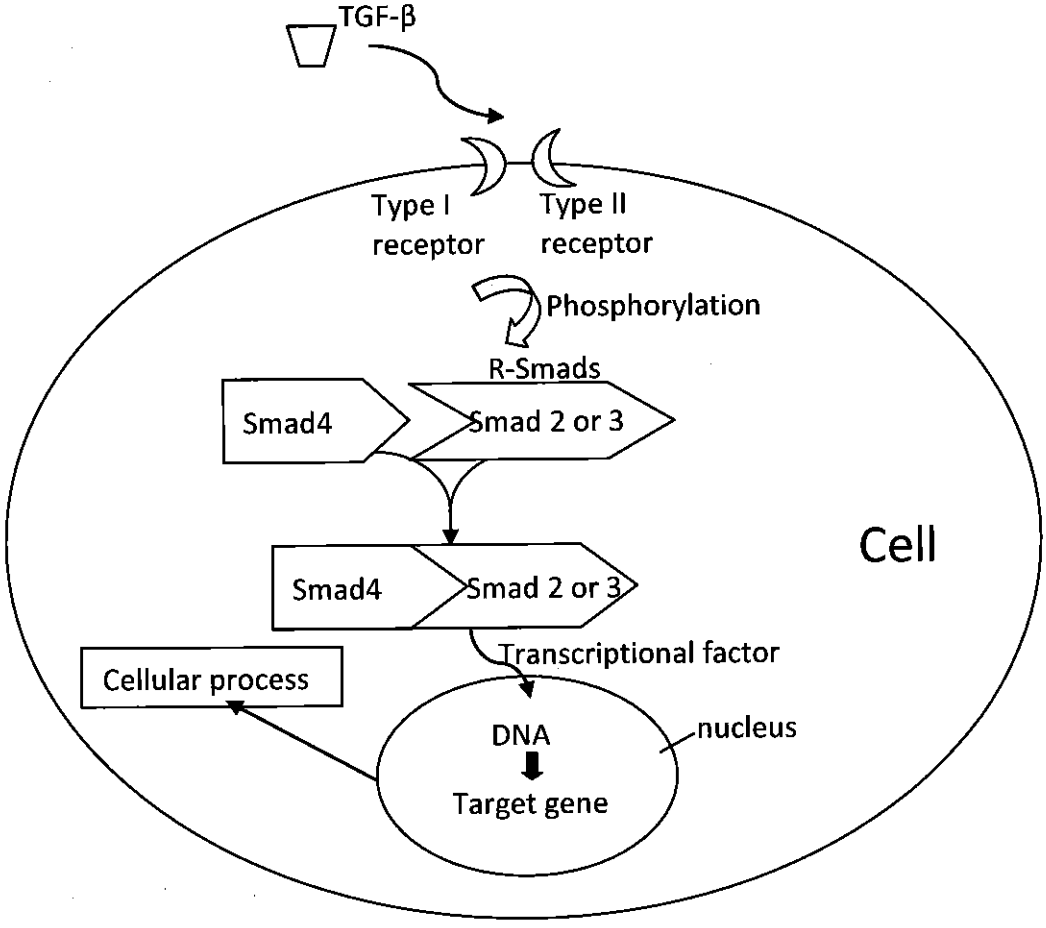

Figure 1.10. The intracellular signaling pathway of TGF-β.

The family of cytokine such as TGF-β1, TGF-β2 and TGF-β3. Particularly, TGF-β1 which is isolated from human platelets, human placenta and bovine kidney is an

attractive cytokine because of the contradicting role of the cytokine in wound therapy and abnormal epidermis formation (for example, scar formation and psoriasis) (Li et al., 2004a, Zhang et al., 2003, Gailit et al., 1994) which is still not clearly understood. However, treatment of keratinocyte in in-vitro cultures with TGF-β1 > 10 ng/ml was reported to promote cell motility, and rapidly increase the Rho activity and β-actin formation (Decline et al., 2003, Coffey et al., 1988, Bhowmick et al., 2001, Shen et al., 2001).

Contractility of cells is the basic causal event which regulates the integrity of tissue (Deugnier et al., 2002, Heida et al., 1996), cytokinesis and morphogenesis (Singer et al., 1999, Horwitz and Parsons, 1999, Kirfel and Herzog, 2004). Due to the importance of the cellular contractility, many polymer based cell traction force measurement techniques have been developed to probe the mechanics and physiological changes that occur in contracting cells (mainly fibroblasts).

Research in mechanobiological measurement systems is expanding fast. Seven methods of probing cells mechanically have been developed as shown in Figure 1.11. These methods are classified as the atomic force microscopy (AFM) nano-indentation, magnetic twisting cytometry, micropipette aspiration, optical trapping, shear flow, soft substrate stretching (Bao and Suresh, 2003) and quartz crystal microbalance systems (Pax et al., 2005, Kang and Muramatsu, 2008). Except for the use of soft substrate techniques, the majority of techniques is either very time consuming or requires considerable investment in expensive equipment, and thus these techniques are not suitable for high throughput drug screening. In comparison, the systems based around soft substrates seem to be the most promising tool for pharmacology application because of their low development cost and potential in high throughput screening.

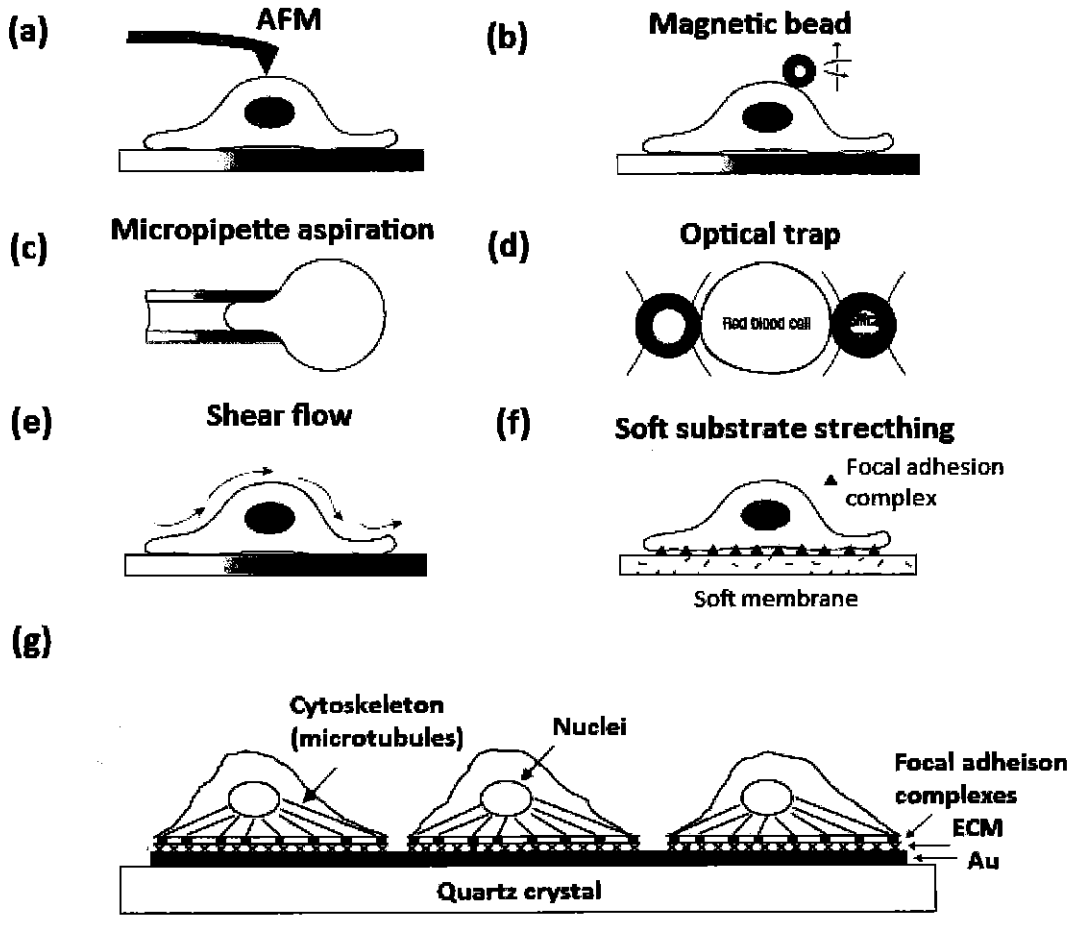

Figure 1.11. Seven experimental techniques used in probing mechanical response of a cell. (a) Atomic force microscopy (b) magnetic twisting cytometry, (c) micropipette aspiration, (d) optical trapping, (e) shear flow, (f) soft substrate stretching (Bao and Suresh, 2003) and quartz crystal (Marx et al., 2005).

The most commonly used soft materials to monitor cell forces are silicone rubber, collagen sheets, polyacrylamide (PAA) and polydimethylsiloxane (PDMS). For example, measuring the shrinkage of a collagen sheet caused by adherent contracting cells is a widely used technique. This type of soft substrate system was applied to assess the effect of Endothelin-1 on smooth muscle cells (Dallot et al., 2003) but it does not provide any information about the contractility characteristic of cells such as morphology changes, force distribution, cell mechanics and traction pattern for single cells. Therefore, more sensitive methods are required to probe the biomechanics of a single cell.

1.2.5 Force Sensor Based on Silicon Sheet

The first use of a thin flexible silicon sheet in monitoring cell traction force can be traced back to the work of (Harris et al., 1980). Buckling effects were observed on a thin sheet of silicon rubber seeded with chicken heart fibroblasts as shown in Figure 1.12a. Although fibroblast induced long lasting wrinkles on the silicone substrate, the most intense indentations did not correlate with the focal contact points (Hinz and Gabbiani, 2003). In order to measure the traction force correlated with the contraction activity of the α-smooth muscle actin, the indentation should be formed at the focal contacts where the anchoring of actin filament heads produce the most abundant stress. Numerous efforts have been made to improve the sensitivity and resolution of this material by increasing the flexibility of the silicon sheet by ultraviolet light treatment (Burton and Taylor, 1997). However, the enhancement on the flexibility of the silicon sheet did not significantly improve the efficiency in measurement (for example, isolation of localised stress) (Figure 1.12b) (Beningo, 2002).

The silicon sheet used for detection of cell forces is based on a continuum. This technique does not limit the cell spreading but allows dispersal of cells focal contacts and distribution of cell traction force in arbitrary directions (Beningo, 2002). However, there are two problems arising from this technique. Firstly, the quantification of forces involves the direct use of a longitudinal deformation line in which, the force was calculated by multiplying the length of the longitudinal deformation line (wrinkle) by the stiffness of the material (Oliver et al., 1995). The stiffness of the silicon substrate used in previous work was determined by measuring the lengths of the wrinkles induced by a calibrated deflection force (Burton et al., 1999).

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