Collective Migration Study of Breast Cancer Cells in 2D

Earnest Mendoz

(MBBS, GMC, Mysore, India

MMST, IIT Kharagpur, India)

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Abstract

Cancer related deaths are mainly attributed to the metastatic spread of cancer cells to distant organs resulting in the formation of secondary tumors. Collective cell migration is one of the factors among many others that are strongly implicated in the growth and progression of cancers. Collective cell migration has been studied by various techniques in 2D and 3D, including wound healing assays which remain among the most commonly used techniques. To improve the information obtained from these methods, various modifications have been employed such as time-lapse microscopy and analysis of cell migration by cell tracking. The latter achieved considerable success with cell migration parameters derived from cell track data. However, these methods do not analyze collective migration in detail at the individual cell level, nor delineate the underlying mechanisms involved in the development of the collective behavior. Moreover, collective cell migration of breast cancers has not been studied in detail.

We utilized a modification of the ring assays for the study of collective migration of breast cancer cell lines on 2D surfaces. We also improved this method by introducing kinematic and kymographic analysis including quantitative estimation of parameters for single cell motility mechanisms. Furthermore, with these assay methods and analytical technique, we have analyzed the migratory behavior of breast cancer collective cell migration on collagen IV coated surfaces. Results showed that collagen IV, a component of the basement membranes, increased the migration of the highly invasive MDA-MB-231 cells. Analyses also revealed that the abnormal collective cell migration in MCF-7 cells was due to altered lamellipod polarization, while the haphazard collective migratory patterns of the highly invasive MDA-MB-231 cells

were the result of mesenchymal modes of migration that was facilitated by lack of intercellular adhesions and directional freedom of lamellipod polarization. The method of assay was also used to analyze two different protein knock down systems to evaluate the capabilities of this system. Finally, we tested the hypothesis that lamellipod polarization affects the collective cell migration in epithelial cell lines. In the course of these findings, it was also shown that the enzyme Rho Kinase was involved in the regulation of lamellipodial polarization in epithelial cells, and inhibition by the ROCK inhibitor resulted in disruption of collective cell migration.

In conclusion, this study emphasizes the usefulness of 2D non-wounding assay methods in studying the collective migration of cells. The improved analysis methods also helped in revealing the details of collective migration of breast cancer cells as well as elucidating lamellipod polarization as one of the crucial factors in the development of individual cell motility, and therefore collective cell migration.

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Chapter 1
Introduction

Cancers are a class of diseases that are characterized by an uncontrolled proliferation of cells that have become abnormal due to accumulation of genetic mutations. The clinically aggressive variants called malignant cancers can be differentiated from the milder and localized benign tumors by their ability to spread to adjacent tissues and distant organs by a process termed metastasis. A majority of deaths due to solid tumors have been attributed to their eventual spread to distant sites leading to disruption of organ function. Several important molecular mechanisms are dysregulated in cancers and contribute to the success of this phenomenon. Mainly these include adhesion (intercellular and cell-substrate), proliferation, angiogenesis (formation of new blood vessels), migration (individual cell and group or collective cell), invasion and avoidance of cell death (apoptosis and anoikis).

Often of interest to researchers, and to us in this work, is cell migration in groups, a key process that underlies crucial physiological processes such as embryogenesis, inflammation, and wound healing. Cancers are phenomenal in that they represent a pathological but biocompatible deviation of normal physiologic processes in healthy cells. Hence it is obvious that physiological processes, like cell migration, can be recruited by the accumulated chance mutations in cancers resulting in untoward clinical effects including pressure effects and tissue destruction, and hence untimely death of affected individuals. Therefore, a study of these processes can add to our understanding of cancers and in the long term, help in improving the treatment strategies against cancers and other diseases.

This chapter will consider the importance and relevance of collective migration in the setting of cancer growth, progression and metastasis. But, for a better understanding of the abnormality of cancers and their dysregulated migration, it is necessary to know the basic structure of normal tissues and the nuances of individual cell motility. Therefore, in these sections, the properties and 3D architecture of normal tissues will be described followed by a brief description of the common methods used to analyze cell migration.

1.1 Tissue architecture and Normal epithelia

The average human organism is composed of over a 100 trillion cells, with more than 200 different types of cells based on structural and functional capabilities. Combinations of different cells coordinate together in function to form complex organs. This unique feature which differentiates multicellular organisms from colonies of individual cells is the result of a compact and well organized structure (Figure 1.1). Among the several characteristic properties of individual cells that contribute to such an organized structure, intercellular and cell-substrate adhesions are the most important. For example, apical cell-cell junctions in epithelial cells are responsible for the tight physical coupling that is necessary for formation of well packed monolayers (Bryant and Mostov 2008; Martin-Belmonte and Mostov 2008). Intercellular adhesion facilitates the grouped movements of cells during cellular organization and rearrangements that are necessary for maintaining the stable structure of organs. Cell proliferation and rearrangements are responsible for driving complicated physiological processes such as embryogenesis, organogenesis and wound healing (Lecuit and Le Goff 2007; Lecuit and Lenne 2007; Montell 2008) as well as maintenance of the lining epithelium of the respiratory and the gastro-intestinal tracts and other organs.

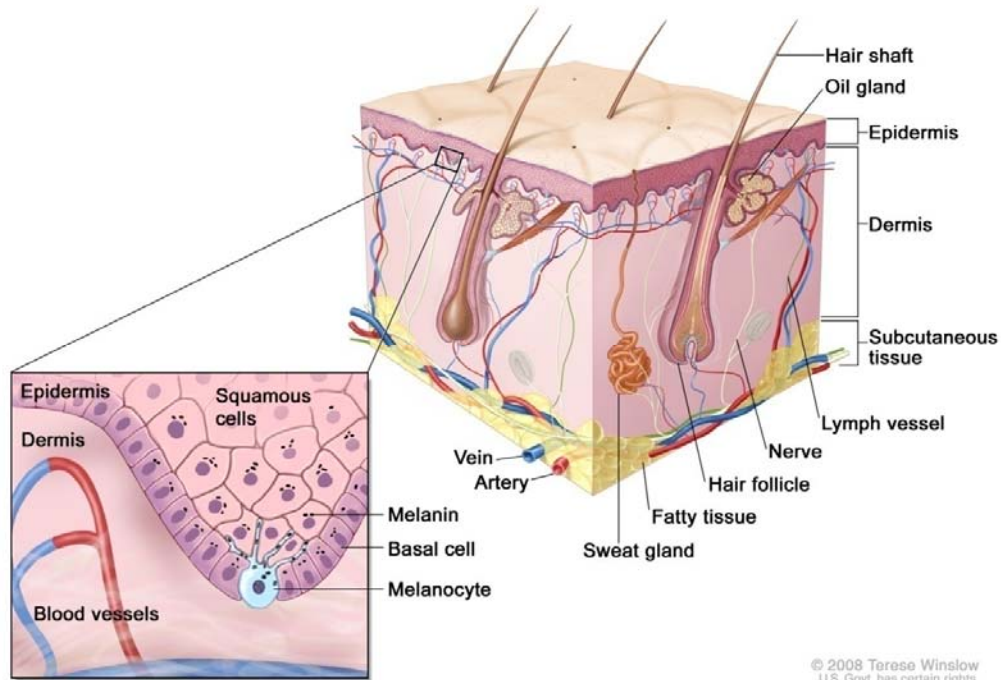


Figure 1.1 Normal Tissue Architecture. This example of the skin shows the compactly organized epithelial cell layer overlying the connective tissue (dermis) and separated from it by a basement membrane. Melanotic tumors arising from melanoma cells and other skin carcinomas like squamous cell and basal cell carcinomas have to breach the basement membrane for invasion in to the dermis. (http://visualonline.cancer.gov)

A large family of proteins, called cell-adhesion molecules (CAMs), mediates adhesion between cells in tissues. CAMs comprise four major families: the cadherins, immunoglobulin (Ig) superfamily, integrins and selectins. For instance, intercellular adhesions in epithelial cells are modulated by E-cadherins, which are calcium dependent transmembrane proteins that are anchored intracellularly by Actin based adherens junctions (Harvey Lodish 2003). This type of intercellular adhesion formation is so basic to the cell communication that, even in *in vitro* cell culture, epithelial cells frequently form groups or patches particularly when the environmental conditions favor growth in confluence. Therefore, studying collective migration of cell sheets is much more important than sparsely seeded cells for a meaningful correlation to the physiological environment.

Epithelial cells that line tissues and organs are always found over a layer of extracellular matrix called the basement membrane (Figure 1.1and 1.2). Major components of the basement membrane include collagen IV, laminin-entactin/nidogen complexes and proteoglycans (Paulsson 1992; LeBleu et al. 2007). Collagen IV, that is found in the basal lamina, a sub-layer of the basement membrane, provides a scaffold for the other structural macromolecules by forming a network of interaction between specialized N- and C- terminal domains (Paulsson 1992; LeBleu et al. 2007). Thus it is more likely the initial substrate component for all epithelial cell-matrix interactions including cancers that arise from epithelial cells.

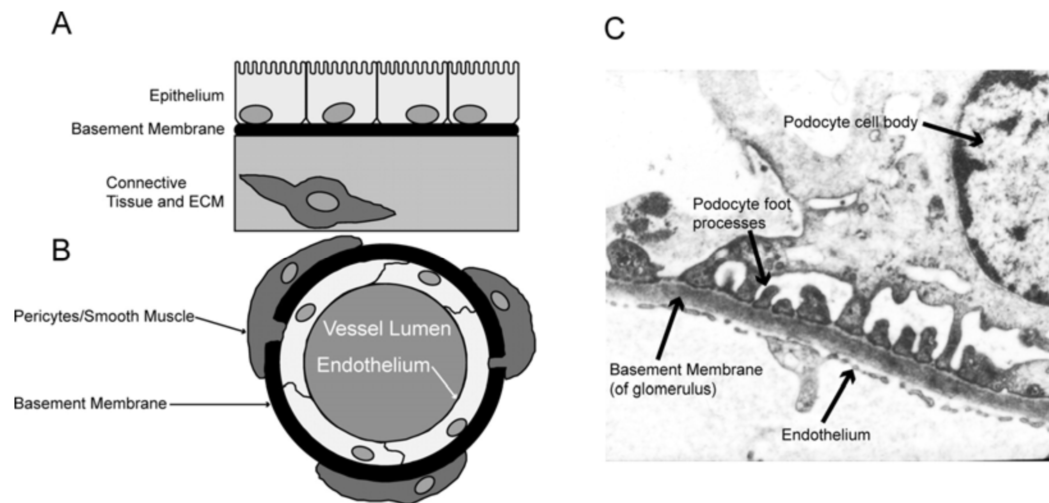


Figure 1.2 Schematic representations of (A) epithelial and (B) endothelial cells in relation to extracellular matrix. The basement membrane underlying the cells comprises Collagen IV and other matrix molecules. (C) Transmission electron micrograph of human glomerular basement membrane supporting the glomerular epithelium (podocytes) and endothelium. (Figure reproduced from (LeBleu et al. 2007))

Thus, it is clear that the basic 3D structure of solid and static tissues in a biological environment consists of compact organization of cells layered on a basement membrane overlying connective tissue. Whatever the organ, compactness is the key to the functional integrity that is observed in multicellular organisms. Cell migration in

groups is very critical in maintaining the compact structure of organs and the physiology of the whole organism. Also, the basement membrane can be viewed as similar to a 2D surface except for the absence of a perfectly flat interface. Further, some components of the basement membrane as well as properties inherent to the normal cells restrict them to these boundaries, ensuring an interface that is continuous without breaks. In malignancies, these basic processes are affected leading to lethal consequences and a study of collective migration in cancers can provide information for a better understanding of these mechanisms and for improving the therapeutic strategies for the effective treatment of these diseases.

1.2 Cancers

Cancers are a group of diseases that are characterized by an accumulation of genetic mutations in cells leading to uncontrolled proliferation and spread to adjacent tissues by local invasion or to distant organs by a process termed metastasis. They represent the leading cause of death in economically developed countries and the second leading cause of death in developing countries (WHO 2008; Jemal et al. 2011). About 12.7 million new cancer cases and 7.6 million cancer deaths occurred in 2008 worldwide (Ferlay et al. 2010). Environmental factors such as tobacco, diet, obesity, alcohol consumption and infectious organisms are responsible for 90-95% of the cancers with a genetic predisposition observed in only 5-10% of the cases (Anand et al. 2008) making cancers arguably and largely a preventable disease.

Transformation of a normal cell into a cancer cell occurs through a sequential process of accumulation of mutations in several genes such as oncogenes, tumor-suppressor genes and microRNA genes, which normally regulate cell growth and differentiation (Croce 2008). All the causes of cancers contribute to this process of Darwinian

microevolution, either by a direct or indirect mechanism. Before progressing into a malignant form, normal cells generally transit into the milder stage of benign tumors which lack the aggressive properties of malignant tumors such as invasion and distant spread (Figure 1.3) (Foulds 1954; Nowell 1976; Hanahan and Weinberg 2000). The benign tumors (solid) closely resemble the normal mature cells and are also characterized by a fibrous sheath or a capsule and typically remain localized as a lump or swelling (Stephens and Aigner 2009). The latter property allows for effective treatment through surgical removal.

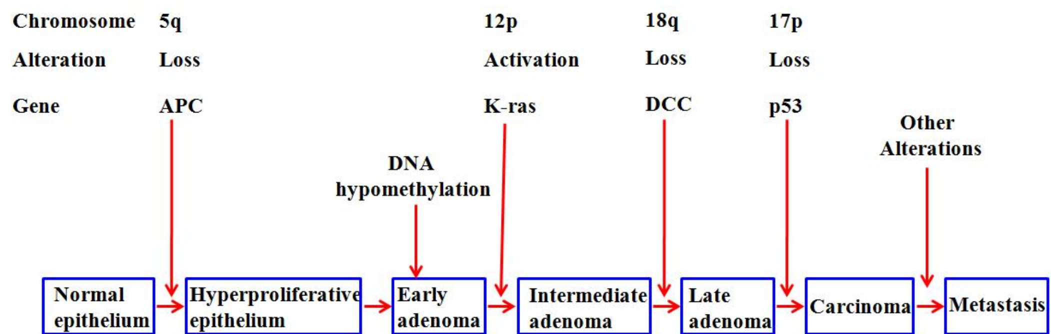


Figure 1.3 Stages of cancer development with sequential accumulation of somatic mutations. Schematic shows the development of colon carcinoma, a well studied example, through various stages of benign tumors and the accompanying genetic mutations. (Figure redrawn and reproduced from (Vogelstein and Kinzler 1993)

Malignant cancers are characterized by complete loss of the distinctive features of the native cells from which they originate, a condition termed anaplasia (Stephens and Aigner 2009), and a severe loss of structural organization. They also undergo a transformation to fibroblastic phenotype by a process called epithelial to mesenchymal transition (EMT) that is thought to be important for the invasion and metastasis (Ingber 2002; Thiery 2002; Thompson et al. 2005; Lee et al. 2006; Guarino 2007; Hotz et al. 2007; Hugo et al. 2007), although this concept is viewed with scepticism by some researchers (Tarin et al. 2005). However EMT is only one of the mechanisms and is not necessary since many cancers do not manifest it during their

progression (Tan et al. 1999; Rubin et al. 2000; Ng 2002; Christiansen et al. 2005; Christiansen and Rajasekaran 2006). Malignant cancers also recruit normal connective tissue cells and blood vessels (angiogenesis) to their advantage and acquire the potential to avoid deaths by apoptosis and anoikis (apoptosis at sites other than region of origin) (Hanahan and Weinberg 2000). Acquisition of all these properties, along with the several genetic mutations, facilitates the spread of cancer cells to distant organs, a feature that differentiates them from benign tumors.

A benign tumor can be less frequently lethal due to pressure effects resulting from its location, such as in a meningioma arising from the meninges surrounding the brain (Stephens and Aigner 2009). But a malignant tumor almost always results in early mortality that can be attributed, in majority of cases, to organ dysfunctions caused by their spread to distant sites. Although cell migration or rearrangement is inevitably present during majority of cellular events, logically and by exclusive principles, bulk growth of a tumor is related to the proliferation-nutrition-attrition dynamics of individual cells while its spread through tissues is a function of properties such as cell migration and invasion. Thus, tumor cell migration is crucial for the success of metastasis.

In view of the above information, a thorough study of collective cell migration of cancers is necessary for a better understanding of the metastatic process and for effective analysis of anticancer agents.

1.2.1 Metastasis

Metastases are responsible for majority of deaths due to cancers. Metastasis consists of several steps (Figure 1.4). The initial stages are characterized by the release of cancer cells from the primary tumor due to defective intercellular adhesion and acquisition of the mesenchymal migratory phenotype.

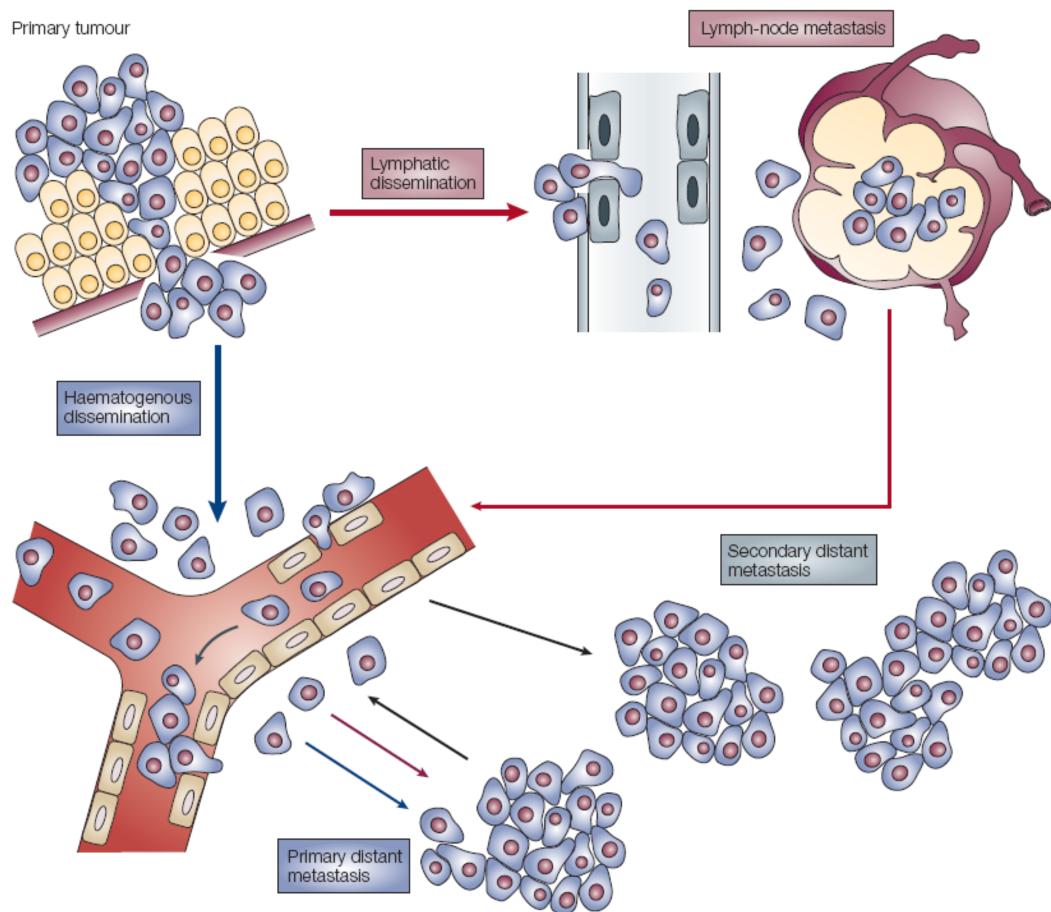


Figure 1.4 The Metastatic Cascade. Cancer cells get detached from a primary tumour, and are transported to secondary sites either through breach into lymphatic (red arrow) or blood vessels (blue arrow). The intermediate steps between these extremes consist of cancer cell invasion of the organ basement membrane, migration towards and invasion of the basement membranes of the vessels (lymphatic or blood) followed by entry (intravasation), transit and survival in circulation, adhesion to endothelium and extravasation at the distant site. (Figure reproduced with permission from Ref: (Pantel and Brakenhoff 2004))

This is followed by migration of the cancer cells towards the nearest lymph (lymphatic) or blood (hematogenous) vessel. Since lymph vessels ultimately drain into blood vessels and distant spread can only be explained by hematogenous route, the following stages are described in reference to the latter mode of metastasis.

Intravasation or entry of the cancer cells into the blood vessels is facilitated by their invasive properties, although a spillage into the vessels in a highly vascular tumor can also be the case. Tumor cells in circulation have to undergo mechanical stresses including compressive forces in capillaries of the lungs and survival in circulation is a function of their adaptive capability to these forces as well as an escape mechanism from apoptosis that affects cells escaping from their native environment (anoikis). The circulating tumor cells can either adhere to endothelium or be arrested in blood vessels by size restriction resulting in extravasation or exit from the blood vessels at secondary sites. Cancer cell migration and growth at secondary site will follow depending on the gene signatures of the cancer cells and whether the environment is conducive in the secondary organs.

Several factors including alterations in gene expressions (Backus et al. 2005; Minn et al. 2007; Tavazoie et al. 2008; Klein et al. 2009), inflammation (Coussens and Werb 2002; de Visser et al. 2005), apoptosis, angiogenesis and tumor cell motility regulate the onset of metastasis and its progression (Brooks et al. 2009). But metastasis is an inefficient process (Chambers et al. 2002; Cairns et al. 2003; Fidler 2003; Minn et al. 2005). Several groups have shown that the inefficiency occurs at the secondary growth sites with the earlier steps being achieved with remarkable efficiency (Luzzi 1998; Cameron 2000; Orr and Wang 2001). Therefore, it is possible that some of the best therapeutic targets to control the progression of cancers should lie in the earlier stages of the metastatic cascade.

Further, cancers which are non-invasive are still known to metastasize, indicating that migration and multiple other factors like angiogenesis play crucial roles. For example, both the cancer cell lines MCF-7 and MDA-MB-231 were isolated from metastatic pleural effusions (ATCC), but MDA-MB-231 alone has been shown to be highly invasive. Also estrogen negative variants of MCF-7 have been shown to be as invasive as MDA-MB-231, and more invasive than the regular MCF-7 cells (Gozgit et al. 2006). Therefore, cell migration seems to be as critical as invasion, although the latter would definitely add to the rapidly detrimental outcomes of cancers. In this context, it is clear that cell migration is important in at least two different stages of metastasis, before intravasation and after extravasation, in addition to its role in the dynamics of local invasion and spread.

1.2.2 Cell Migration in Metastasis

Increased cell motility is an important characteristic of cancers (Brooks et al. 2009) and tumor cell migration is a crucial factor for the spread of cancer cells to adjacent and distant organs through cancer invasion and metastasis (Friedl and Wolf 2003; Guo et al. 2008). Cell migration is more important than cell division in the growth and expansion of tumors (Enderling et al. 2009). The role of individual cell migration in the spread of cancers, at the regulatory level of involvement, have been documented by several researchers (reviewed in (Condeelis et al. 2005; Kedrin et al. 2007; Machesky 2008)). Specialized conformations in the cell membranes such as lamellipodia, filopodia and the invadopodia (described in the literature review) assist in the migration of both normal and cancer cells. For example, proteins such as Arp2/3 complex and SCAR/Wave proteins in lamellipodia and fascin, diaphanous and Mena/VASP in filopodia are important regulators of cell motility and have been

linked to metastases (Condeelis et al. 2005; Di Modugno et al. 2006; Kedrin et al. 2007). Similarly N-WASP, Arp2/3, dynamin and cortactin in invadopodia regulate cellular invasion (Buccione et al. 2004; Ayala et al. 2006; Gimona and Buccione 2006). The SCAR WAVE2 and Arp2 have also been linked to metastatic and aggressive cancer phenotypes in lung, breast and colorectal adenocarcinomas (Semba et al. 2006; Iwaya et al. 2007; Iwaya et al. 2007). Overexpression of the Rho GTPase proteins in many cancers are associated with enhanced cell migration and metastatis (Boettner and Van Aelst 2002; Sahai and Marshall 2002; Aznar et al. 2004). Cell adhesion molecules are also altered in cancers affecting their collective migration and adhesion to extracellular matrix. For example, it is well known that E-cadherins are downregulated and integrin molecules are overexpressed in many cancers (Berx and Van Roy 2001; Cavallaro 2004). Diffuse infiltrating cancers are associated with mutations in the genes encoding E-cadherin and catenins, which are proteins linking cadherins to the cytoskeletal Actin (Hirohashi and Kanai 2003). Thus it is clear that cytoskeletal protrusions and the proteins controlling them as well as adhesive interactions are involved in the regulation of metastasis. However, the contributions of both individual and collective cell migration to the dynamics of metastatic progression in cancers, is still not well known to researchers. Furthermore, studying the role of individual cell migration in the process of cancer growth and spread is important for identifying potential and promising therapeutic targets. This thesis will therefore be aimed at the study of collective cell migration of cancer cells and with the contributions of individual cell motility to cancer progression.

1.2.3 Breast Cancers

Breast cancer is the malignant proliferation of epithelial cells lining the ducts or lobules of the breast (Lippman 2008). It is one of the most frequent cancers (excluding skin cancers) occurring among women with an estimated 1.38 million new cancer cases diagnosed in 2008 (23% of all cancers), and ranks second overall (10.9% of all cancers) (Ferlay et al. 2010). It is now the most common cancer both in developed and developing regions with around 690 000 new cases estimated in each region (population ratio 1:4)(Ferlay et al. 2010). Infiltrating ductal adenocarcinomas form about 75 % of the clinically diagnosed cases of breast cancers (emedicine.medscape.com; www.cancer.org (American Cancer Society)), while infiltrating lobular carcinomas account for about 15% of the cases.

Human breast cancer is a clonal disease that results from a single transformed cell with sequential accumulation of somatic (acquired) or germline mutations; breast cancers can therefore exist either as noninvasive diseases or an invasive but nonmetastatic disease for a prolonged period of time! (Lippman 2008). About 10% of human breast cancers can be directly linked to germline mutations in genes such as BRCA-1 and BRCA-2, which are associated with an increased incidence of breast cancer in both men and women. Inherited mutations in p53 tumor suppressor gene (Li Fraumeni syndrome) and PTEN have also been reported to increase incidence of breast cancers (Lippman 2008).

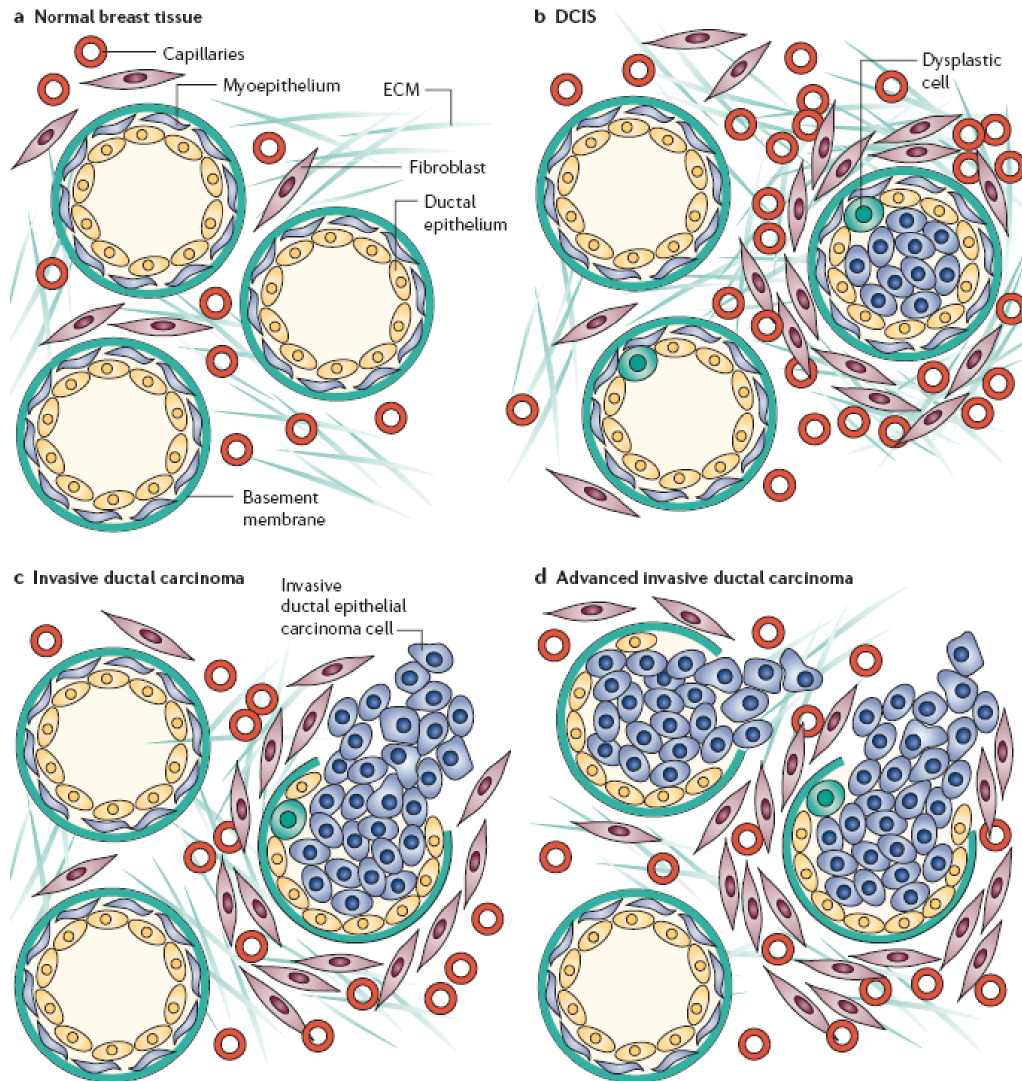


Figure 1.5 Progression of normal breast ductal epithelial cells to invasive ductal cancrcinoma. Note the areas of altered but intact basement membranes and the normal ductal architecture in the 'in situ' form which may persist for a period before the complete progression. (Figure reproduced with permission from (Kalluri and Zeisberg 2006))

A majority of deaths due to breast cancers are due to metastases. Metastases in breast cancers occur in high frequencies to the skeletal system followed by the visceral organs such as liver, lungs, brain and ovaries (Solomayer et al. 2000). Gene expression signatures have been identified that differentiate metastastatic breast cancers from primary tumors (Ramaswamy et al. 2003; Woelfle et al. 2003; Nagaraja et al. 2006) and have been used to assess the prognosis in breast cancers

(O'Shaughnessy 2006). But acquisition of important genetic mutations is one of the strategies adopted by cancer cells to be successful in invasion and metastasis (Hanahan and Weinberg 2000), and cancer cell migration is an important determinant for both the outcomes (Keely et al. 1998; Chin et al. 2005; Sahai 2005; Wolf and Friedl 2006; Machesky 2008; Parri and Chiarugi 2010; McSherry et al. 2011).

Breast cancer cell migration has been studied by many researchers. For example the junctional adhesion molecule, JAM-A has been shown to regulate cell migration in MCF-7 breast cancer cells through Rap1GTPase and β1-integrin activation (McSherry et al. 2011). The effect of fibronectin and vitronectin substrates on the migration of invasive breast cancers MDA-MB231 and MDA-MB-435 were shown to be dependent on different combinations of integrin subunits, indicating that multiple agents may be required for therapy against these proteins (Bartsch et al. 2003). Similarly, several others have reported on the effects of various protein expressions, growth factors and drugs on breast cancer motility (Hirsch et al. 2006; Dulyaninova et al. 2007; Timoshenko et al. 2007; Brew et al. 2009). But collective cell migration of breast cancer cells has not been well studied and the contributions of individual cell motility mechanisms to the collective behaviour have not been reported so far. Therefore, in this work, one of our objectives is to study the collective migration behaviour in both benign and malignant breast epithelial cells, so as to achieve a better understanding of the contributions of these processes to the growth and progression of breast cancers.

1.3 In Vitro Migration Assays

A variety of migration assays have been used in research to perform migration based experiments and report effects of drugs and proteins on the cell migration. For

instance, in vitro scratch assays or wound healing assays involve scratching a confluent monolayer and measure the time required by the cells to fill the gap (Liang et al. 2007). This relatively inexpensive method of 2D migration assay is very common and has been used to assess various experimental scenarios. For example, it has been used to analyse the effect of expression of cytoplasmic proteins on collective cell migration in epithelial and cancer cells (Long et al. 2006; Shin et al. 2007) or the effect of antibodies and drugs on cancer cell migration (Herrera-Gayol and Jothy 1999; Hunter et al. 2001; Yoneda et al. 2001). Several methods have been introduced to improve on the details of observation in wound healing assays (Yarrow et al. 2004) and used to perform high throughput screening (HTS). For example, time lapse microscopy has been combined with the wound healing assays (Yarrow et al. 2004) for increasing the throughput, and automated tracking has been used to study cell migration during scratch assays (Fotos et al. 2006). Although several assay methods have been reported in literature (Decaestecker et al. 2007), none of them attempt to study the migratory track patterns in relation to the individual cell mechanisms or behaviour that is actually responsible for the development of grouped migratory patterns. Therefore this work focuses on developing an assay method that is easy to perform and could be useful in assessing cell migration in a more detailed manner. A detailed discussion on the migrational assays will be considered in the corresponding section in the next chapter.

1.4 Objectives and Scope of Work

In the view of this introduction, the working hypothesis for this dissertation is that "cell motility mechanisms, intercellular adhesion and surrounding environment together influence the collective migratory behavior of cancer cells in 2D". Therefore,

studying individual cells in terms of the exploratory machineries (lamellipods and protrusions), migratory mechanism (contractility) and interactions with adjacent cells (intercellular adhesion) as well as substrate (substrate adhesion) can be critical to predicting their collective migratory behavior. The following are thus the objectives of this thesis:

1. To develop a reliable 2D migrational assay method for evaluating cell migration using a non-wounding approach and to study the cytoskeletal organization as well as collective cell migration in breast epithelial cell lines. The cytoskeletal organization will be studied for the stress fiber formation, and correlated with the migrational capacity of the cell lines. The effect of human Collagen IV on the migration of these cell lines will also be explored.

2. To evaluate the non-wounding migrational approach in protein knock down systems in epithelial and malignant cell lines.

3. To elucidate the contribution of the mechanisms of individual cell motility to the evolution of collective behavior in the breast cancer cell lines by developing parameters to assess individual cell motility. Such parameters would allow us to identify critical factors, in individual cells, that are responsible for the collective migration patterns. Further, the involvement of such factors can be validated by inducing the collective migration patterns of cancers in epithelial cell lines by targeting some of them.

By achieving these objectives we hope to be able to contribute to a better understanding of collective cell migration in breast cancer growth and progression as well as a reliable method for the analysis of collective cell migration.

Chapter 2
Literature Review

This chapter will focus on the background information relating to experimental approaches towards 2D cell migrational assays in the study of collective cell migration in cancers. In particular, the sections will deal with the information on components of cytoskeleton, and their cooperative actions leading to individual cell motility. The impact of individual cell motility mechanisms on the collective cell migration of cancers will be discussed, to emphasize their importance in conjunction with environmental factors in cancer growth and progression. One of the sections will also deal with differences in cell migration in 2D vs. 3D since it has been held in view that migration in these dimensions are entirely different and unrelated, and is erroneously considered to be incomparable. The final sections will introduce the various methods available for the analysis of cell migration and the rationale behind the selection of the particular cell lines used in this work.

2.1 Cytoskeletal Organization

Actin forms the major component of the dynamic cytoskeleton of all cells(Weber et al. 1975). It exists either in the monomer form (G-Actin) or the filamentous form (F-Actin). The polymerisation (G to F) and depolymerisation (F to G) cycles of Actin that balances these states, drive the constantly changing shapes of cells and also their motility and adhesion. The contribution of Actin to the movement of individual cells relates to its involvement in the formation of protrusive structures like lamellipodia and filopodia (considered in the subsequent sections) and filamentous structures that facilitate contractility inside the cytoplasm called stress fibers (Ridley et al. 2003; Hotulainen and Lappalainen 2006). Structures like Actin stress fibers, although

associated with static cells, are capable of undergoing dynamic reorganization in motile cells (Wang 1984) and therefore affecting the cytoskeletal structure as well as contractility of the cells (Hotulainen and Lappalainen 2006). In this section, we will consider the role of stress fibers in the cytoskeletal organisation and the motility of cells. The lamellipodial protrusions will be dealt in a later section.

Stress fibers are parallelly arranged bundles of Actin filaments in the cells (Lazarides and Weber 1974; Byers et al. 1984). As studied in fibroblasts, stress fibers are composed of bundles of short Actin filaments with alternating polarity and are distributed within the body of the cell.(Cramer et al. 1997). The formation of stress fibers is initiated by the filaments formed in lamellipodia (Small et al. 1998). Three types of stress fibers have been observed in cells: ventral stress fibers, arcs and dorsal stress. The ventral stress fibers extend from focal adhesions at the leading edge of the cell to the perinuclear regions and are contractile in function (Hotulainen and Lappalainen 2006). Arcs are convex arrays of Actin filaments, observed on the dorsal surface of spreading and migrating cells, and are not anchored at focal adhesions but are nevertheless contractile and are responsible for the centripetal flow of cortical receptors. The dorsal stress fibers arise from focal adhesions at the leading edge and terminate on to the arcs (Soranno and Bell 1982; Heath 1983; Wang 1984; Heath and Holifield 1993; Small et al. 1998). Stress fibers are formed by two separate mechanisms of Actin assembly. The dorsal stress fibers are assembled through formin (mDia1/DRF1)–driven Actin polymerization at focal adhesions, while transverse arcs are formed by endwise annealing of myosin bundles and Arp2/3-nucleated Actin bundles at the lamella (Hotulainen and Lappalainen 2006). Dorsal stress fibers and arcs together give rise to ventral stress fibers.

The polarity of Actin filaments have been observed to change from unipolar in the focal adhesions to mixed polar composition along the stress fibre (Begg et al. 1978; Sanger and Sanger 1980; Cramer et al. 1997). This raised speculations that preformed Actin filaments might be added to stress fibers during assembly, rather than simply arising through elongation from the contact site, since microinjections of G-Actin into cells resulted in slow incorporation into stress fibers (Wang 1984). Furthermore, Wodnicka and Burridge have shown that Rho mediated contractility in fibroblasts precedes the formation of stress fibers and focal adhesions (Chrzanowska-Wodnicka and Burridge 1996). As explained by their model, activated rho facilitates phosphorylation of myosin light chains (MLCs) leading to assembly of myosin filaments and their subsequent interaction with Actin. The resulting tension aligns the Actin filaments into stress fibers and aggregates integrins stimulating FAK activity and elevating tyrosine phosphorylation, and thus focal adhesion formation.

Furthermore, individual stress fibers have been shown to convey tensile forces as evidenced by their viscoelastic recoil following laser induced disruptions (Kumar et al. 2006). The recoil of individual stress fibers following such disruptions were completely abolished by inhibition of myosin light chain kinase (MLCK), while being only partly affected by Rho-kinase (ROCK) inhibition (Kumar et al. 2006). All these results point to the crucial role of stress fibers in maintaining the cytoskeletal organization and contractile tension in the cells. The proposed current model holds that free Actin filaments are severed from the base of the lamellipodium and that these filaments contribute to a cytoplasmic pool, that is recruited for stress fibre assembly (Small et al. 1998). Therefore, stress fiber organisation in cells might give a clue about the contractility machinery and migratory potential of cells, although prominent stress fiber assembly might be a late stage phenomenon.

2.2 Collective Cell Migration

Collective migration is a common phenomenon in biological systems that can be appreciated at macroscopic as well as microscopic levels (Camazine et al. 2002; Deisboeck and Kresh 2006). Even in randomly migrating organisms, an increase in concentration of individual units above a certain threshold level induces organized behaviour resulting in collective migration patterns (Buhl et al. 2006). The resulting behaviour is complex, but the patterns of behaviour, especially overall outcomes must be largely predictable, although limited by the availability of computational resources.

In epithelial cell sheets, the presence of strong adhesive connections between the cells is crucial in coordinating the migration of groups of them to result in an organized collective migratory behaviour. Collective cell migration is a characteristic feature of important physiologic processes such as embryogenic morphogenesis (Armstrong 1985), wound healing (Martin and Parkhurst 2004), immune responses (Henrickson et al. 2008; Jacobelli et al. 2009; Tooley et al. 2009) and pathologic consequences such as metastasis in malignancies (Hanahan and Weinberg 2000; Schafer and Werner 2008). Certain key proteins and signals are required for the regulation of each of these processes in the biological environment. For example, adhesion of fibronectin to $\alpha5\beta1$ integrin is necessary for the effect of the cytokine TGF-β on collective migration of hepatocytes (Biname et al. 2008), and its synergistic activity along with hepatocyte growth factor (HGF) on colorectal carcinoma cells (Shimao et al. 1999). Glutamate, an excitatory neurotransmitter has been shown to induce collective migration in highly invasive tumours of the brain (Sontheimer 2003; Lyons et al. 2007).

Collective cell migration can occur in different patterns (Friedl 2004; Rorth 2009). For example, collective cell migration during border cell migration and tracheal

development in drosophila embryo (Montell 2003), vascular sprouting and renal morphogenesis (Teddy and Kulesa 2004; Vasilyev et al. 2009), and the development of the sensory lateral line in zebra fish (Ghysen and Dambly-Chaudiere 2004) follow sheet or cluster like patterns (Aman and Piotrowski 2010). Sheet migration is also observed when scratch assays are performed with primary cells and cell lines of epithelial or endothelial cell origin (Rorth 2009). Sprouting and branching type of collective migration have been observed when cells were seeded in 3D matrigels (Pollack et al. 1998; Bruyere et al. 2008). However, cell migration during gastrulation and neural crest cell development occurs in a stream-like fashion due to the mesenchymal phenotype of the cells. This type of migration is also found in some types of epithelial cancers where some leading edge cells facilitate the invasion process and guide a cohort or stream of followers (Deisboeck et al. 2001; Hegerfeldt et al. 2002; Friedl and Wolf 2003; Friedl 2004).

Cell adhesion molecules distributed over the surface of the cells also play an important role in collective cell migration. They induce spontaneous aggregation of like cells in a mixture of cells (Glazier and Graner 1993; Beysens et al. 2000; Deisboeck and Couzin 2009). This phenomenon is interesting and significant in the setting of a heterogeneous tumour population with just a few potentially metastatic clones. Collective cell migration in the normal epithelial tissues in a healthy individual can be envisaged in two different situations: regular rearrangements during the normal replacement of cells that occurs during division and the patterned mesenchymal migration that occurs during wound healing. A patterned migration like that observed during physiological wound healing can also be expected in a malignant tumour that is growing in size as well as breaching the adjacent tissues.

2.2.1 Collective Cell Migration in Cancers

Collective migration is important in the growth and spread of cancers. It is being recognized as being important for invasion in many cancers (Friedl and Wolf 2003; Bidard et al. 2008; Ewald et al. 2008). Collective migration in cancer cells can follow different patterns due to the diverse nature of the disease, since it can arise from many different cell types. For example, tumor cells in 3D culture systems can spread as groups or sprouts and such growths have been observed in biopsies from cancer patients (Rorth 2009). Also expression of proteins such as podoplanin facilitates such growths in cancer cells, as observed in in vivo models of pancreatic cancers expressing podoplanin (Wicki et al. 2006). Similarly leading cells in colorectal carcinomas show enhanced expression of Fascin, a protein which regulates Actin (Vignjevic et al. 2007). Frequently, cancers acquire mesenchymal phenotype, but the transition is not an inevitable requirement (Christiansen and Rajasekaran 2006). However, mesenchymal type of collective migration and swarm-like migratory patterns can provide cancers with certain advantages that aid in its growth and progression (Deisboeck and Couzin 2009).

The role of collective migration of cancer cells in tumour growth and spread has been extensively studied, but the kinematics involved has not been clearly documented. And although, various patterns of collective migration have been mentioned, very few attempts have been made to describe the role of individual cells in the development of the collective migration patterns. In this work, we are interested in the contributions of individual cell mechanisms on the collective migratory behaviour of breast cancer cells, and how differences in the mechanisms can change the kinematics of the collective behaviour and its implications in the biological environment.

2.3 Individual Cell Motility and Migration: Lamellipod Formation and Cell Contractility; Role of Rho/Rac and ROCK.

Motility is inherent to almost all types of cells. Cytoskeletal reorganization and individual cell motility are regulated by Rho GTPases, members of the Ras superfamily of GTP binding proteins that include the well characterized forms of Rho, Rac and Cdc42. But cancers are known to harbour mutations in these genes thereby providing enhanced cell migration potential and hence their metastatic capabilities (Boettner and Van Aelst 2002; Sahai and Marshall 2002; Aznar et al. 2004) (Figure 2.1). Therefore an understanding of individual cell motility is necessary to realize its relevance in cancer growth and progression.

Individual cell migration is considered to be a cycle of lamellipodial protrusion formation, adhesion to extracellular matrix by means of focal adhesions, contractility of myosin motors necessary for cell movement and for detachment from the focal adhesions at the rear end of the cell (Lauffenburger and Horwitz 1996). Epithelial cell migration on 2D surfaces is initiated by the formation of membrane protrusions such as lamellipodia and filopodia at the peripheral edges of the cells. Continuous waves of polymerisation and depolymerisation of Actin are required for the formation of these protrusions and the constant reorganisation of the cytoskeleton of cells that leads to shape changes and cell motility. Actin polymerisation also provides necessary force for the movement of cells (Mogilner and Oster 1996; Loisel et al. 1999; Footer et al. 2007; Huber et al. 2008).

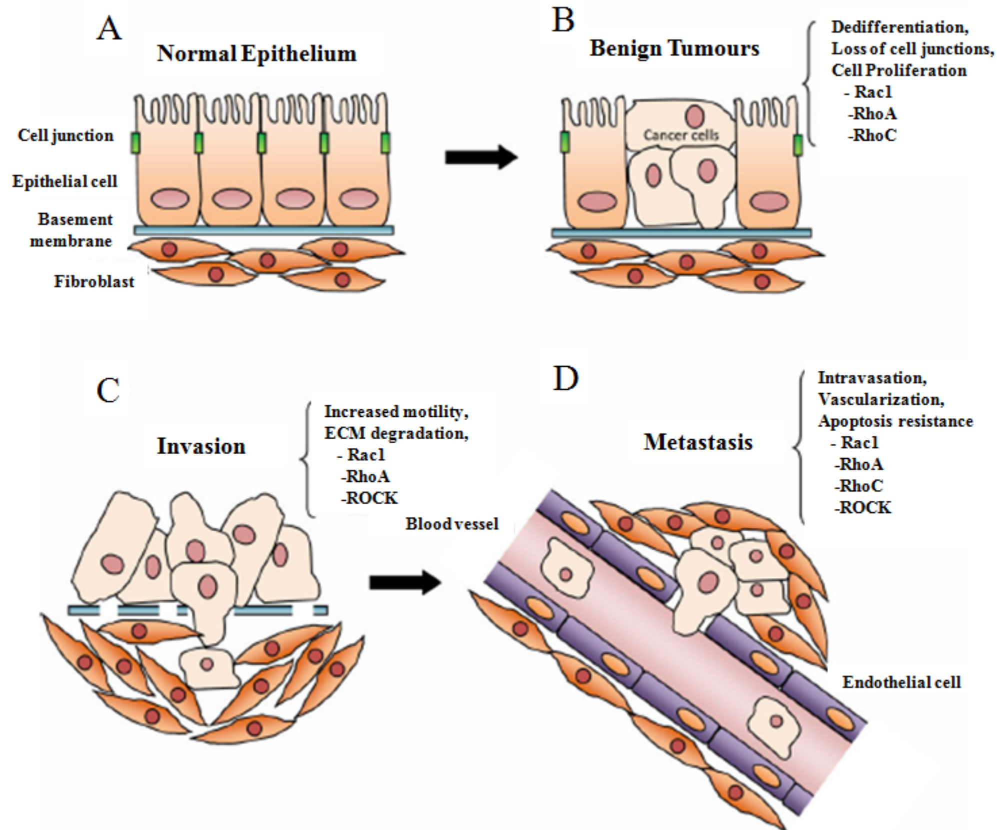


Figure 2.1 Rho proteins in tumor progression. Rho proteins are involved in the development of benign tumors (B) by affecting dedifferentiation, cell proliferation and cell junctions. In locally invasive tumors (C), Rho proteins and ROCK increase motility, disrupt cell-cell and cell-matrix adhesion and enhance ECM degradation. Rho and ROCK also promote metastasis (D) by influencing angiogenesis and transendothelial migration. (Figure reproduced from (Parri and Chiarugi 2010, Biomed Central))

Lamellipodia are meshworks of filamentous Actin that are responsible for the movement of leading edges of migrating cells (Tilney and Saunders 1983; Zigmond 1993). A stable assembly of seven protein subunits called the Arp2/3 complex induces the formation of a branched network of Actin filaments in the lamellipodia by promoting nucleation of of new Actin filaments from the sides of pre-existing filaments (Mullins et al. 1998; Svitkina and Borisy 1999; Bailly et al. 2001). The elongation of such filaments is subsequently inhibited by capping proteins to ensure short, stiff filaments as well as to concentrate polymerization to the protruding region

close to the plasma membrane (Pantaloni et al. 2000; Nicholson-Dykstra et al. 2005). Several other Actin-binding proteins are also involved in regulation of the Actin polymerisation in protrusions (dos Remedios et al. 2003; Pollard and Borisy 2003). Actin polymerisation and depolymerisation activities are significantly higher in the lamellipodia compared to that in the lamellar region of the cells (Ponti et al. 2004). Lamellipodial protrusions are controlled by Rac activity (Ridley et al. 2003) and decreased levels favour directional persistence in epithelial cells and fibroblasts (Pankov et al. 2005). Polarisation of lamellipodial protrusions is therefore required in migrating cells for them to persist in a particular direction.

Lamellipodium of migrating epithelial cells are stabilized initially by the formation of small cell-substrate adhesions, known as focal complexes, under the control of Rac and Cdc42 (Nobes and Hall 1995; Allen et al. 1997; Hall 1998; Rottner et al. 1999). Maturation of these focal complexes to focal adhesions that contain large clusters of integrins is mediated by Rho as well as myosin induced contractility. Rho activity is important for the contractility and tension inside the cells (Ridley et al. 2003). Contractile forces within the cells, which are necessary to drive cell motility, are generated by the interaction of myosin II with Actin filaments. The activity of myosin II is positively regulated by phosphorylation of myosin light chains (MLC) by myosin light chain kinase (MLCK) and the Rho kinase (ROCK). Contractile forces developed against the focal adhesions on extracellular matrix are responsible for the migratory speed of cells (Galbraith and Sheetz 1998). For rapid cell migration to occur, an optimal balance should exist between Actin filaments, myosin and focal adhesion dynamics (Gupton and Waterman-Storer 2006). Lamellipodial Actin polymerisation establishes a mechanical link between the contractility of myosin to the initiation of focal adhesion sites, thereby driving cell motility (Giannone et al. 2007).

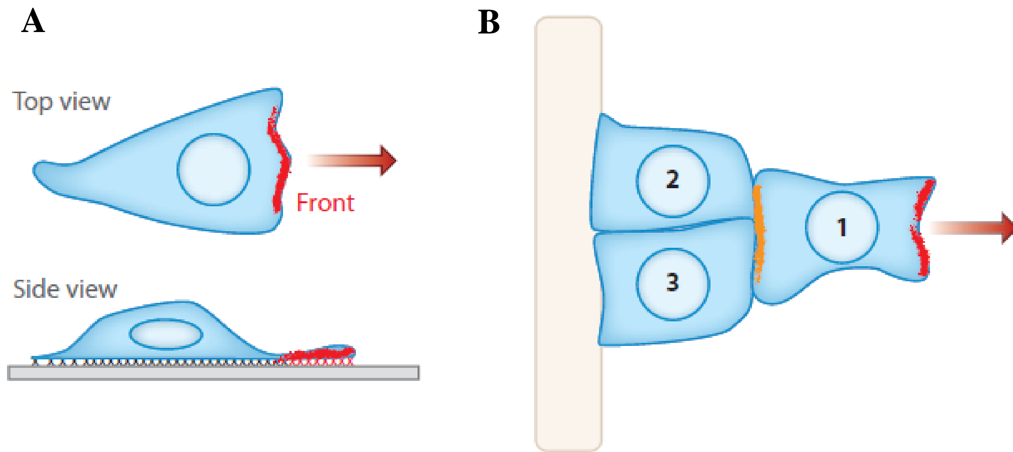


Figure 2.2 Basics of individual and collective cell migration. (A) A single cell migrating on a 2D flat surface. Actin based protrusions (red) formed on the leading edge of the cell and substrate adhesion (spikes on side view) help in exploration of the environment as well as subsequent migration. (B) Collective cell migration depends on inherent motility of individual cells as well as interaction between adjacent cells. Thus motility of the cell 1 is dependent on its strength of adhesion with the cells 2,3 as well as their relative movement. For example, if cells 2,3 show propensity to move in the forward direction (red arrow), then the net movement of the cell 1 will be enhanced. (Figure reproduced from (Rorth 2009))

During collective migration, cells of the leading row are usually well spread and show clear and broad lamellipodial protrusions, due to free space availability (Figure 2.2). But, lamellipodia are not restricted to the leading edge cells alone, and cryptic lamellipodial protrusions have been shown to contribute to collective migration of several rows of cells from the leading edge of wounded epithelium (Farooqui and Fenteany 2005). Therefore, lamellipodial polarisation in individual cells is required to develop the polarisation of the monolayer that is observed during sheet migration of cells.

All these known mechanisms responsible for individual cell motility are necessary for collective migration and invasion, and are important regulators of metastasis. Studying the contributions of individual cells in collective migration of malignant cells can therefore help in analyzing the pattern of cancer growth and dissemination. These patterns of collective migration can be of significant importance in the erosion

of adjacent tissues if the cancer cells are sufficiently invasive, and in the development of distant metastases if they acquire higher migratory capabilities, and ability to control them can be helpful in therapeutic strategies against cancers.

2.4 Cell Migrational Assays

Cell migration is an essential both in normal physiological processes and in the pathology of diseases. To study cell migration in vitro, various methods have been used including 2D migration assays, 3D invasion assays and filter assays (Frow et al. 2004; Entschladen et al. 2005; Decaestecker et al. 2007). Some of the 2D migrational assays include microcarrier bead assays (Rosen et al. 1990; Busso et al. 1994; Kjoller et al. 1997), wound healing assays (Liao et al. 1995; Kornyei et al. 2000; Fahmy and Khachigian 2002; Watanabe et al. 2003; Andre et al. 2004), barrier migration assays (Van Horssen and ten Hagen 2011) such as those using Teflon or glass rings (Horodyski and Powell 1996; Cleek et al. 1997; Cai et al. 2000), colloidal gold migration assays (Albrecht-Buehler 1977), and chemotaxis assays performed using the Dunn chamber (Zicha et al. 1991; Webb et al. 1996; Zicha et al. 1997; Allen et al. 1998; Debeir et al. 2004). Assays for cell migration in 3D include transwell cell cultures or modified Boyden chambers (Ohnishi et al. 1998; Worthylake et al. 2001; Connolly and Maxwell 2002; Lee et al. 2003; Taraboletti and Giavazzi 2004) and collagen gel invasion assays (Bracke et al. 2001).

Invasion assays attempt to simulate the invasion and migration of cells in 3D along fibers and fibrils within connective tissues. 2D migrational assays, on the other hand, assess cell adhesion and migration which is a key factor for growth and progression as well as spread of cancers to distant sites. While 2D migrational assays are mainly used for screening purposes, 3D assays are generally more complex in their experimental setup and are mostly used to analyze the migration process per se

(Entschladen et al. 2005). Newer approaches to cell migration study involve combining the technique of time lapse microscopy (discussed later) which allows for powerful analysis including single cell measurements, improving the strength of the experiment.

Wound healing assays have been one of the most commonly used methods for studying cell migration in 2D, and combined with strong analysis techniques they can be powerful tools. The following section will describe the wound healing and barrier methods of assays which are particularly important in in vitro collective cell migration studies.

2.5 Wound Healing and Non-Wounding Migrational Assays

Collective cell migration due to chemotaxis is an important process in the healing of cutaneous and organ injuries (Figure 2.3), where a breach of the epithelial continuity initiates rapid cell migration at the wound edges in an effort to close the gap (Clark 1996). Wound healing assays were developed to study such cell migration in an environment that simulates an actual wounded site. A wound healing assay or scratch assay is one of the most common procedures used to assess cell migration in vitro that is easy to perform and is a low cost alternative that demands lesser sophistication than other methods. Usually performed over several hours to 24 hours, it requires preparation of the sample that includes seeding of cells on the observing platform for a few days to achieve confluence that is then followed by scratch wounding the surface of the monolayer (Liang et al. 2007). This method has been persistently used in research to assay cell migration in transfected samples for evaluating the functions of proteins (Fukata et al. 1999; Etienne-Manneville and Hall 2001; Abbi et al. 2002; Saga et al. 2003), effect of drugs on cells (Fischer et al. 1990; Mc Henry et al.

2002)and effects of substrate coating (Newgreen et al. 1982; Thomas et al. 1992; Masiero et al. 1999) on cell migration through cell-substrate interaction, to mention a few. Overall, this has been an acceptable method for rapid assessment of drug and protein effects on cell migration as well as in the assessment of drug toxicity.

On the other hand, a non-wounding migrational assay, referred in literature as barrier assay (Van Horssen and ten Hagen 2011) (Figure 2.4), is one where various types of barriers are used to restrict cells to an area before lifting the barrier and allowing the cells to migrate. These modifications were tried by various researchers in view of the reason that not all biological situations are necessarily similar to wound healing (cancer migration for example) and also to explore new methods of investigating cell migration.

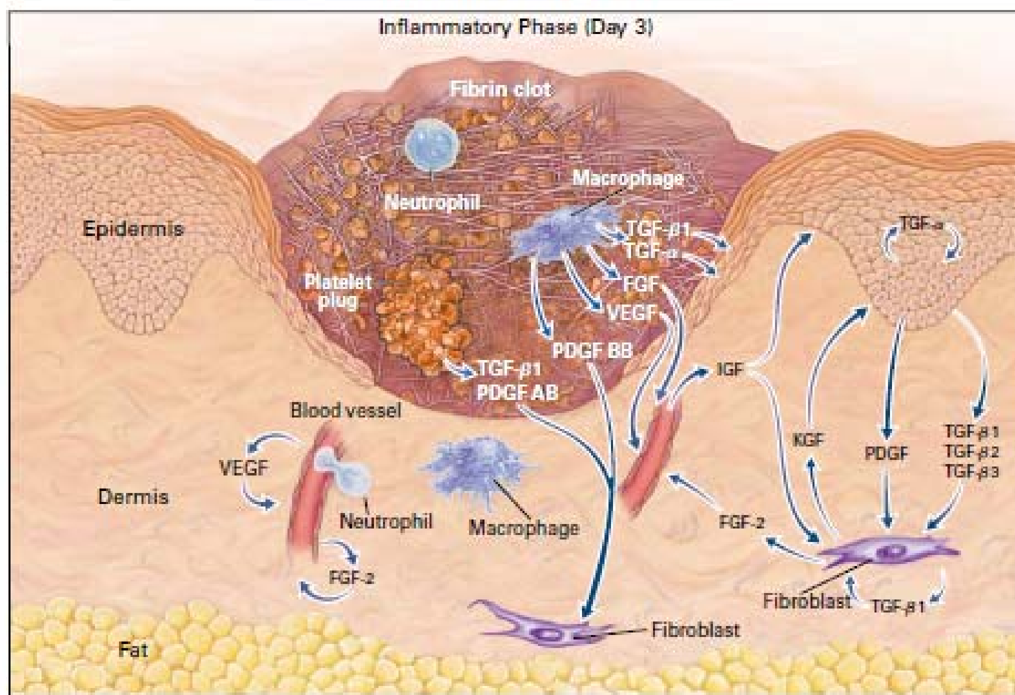


Figure 2.3 Typical Process of cutaneous wound healing. Chemotactic cell migration along the edge of a healing wound with the necessary growth factors is shown in this schematic. TGF transforming growth factor ; FGF fibroblast growth factor; VEGF vascular endothelial growth factor; PDGF platelet-derived growth factor; IGF insulin-like growth factor; and KGF keratinocyte growth factor. Figure reproduced from the reference (Singer and Clark 1999)

Several differences can be easily noted between the two methods. For example, the advantages in the latter method include the following:

1. Cell migration can be consistently assessed by monitoring within 12 hours post seeding and settling time/doubling time (which is usually 2-8 hours to 24 hours for most cell lines)

2. Absence of denuded cell debris, if particular care is maintained during sample preparation, adds to the difference; scratches in wound healing assays invariably results in the release substances as well as activation of intracellular proteins that affect cell migration through signal transduction pathways (Matsubayashi et al. 2004) leading to unintended chemotaxis of the cells at the edge. For example, Nikolic et al. not only showed that ERK1/2-MAPK activation coordinates individual cell motility but also that reactive oxygen species generated at the wound edge are involved in the propagation of the MAPK waves (Nikolic et al. 2006) that were previously reported by Matsubayashi (Matsubayashi et al. 2004).

3. Detailed observation can be made at single cell level at the leading edge, with no interference to the migrating cells from cell debris.

4. Distance covered in time monitored vs. time taken for the cells to close the wound. Although, alternatively, cell edge distance has also been measured in wound healing assays, they are not as accurate as individual cell measurements.

Interestingly, the effects of injury on the migration of individual cells in monolayers was investigated in the above mentioned study (Nikolic et al. 2006) which compared classical wound healing assays with the non-wounding variants.. It was found that in the case of uninjured conditions, the initial position of the cells with respect to the

wound edge was not important in determining the final displacement of the cell. This indicates that wounding actually contributes additional effects on migration of cells in a monolayer. Therefore, for effectively studying collective migration in native physiological conditions, a non-wounding setup would be ideal.

Wound healing assays and their modifications do allow study of collective cell migration found in monolayers (Figure 2.5). But theoretically, one variant of cells can be highly motile and yet due to swarming and tortuous movement cover lesser distances via cell monolayer growth (increase in cell monolayer dimensions) while another variant of the same cells could be less motile but well coordinated in their migration due to strong intercellular adhesions resulting in sheet migration and thus a larger cell monolayer growth. This effect would not be discernible on an ordinarily performed 2D wound healing assay which can only record more migration in the latter condition. Also, the effect of proliferation on monolayer edge growth complicates the situation. For example, a high throughput method of analysis using a wound healing assay was reported which relied on obtaining differences in the gap areas of the scratch wound in the cell monolayer, during the period of monitoring , to assess cell migration (Yarrow et al. 2004). The method allowed for quantitating more than 10, 000 scratch wounds per day, but could only report a difference in migration with no reference to individual cell motility or its effect on the collective migration, essentially highlighting the drawbacks mentioned above.

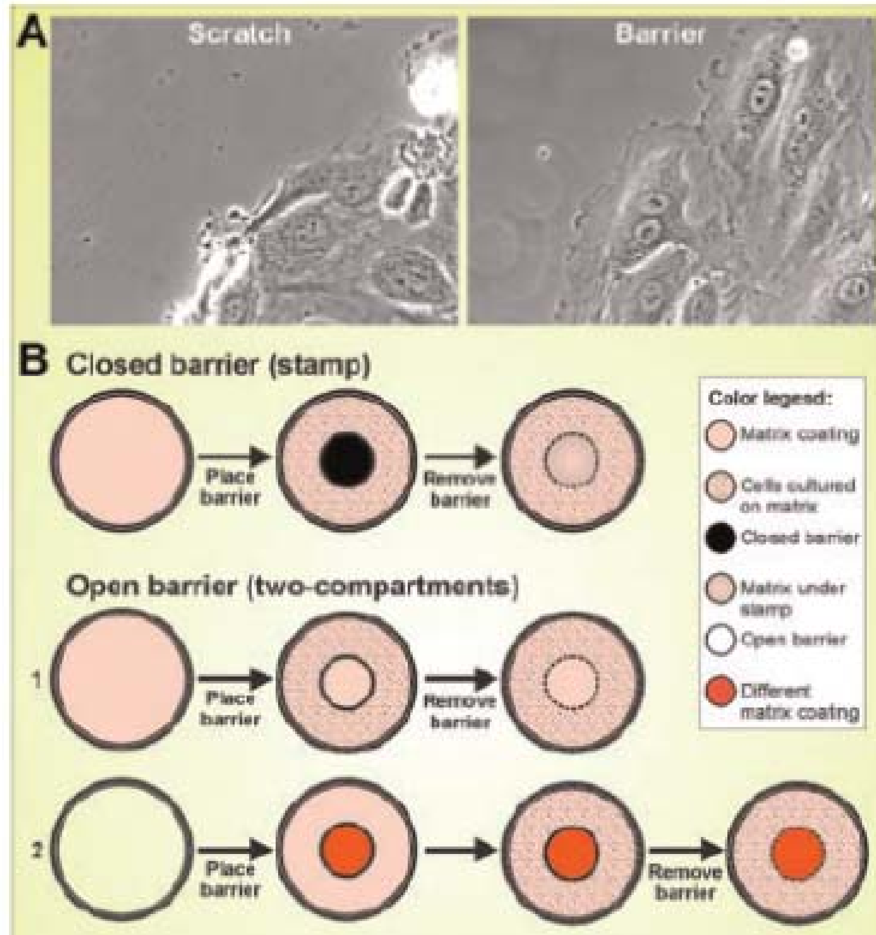


Figure 2.4. In vitro scratch assays and barrier migration assays. (A) Dead cell debris in classical wound healing assay compared with a clean monolayer edge in a barrier method of assay (Figure reproduced from (van Horssen et al. 2006)) (B) Variations of barrier migration assays. Closed barrier migration assays restrict coating to a single layer and might damage the coating . Open barrier assays allow multiple compartment set-ups (figure shows two) with as many different matrix coatings. Figure reproduced from the reference (Van Horssen and ten Hagen 2011)

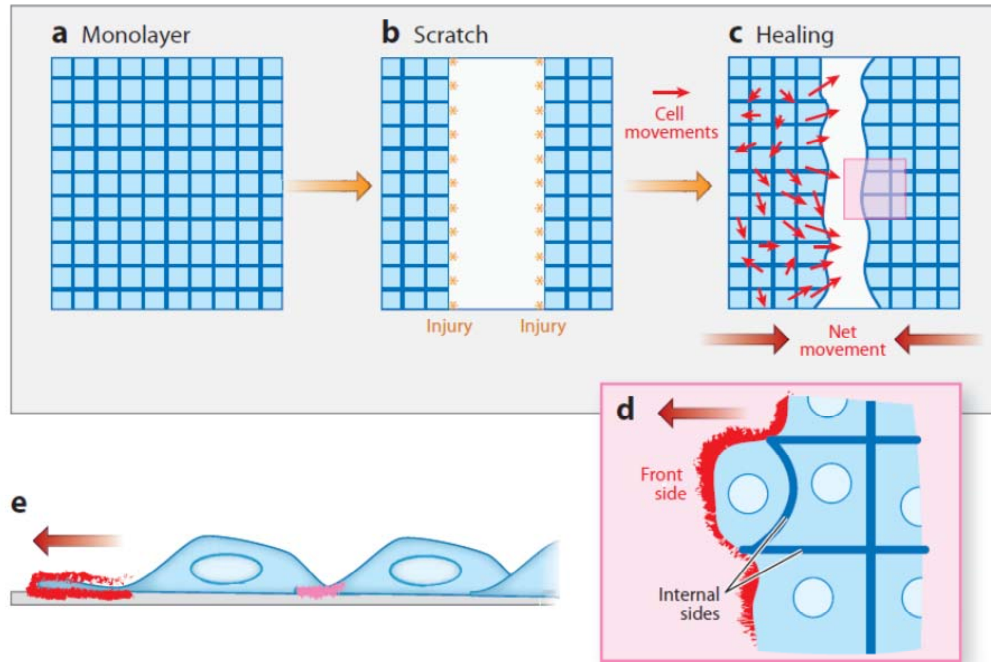


Figure 2.5 Collective Migration in 2D wound healing assays. Injuring a monolayer (b) creates free space and deconstraining of cells resulting in forces leading to migration of the cells in an effort to seal the gap (c). Cells at the leading edge (d) adapt to the free space by gaining polarity in this direction. The cells behind the front row cannot polarize but have the capability of forming cryptic lamellipodia (Farooqui and Fenteany 2005) that leads to collective migration towards the free space. (Figure reproduced with permission from (Rorth 2009))

Thus, if a usual wound healing assay was performed for the effect of a drug, the resulting erroneous conclusion is now obvious. The implication too would be less clear unless correlated with the in vitro environment. For example in a 3D environment having guiding cues such as fibers and fibrils, the migration of highly motile cells, but with affected intercellular adhesion, could become dangerously significant (cf. Section on 2D vs 3D migration below) when compared to their apparently less recorded migrational status on a 2D Assay.

Further, results of scratch assays are effects of both cell migration and proliferation and it is impossible to know the true effect of drugs that have been tested using this method (Decaestecker et al. 2007). However, observing and quantitating the migration at individual cell level helps in isolating the effects on cell migration. Also,

since wounding would be artificial and non-physiological event in the setting of a cancer, non-wounding migrational assays with advanced analytical methods can prove to be immensely helpful in studying the effects of various factors on cell migration. These are some of the critical reasons for explaining why a detailed observation of 2D migration can be useful in the study of collective migration.

Alternatively, particle image velocimetry (PIV) has been used to analyse collective migration in MDCK (Madine Darby Canine Kidney) cell samples prepared with a similar non-wounding technique using crosslinked polydimethylsiloxane (PDMS) microstencils (Poujade et al. 2007). This method however retrieves the displacement vectors at local regions (pixel windows) by cross-correlation of successive images in the video sequences/stack (Raffel et al. 2007). The resulting information is a continuous snapshot of displacement vector fields that considerably dissects the collective migration process, but ignores the movement of an individual cell over the entire sequence.

In conclusion, in vitro wound healing and other assays have been combined with individual cell motility measurements to study collective cell migration and invasion, sometimes with attempts to show the effect on single cell motility. But to our knowledge, no studies have documented the correlation between measured parameters for individual cell motility mechanisms and the development of the collective migratory patterns. In this work, we will approach the problem of collective migrational behaviour of breast cancer cells using a non-wounding migrational assay, and focus on individual cell motility parameters that can help in explaining the collective migratory patterns.

2.6 2D vs 3D migration

2D migrational assays have been used to study cell migration in the setting of various research problems as mentioned earlier. But one important drawback with a 2D setup is that it doesn't entirely simulate the complex in vivo 3D environment of the multicellular organisms that is experienced by most of the cells. Therefore, conclusions from 2D experiments are subject to considerable skepticism. In this section, we will introduce some of the works that have provided considerable details in regard to migration in these dimensions.

Cell migration on 2D surfaces involves various processes such as formation of active membrane protrusions at the leading edge, adhesion of the cells to the underlying substrate via focal adhesions, signal transduction to the cytoskeleton via adhesion receptor coupling, contractility of the lamella for cell movement, and the subsequent cell-substrate detachment at the trailing edge (Lauffenburger and Horwitz 1996). Events occurring in the biological environment such as embryonic morphogenesis and wound healing essentially involve this type of cell movement (Friedl et al. 1998; Aman and Piotrowski 2010). Migration in 3D however introduces factors such as matrix resistance, low affinity and non-adhesive interactions (Schor et al. 1983; Mandeville and Maxfield 1997; Friedl et al. 1998) and fibre-guidance for cell movement (Even-Ram and Yamada 2005) which makes the process quite different, and complicated. Examples include directional migration of mesodermal cells during gastrulation (Nakatsuji and Johnson 1984), and migration of primordial germ cells and leukocytes (Friedl et al. 2001). Guided motility is characteristic of migration in 3D matrices, and particularly important in pathogenesis of diseases like cancers. For example during cancer cell invasion, tumor cells can move in a stream like fashion

utilizing the guidance cues provided by fibres of the extracellular matrix (Farina et al. 1998; Wyckoff et al. 2000; Friedl and Wolf 2003; Sahai 2005).

2D surfaces favor various cell shapes such as amoeboid or simple –shape forms in Dictyostelium and leukocytes, spread-out forms in keratinocytes or bi- to multipolar morphologies of fibroblasts and myoblasts (Friedl et al. 1998). But introduction into 3D matrices induces a characteristic spindle-like elongated shape in the cells, as reported by various researchers (Weiss and Garber 1952; Nakatsuji and Johnson 1984; Heath and Peachey 1989; Niggemann et al. 1997). A recent study has shown that 1D fibrillar patterns induce migration in cells that is quite similar to that observed in 3D involving uniaxial cell movement dependent on myosin II contractility and microtubules (Doyle et al. 2009) . The same study also reported that both 1D and 3D migration are independent of extracellular matrix ligand density. This is interesting since varying ECM density produces a biphasic response in cell migration on 2D surfaces. Closer observations of the individual cells on the 1D pattern reveal that some cells show lamellipod like protrusions at their ends (Figure 2.6) which was not considered in the study. This could mean either a local defect in the patterned topology leading to availability of a 2D surface or the aggressive attempt of some cells to explore further with protrusions. In both cases, it is evident that a flat topology favors the normal cellular motility mechanisms to express in the form of protrusions, especially if the surface facilitates substrate adhesion. Also, both 3D and 1D essentially restrict the formation of cell-matrix contact points (focal complexes and focal adhesions) to two regions of the cells resulting in the spindle shapes that have been reported, and explaining why such migration is independent of ECM density. In case of multiple contact points, alignment of these points provides for the directional restriction.

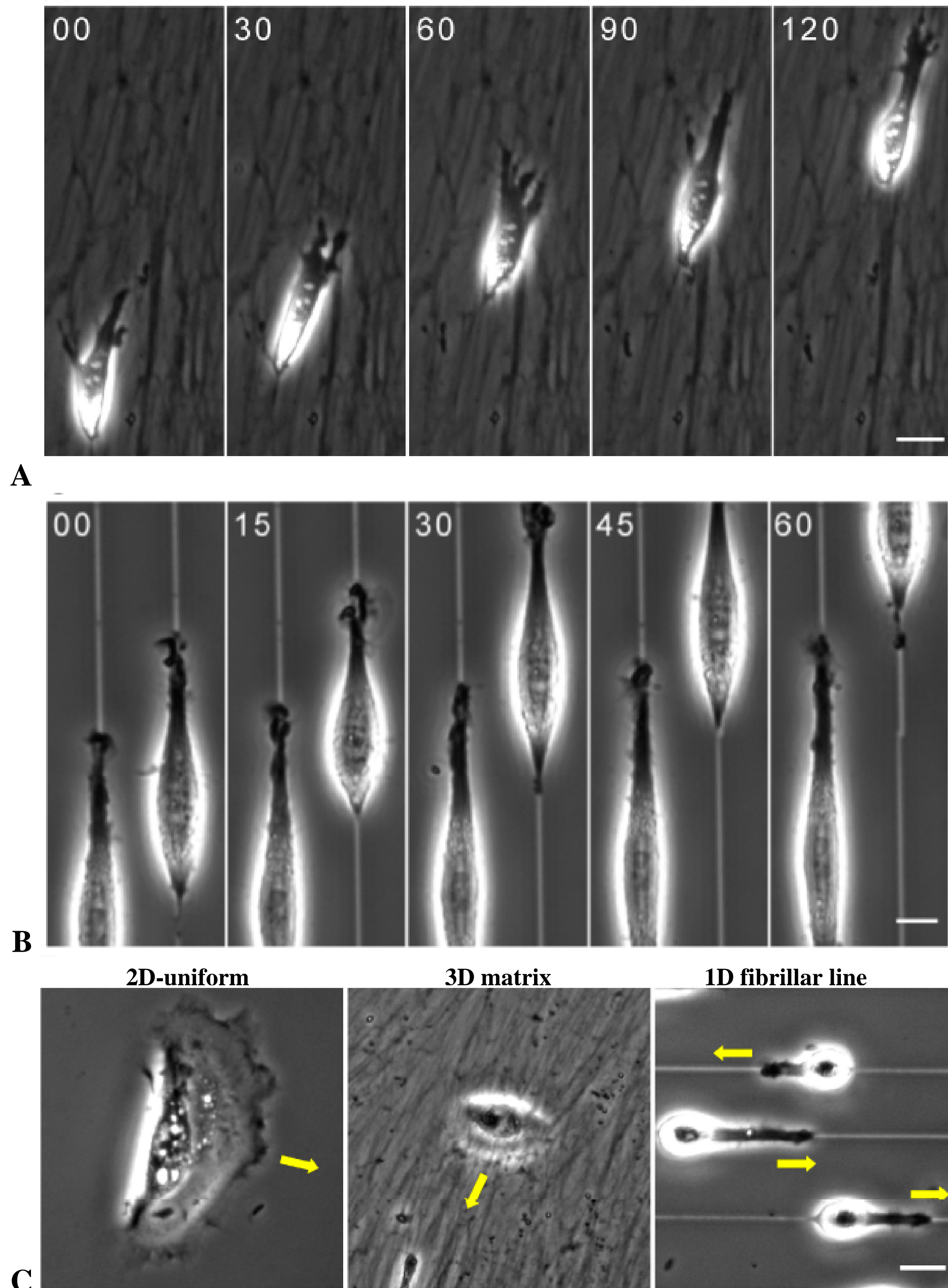


Figure 2.6 Effect of different topologies on cell migration. (A) NIH-3T3 fibroblasts in a 3D cell-derived matrix. (B) Migrating phenotype of fibroblasts plated on single lines (approximately 1.5μm) mimic those in the 3D matrix. (C) Morphological appearance of human keratinocyte cells migrating on 2D surface, 3D matrix, and 1D fibrillar lines. (Figure modified and reproduced from the reference (Doyle et al. 2009) (© 2009 Rockefeller University Press))

Thus, in the light of this information combined with our understanding of the normal epithelial structure and the similarity of the intracellular motility machinery regardless of the dimension of the environment, it is clear that a study of collective migration on 2D surfaces could be important, especially in the setting of cancer growth and progression. Furthermore, collective cell migrations and rearrangements during embryonic morphogenesis, wound healing and initial stages of cancer development prior to breaching of basement membranes would still follow rules similar to migration over 2D surfaces.

2.7 Time Lapse Microscopy and Cell Migration Analysis

Cell migration has been studied by various methods including the migrational assays mentioned in the earlier sections. But migrational assays do not help in understanding the complex interactions and processes involved in cell migration because they capture snapshots of real events for analysis. Time lapse video microscopy is a technique which allows capturing the dynamics involved in both individual and collective cell migration and helps in better understanding the details involved in the process (Entschladen et al. 2005).

Time lapse video microscopy has been used to study cell migration of immune cells such as lymphocytes (Bousso et al. 2002; Miller et al. 2002; Stoll et al. 2002; Lindquist 2004; Mempel et al. 2004; Allen et al. 2007), germ cells (Molyneaux et al. 2001), cancers (Harms et al. 2005) as well as chemotaxis in macrophages. The need for accurate methods for studying cell migration has necessitated development of techniques for automating the process (Fotos et al. 2006). Time lapse also improves on the conventional wound healing assays by allowing measurements and analysis of individual cell movements. Combined with analytical methods for cell migration such

as cell tracking, time lapse imaging can be a powerful tool for studying the kinematics and dynamics involved in both individual and collective cell migration.

Collective cell migration analysis of time lapse video sequences is usually performed by tracking of individual cells. Methods including manual (Michl et al. 2005; Wolf et al. 2007; Boldajipour et al. 2008; Henrickson et al. 2008), automated (Demou and McIntire 2002; Bahnson et al. 2005) and interactive (semi-automatic involving human operator) tracking (Zicha et al. 1998) have been used for cell migration analysis in research. However, one of the troublesome manifestations of cells in confluent monolayers is the inability to distinguish most of the cell contours making it almost impossible to perform efficient segmentation for image processing. Complicating the issue further, is the haphazard migration of some cell lines (like the MDA-MB-231) which contributes to erroneous measurements. Also, an essential drawback of automated tracking systems is the need for clear contrast among the cells in the images; thereby necessitating the use of sparse cell populations (Bahnson et al. 2005; Li et al. 2008; Chen et al. 2009; Huth et al. 2010) in cell migration studies, which undermines the essence of true collective cell migration. Also larger volumes of data require heavy computing resources, thus forcing observations to be restricted to a few frames. For our experiments, we wished to study collective migration of cancer cells over 24 hours duration and for this reason we have resorted to using the manual mode of tracking individual cells. The manual method is reliable in the setting of collective migration, and although tedious, provides valuable information regarding the behavior of cells in monolayers over longer duration periods.

Irrespective of the method of tracking, the data obtained from tracking can be used to evaluate cell motility (cell speed and migratory distance covered by cells), directionality (straightness index and deviation angles in track paths) and the

migratory behavior of the cell population as a whole (track plots) (Beltman et al. 2009). These parameters have been commonly employed for quantifying the cell migration from time lapse images. Some of the studies have even combined tracking with kymographic analysis of single cells for effects on lamellipod protrusions (Totsukawa et al. 2004), but none of the studies have attempted to study the effects of individual cell motility mechanisms in a systematic manner or correlate the results with track patterns and measurements.

2.8 Cell Lines: Differences by ATCC and Previous Proteomic Researches and their Implications

This thesis work involves the use of two different cell types, derivatives of the ductal epithelium of the human breast and human corneal epithelial cells. Majority of this work is mainly focused on the collective migration of both benign and malignant breast cancer cells. However, since we have also developed individual cell motility parameters that can be correlated to the collective migrational patterns, we have tried to investigate the reliability the concept by testing it on normal epithelial cells. For this reason, and also because established normal human breast epithelial cell lines are unavailable, we have used human corneal epithelial cells in a two part experiment involving evaluation of the assay method and the concept of individual cell motility parameters in the development of collective migration patterns.

2.8.1 Breast Ductal Epithelial Cell Variants

The breast epithelial derivatives MCF-10A, MCF-7 and MDA-MB-231 were obtained from our collaborators Dr. Alan Premkumar (Cancer Science Institute, Singapore) and Prof. Ong Choon Nam (NUS Environmental Research Institute). These cell lines have

been frequently used in literature for comparisons between the manifestations and gene expressions in benign and malignant tumors (Meric et al. 2002; Fay et al. 2003; Fox and Kandpal 2004; Nagaraja et al. 2006; Lai et al. 2010). The MCF-10A cells (Figure 2.7A) are adherent variants of a non-tumorigenic cell line derived from benign fibrocystic breast disease cells obtained from a 36 year old female Caucasian, and produced by long term culture in serum free medium with low Ca++ concentration. These cells are commonly used in literature to represent benign tumors.

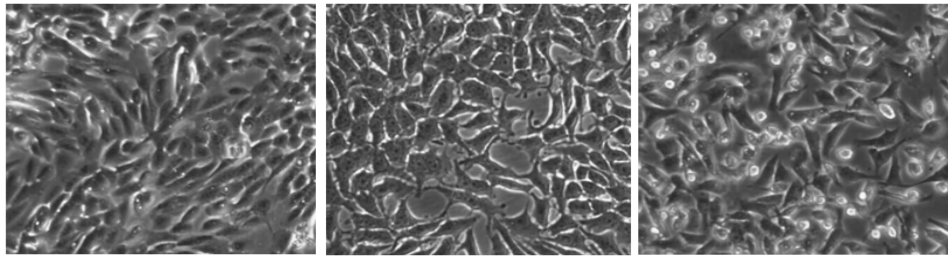


Figure 2.7 Breast ductal epithelial cell variants: (A) MCF-10A cells with almost no gaps between in between indicating strong intercellular adhesion. (B) MCF-7 cells show distinct gaps in between, although appearing well organized as a monolayer (C) MDA-MB-231 cells. Note the disorganized arrangement of the MDA-MB-231 cells. These cells frequently assume rounded morphology and can be found migrating across other cells.

As mentioned earlier, majority of the clinically diagnosed breast cancers are infiltrating ductal adenocarcinomas. Both the MCF-7 and MDA-MB-231 cells (Figure 2.7B and C) are ductal adenocarcinomas of the breast that were obtained from metastatic sites of pleural effusion. The MCF-7 cells were obtained from a 69 year old female Caucasian and have multiple chromosomal abnormalities including hypertriploidy to hypotetraploidy (modal number = 82; range = 66 to 87), absent chromosome 20 was disomy of chromosome X. The MDA-MB-231 cells were obtained from a 51 year old female Caucasian with metastatic breast cancer and also exhibit chromosomal abnormalities such as aneuploidy (modal number = 64, range = 52 to 68) with chromosome counts in the near-triploid range and absent normal chromosomes N8 and N15.

Differential protein expression in the three cell lines have been reported in several research studies (Pucci-Minafra et al. 2002; Varnum et al. 2003; Nagaraja et al. 2006; Pawlik et al. 2006; Lai et al. 2010). Several gene expressions were significantly different among these cell lines. For example, microarray analysis was used to identify altered transcripts that were involved in cell–cell or cell–matrix interaction, Rho signaling, calcium homeostasis and copper-binding/sensitive activities. A set of nine genes were reported to be sufficient to distinguish MDA -MB- 231 from MCF-7 cells (Nagaraja et al. 2006).

Similarly, differential expression of total and secreted proteins were reported by Lai et al. Interestingly, a higher number of cell-motility related proteins were upregulated in the MCF-7 cell lines when compared to MCF-10A and MDA-MB-231 (See Appendix A) (Lai et al. 2010). Proteins that affect contractility such as the Rho-GTPase activating proteins and ROCK 2 were overexpressed in MCF-7.

2.8.2 Human Corneal Epithelial Cells (HCE-T)

Human SV-40 immortalised corneal epithelial cell line (RCB1384, HCE-T, Riken Cell Bank, Ibaraki, Japan) were a gift from Kaoru Araki-Sasaki (Kinki Central Hospital, Hyogo, Japan) and Dr. Louis Tong of Singapore Eye Research Institute (SERI). HCE-T cells (Figure 2.8) were originally obtained from the corneal tissue of a 49 year old female and were immortalized with the SV40 large T antigen (www2.brc.riken.jp). Transfected control and TGM-2 knock-down variants (details in chapter 5) were used to evaluate the Non-wounding 2D migrational assay setup as well as to test the hypothesis that disruptions of intracellular contractility affect the lamellipodial protrusion formations and hence the collective migratory pattern.

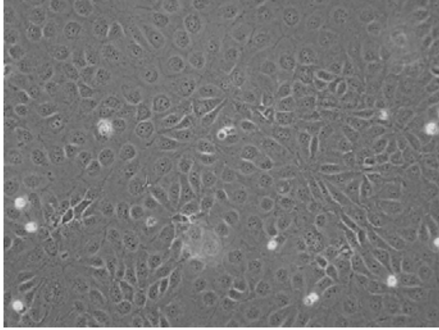


Figure 2.8 Human Corneal Epithelial Cell Line, HCE-T.

To summarize, it is important to study collective migration of cancer cells in order to know how the migration patterns affect cell behavior in groups and their subsequent effects on tumor growth and progression. It will also help in understanding the role of collective cell migration in the development of metastases both during the initial and the final stages of the process. An understanding of individual cell contribution will also be beneficial in our knowledge of the physiological processes involving normal cell migration and rearrangements. Finally, since migrational assays have been used for a long time and have a very important role in determining the effects of drugs and other interventions on cells but yet are insufficient in providing detailed information, it is important to improve on the existing migrational assay techniques so that the deficiencies are appropriately dealt with.

Chapter 3
Materials and Methods

This chapter describes the details involving the techniques and protocols used in studying the cytoskeletal organization and performing the non-wounding migrational assay. Additionally, the methods for analyzing the collective migration track patterns and contributions of individual cell mechanisms to collective migration of cancer cells will be explained. Also since this type of assay was tested on two cell lines using different protein knock down systems, the relevant interventional techniques will be described in the corresponding chapters.

3.1 Cell Culture

MCF-7 (non-invasive, Breast Adenocarcinoma) and MDA-MB-231 (invasive, Breast Adenocarcinoma) cells were cultured in DMEM (Dulbecco's Modified Eagle Medium) with 10% Fetal Bovine Serum (FBS) and 1% antibiotics (Penicillin and Streptomycin). The MCF-10A (Benign, Fibrocystic Breast Disease) cells were grown in MEBM (Mammary Epithelial cell Basal Medium, Clonetics) supplemented with Bullet kit containing growth factors (Insulin, basic Fibroblast Growth Factor, Corticosteroid and Bovine Pituitary Extract). The cancers cells used for the experiments were between passage numbers 15-25.

The human corneal epithelial cells (HCE-T) (passage numbers less than 12) were cultured in DMEM-F12 supplemented with 5% FBS and 1% antibiotics (Penicillin and Streptomycin). The Transglutaminase-2 (TG2) knock down variants (shTG), were cultured in DMEM-F12 with 5% FBS and 0.15ug/ml of Puromycin, which is a selective antibiotic for the TG2 shRNA plasmids.

All the cells were maintained in incubators at a CO_2 concentration of 5% and temperature of 37° C and grown to confluence before using them for experiments.

3.2 Study of Cytoskeletal Organization

Cytoskeletal reorganization is essential for the dynamics of cell movement. Cytoskeletal organization, therefore, is an indicator of the status and orientation of the cell movement. Polymerisation and depolymerisation cycles of Actin drive the motility of cells in combination with the contractility proteins, myosins. A study of Actin filament organization would thus serve to correlate with the motility behavior of cells and explain the reason behind their collective migration pattern. To study the distribution and orientation of Actin stress fibers in both individual cells and cell groups in cell monolayers, we used the standard Actin staining protocols involving use of the F-Actin binding molecule, Phalloidin. The following subsections describe the staining procedures and the mode of acquisition of images for assessing the stress fiber organization in the breast cancer cells.

3.2.1 Actin Staining

Stress fiber response was observed in individual spreading cells as well as cells from the leading edge of the migrating front and the confluent center of the monolayer. Accordingly, samples for cell spread imaging were seeded at lower density while those in a monolayer were imaged by using the samples used for collective migration. Standard protocol for Actin staining with Phalloidin was followed. The cell samples were fixed with 3% paraformaldehyde for 15 minutes and permeabilized with 0.1% Triton X-100 for 6 minutes before staining. Phalloidin–Tetramethylrhodamine B isothiocyanate (Sigma) was used to stain for filamentous Actin present in the stress fibers and 1:1000 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI, Sigma) was

used as a counter stain for the nuclei. The cells were washed twice with 1x PBS after each step of the staining procedure. Mounting medium (PVA-DABCO®, Biochemika, Sigma Aldrich) was then used to fix the stained samples.

3.2.1 Confocal Microscopy

The Laser Scanning Confocal microscope (LSCM) is an important tool that has found major application in biomedical imaging of living and fixed tissues at the level of light microscope. In specimen thicker than 2um, light from focal planes away from the region of interest interferes with the resolution of structures in focus. The Confocal microscope is designed to eliminate the interfering flare and provides an increase in both lateral and axial resolution (Paddock 2000) (Figure 3.1). The LSCM has been used in biomedical sciences to study the details of structures within cells such as filamentous Actin (Ezer et al. 1997) and for accurate localization of proteins (de Almeida et al. 1994; Xia et al. 2003; Mokin and Keifer 2006). It has also been used to study the dynamic changes occurring in cells during migration. For example, the cytoskeletal reorganization in melanoma cells during the process of transendothelial migration (Voura et al. 1998) was shown to be a two step process involving cell attachment and penetration of the monolayer. Since we were interested in the cytoskeletal organization of the breast epithelial derivative cell lines, we resorted to the LSCM in view of its good resolution and reliability. Our objective here was to obtain the stress fiber formation of the cell lines with higher details. For this purpose we used the Nikon TE2000 Confocal Microscope to obtain images of stress fibers under 20x and 63x oil immersion objectives. The proprietary software EZC1 v3.90 was used to handle the microscope and the image data.

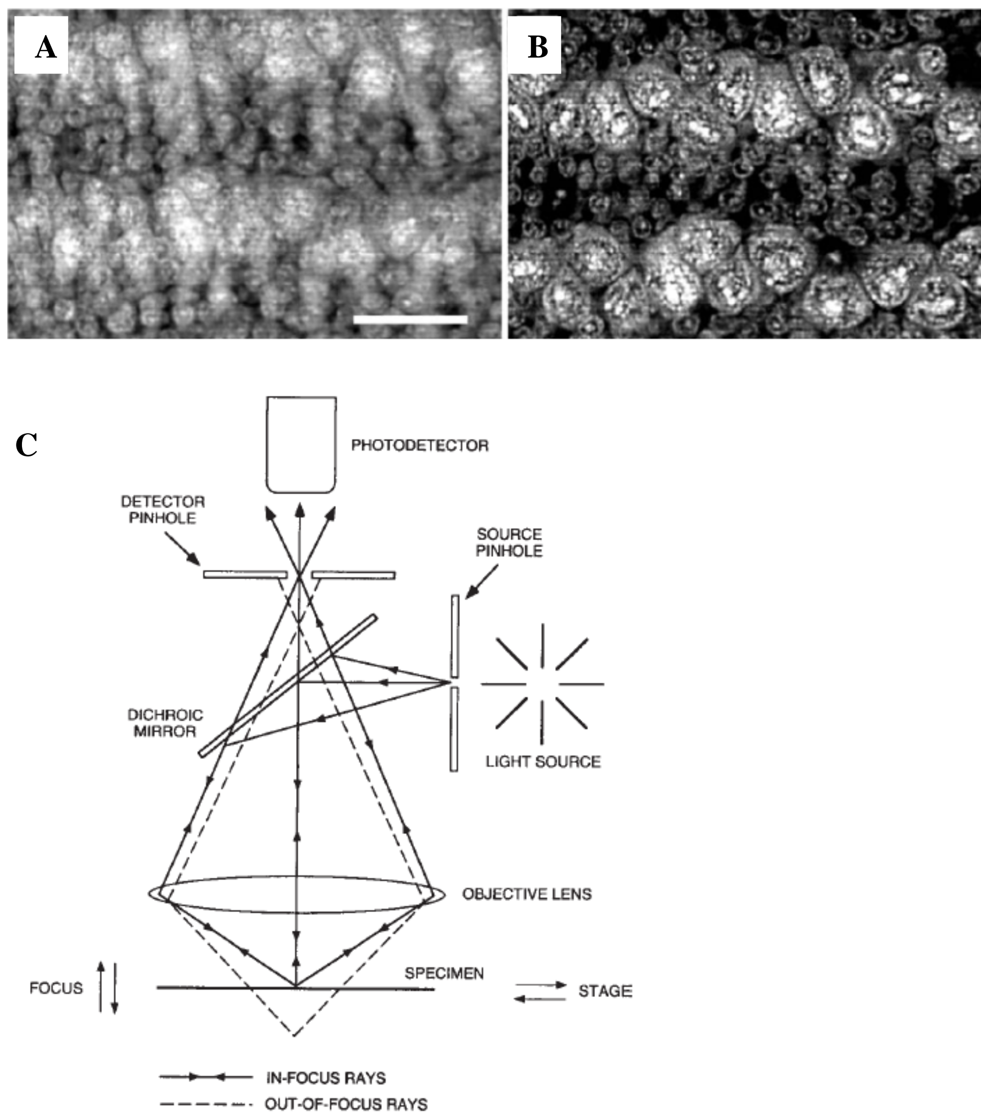


Figure 3.1 Principles of Confocal Microscopy. Epifluorescence image (A) compared with the high resolution confocal image (B) of a similar region of a whole mount of a butterfly (Precis coenia) pupal wing stained with propidium iodide. (C) Schematic diagram showing the optical principle of confocal microscope where light rays that are “out-of-focus” are eliminated via pinholes placed in front of the light source and the photodetector. Figures adopted from the reference (Paddock 2000)

3.3 Non-wounding 2D Migrational Assay Setup

As described in the literature review, one of our main objectives in the studying the collective migration of breast cancer cells was to develop an approach of 2D migrational assay setup with particular emphasis to analytical details. To achieve this, we have resorted to using one of the non-wounding migrational assays called the Ring assay technique. We have used glass cylinders to isolate seeded cells from surrounding areas and produce circular monolayer of cells that can be studied by live video microscopy. We have combined this approach by analysing the data at higher details than the normally used wound healing assays, in order to delineate the mechano-biological processes that are responsible for the outcomes of collective migration. Thus with this methodology, not only are the ordinary details obtained by in vitro scratch gained, but also specific details pertaining to the mechanism of action can be delineated.

The following subsections describe the technique of preparing the samples and the experimental setup along with the live time lapse video microscopy method.

3.3.1 Sample preparation

Glass Culture Cylinders (Bioptechs) (Figure 3.2 A, B) of 6mm inner diameter were placed upright on their highly polished edges in the centre of 35mm culture-coated petri-dishes (Corning). Alternatively, human Collagen IV (Abcam) was used to coat the petri-dishes as described below. The cells were released with 0.05% trypsin, centrifuged at 1200g for 4 minutes, re-suspended in the relevant complete medium and about 15,000-20,000 cells in 100µl of complete medium were added carefully into the upright cylinders. The petri-dish on the exterior of the cylinders was filled with 2ml of medium to support the cylinders. The highly polished edges of the

cylinders allow them to efficiently isolate the cells from the surrounding area so as to obtain a clean undisturbed monolayer edge for monitoring cell migration. The petri-dishes were incubated with the cylinders undisturbed for 24 hours, although a few hours should be sufficient to allow the cells to adhere. The glass cylinders were carefully lifted using tweezers after 24 hours of seeding the cells, and the petri-dishes washed thrice with Phosphate Buffer Saline (PBS), thus exposing a clean edged monolayer of cells. The cell monolayers were then video monitored, beginning from within an hour of the lifting of the cylinders.

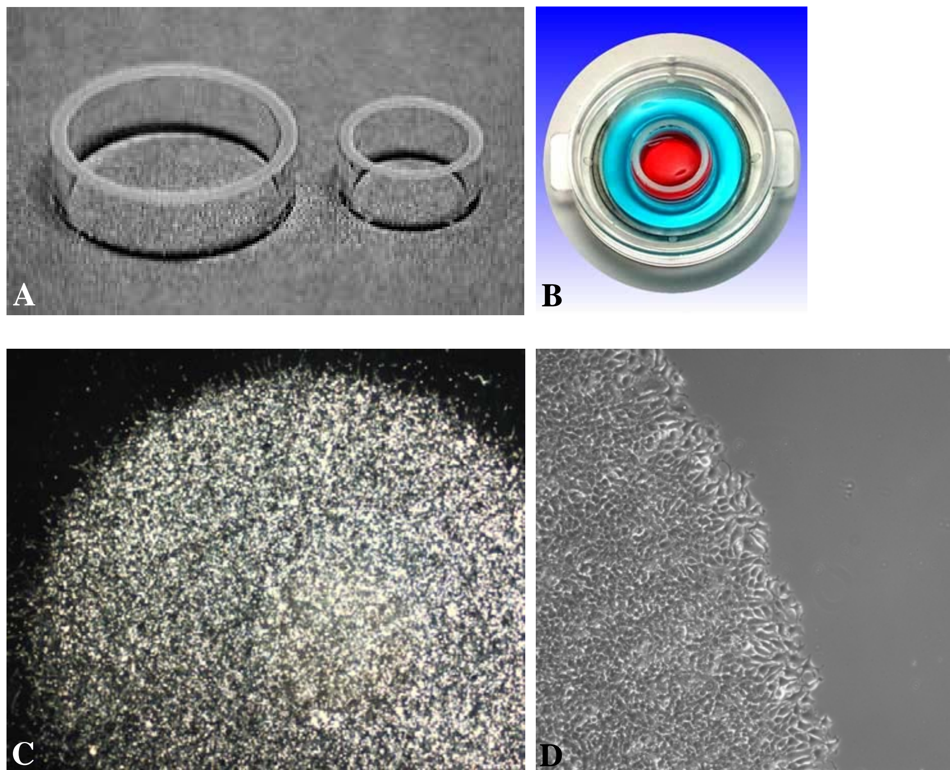


Figure 3.2 Glass Culture Cylinders (Bioptechs) (A). (B) Image shows the cylinder efficiently isolating fluid (red) from the fluid (green) in the surrounding chamber. (C) Circular cell patch immediately after removal of cylinder, under low power magnification (D) The cell patch under 10X objective following PBS wash reveals a clean patch of MCF-7 cells. NB: image was cropped to fit the location in document. The actual crop contains about twice the amount of free space available for video monitoring.

3.3.2 Optimization of Cell Seeding Density

A particular problem with using barrier assays or non-wounding migrational assays is the effect of cell sizes on the number of cells needed to achieve confluence. Since the monitoring is begun within 24 hours of seeding the cells, the cell seeding density has to be precise within a few hundred cells; otherwise the result can either be excessive cells along with dead cell debris or sparse colonies of cell patches. Both outcomes are not preferred for a study of collective migration and furthermore, the former interferes with analysis and interpretations. So a trial and error method by using cell count and the area of distribution in the flask, followed by extrapolation to the area of the inner circle of the cylinders was used. About 15-20,000 cells were required for seeding based on the size of the cells, with the higher cell number found suitable for the smaller sized MCF-7 cells and the lower cell number for the larger MDA-MB-231 cells. Even so, each sample preparation schedule consisted of 3-4 cylinder samples in as many petri-dishes to ensure obtaining a reliable patch of cells for monitoring migration. This is the only tangible drawback of this system.

3.3.3 Human Collagen IV as Substrate

Collagen IV was chosen as a substrate coating to test the collective migration of breast cancer cells in the 2D migration assay set up. The rationale was two-fold. Firstly, and more importantly, collagen IV is a major component of basement membranes(Paulsson 1992) and so, cancers arising from epithelial structures in any organ or duct system encounter the components of the basement membrane very early during the process of migration and invasion. Therefore collagen IV is likely to have a significant impact on the behavior of cancer cells.

Human collagen IV (Abcam), was used at a concentration of 10μg/cm^2 to coat the surface of the petri-dishes used to prepare the sample. The petri-dishes were incubated at room temperature with freshly prepared collagen IV solution for 1 hour, and then washed once with 1X PBS before proceeding as described in the section on sample preparation.

3.3.4 Drug treatment

The Rho Kinase (ROCK) inhibitor Y27632 was used to disrupt intracellular motility of the normal epithelial cells (HCEC) to test the use of individual cell motility analysis in comparison to the collective migration analysis. We have used a concentration of 30-100μM to achieve the disruption of the intracellular contractility and collective migration pattern.

3.3.5 Live Time Lapse Video Microscopy

The Biostation IM (Nikon) was used to obtain time lapse video microscopy of the migration of cell monolayers. The Biostation IM (Figure 3.3) is a live multipoint time lapse imaging system comprising a phase contrast microscope, an in-built incubation system and a CCD camera. The images were acquired at a 10X magnification with 1-minute intervals between the frames over 24 hours for all the cell lines, but were reduced to videos with 10-minute interval frames for tracking purposes. From preliminary observations, it was found that this duration is shorter than the minimum time period required for these cells to change the direction of their movement (persistence time), and hence suitable for tracking and collective migration parameter estimation. Individual cell motility parameters were observed in videos that were obtained at 1-2 min intervals. Throughout the experiments the CO_2 concentration was maintained at 5% and temperature at 37° C. The smaller size of the chamber that

encloses the stage for the microscope allows for accurate maintenance of temperature as well as CO2 supply across the petri-dishes as confirmed by the status of the cells.

Figure 3.3 The Biostation IM (Nikon). The white box on the left resembling a Central Processing Unit (CPU) actually hosts the motorized microscope, incubation chamber with immobile stage and the humidifier unit. The unit in the center is the controls for the movement of the microscope objectives (10X, 20X and 40X) equivalent to the x-y-z axis movement of a usual microscope stage. The monitor shows the proprietary software used to manipulate the Biostation. The CPU is hidden from the view. (www.nikoninstruments.com)

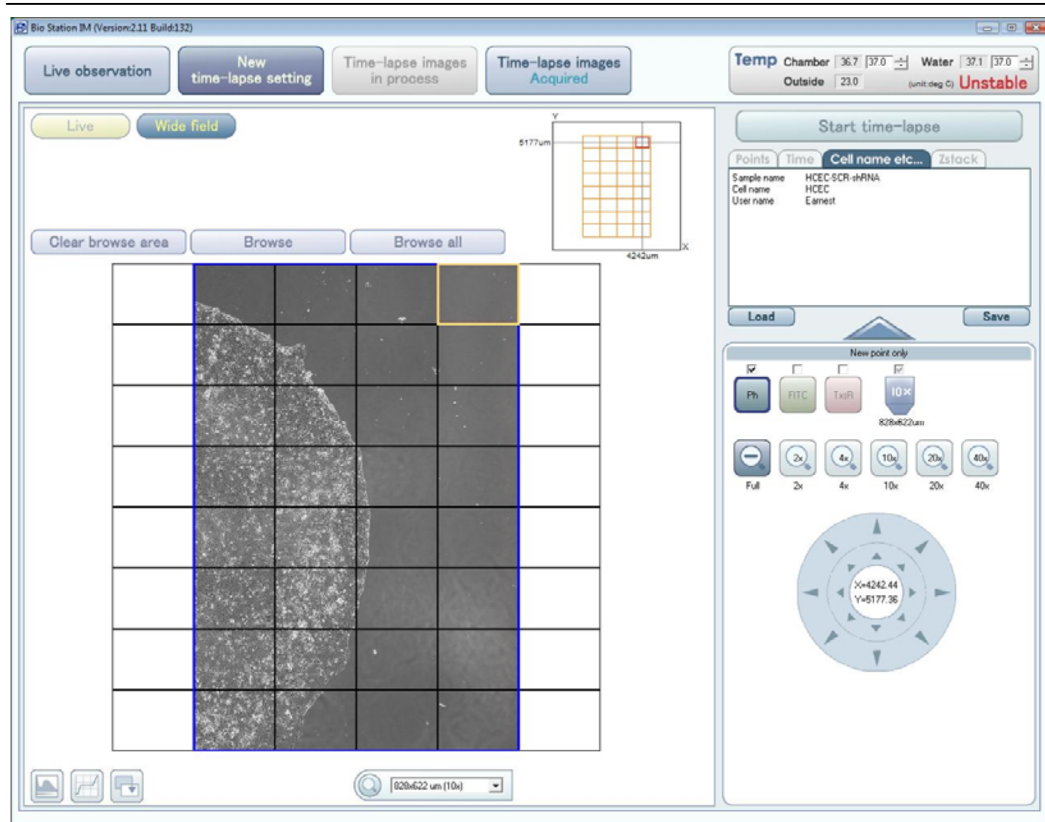


Figure 3.4 A typical sample image representing a circular cell monolayer as viewed by the phase contrast microscope of the Biostation IM (Nikon), after removal of the cylinder. The proprietary software allows for selection of multiple regions for video monitoring.

3.4 2D Collective Cell Migration Analysis

Although individual cells are analysed for migratory parameters, we would prefer to call these analyses as pertaining to “collective cell migration” since they provide information about the overall pattern of movement and characteristics by regarding the cells as a group. Also, the parameters in this section are obtained by measuring cumulative effect of only the movement or periodic (10 minute interval) displacements of individual cells. This method of analysis emphasizes the significance of cumulative effect of individual cell motility in the development of a

collective migration pattern disregarding the details of the motility mechanisms in individual cells.

The following sections will involve a detailed description of the method of tracking cells with the conventions involved and the parameters chosen for estimating the collective cell migration.

3.4.1 Cell Tracking

To circumvent the limitations involved in automated tracking, as discussed in the literature review, and to focus on cell sheet behavior rather than the manner of tracking, we resorted to the use of manual tracking of individual cells. The Image J plugin, MTrackJ (Eric Meijering, Biomedical Imaging Group Rotterdam, Erasmus MC - University Medical Center Rotterdam, Netherlands) (Meijering et al. 2012), was used to track the cells (Figure 3.5) of the leading 6-8 rows at the edge of the monolayer. The approximate centers of the cell bodies (lamellae) excluding the lamellipods were used to mark the cells in each frame. The lamellipods function by helping the movement of the cell body along with the nucleus, and are hence the dynamic components representing the motility machinery of the entire cell. This method of tracking therefore aims to reduce the fluctuation of the marker due to the artifact produced by movements of lamellipods which actually do not result in the movement of the cell (Figure 3.6). This factor however affects only the front rows of well spread cells. Cell lines with intact intercellular adhesion have well spread motile cells at the leading rows but for those with defective intercellular adhesion, the lamellipodial protrusions are visible even for cells in several rows from the leading edge of the monolayer. Only the cells that were present within the field of view for the entire time range were tracked and considered for the measurements. Cell areas

(n>60) were estimated for both the tested conditions with the software NIS*Elements (Nikon)*.

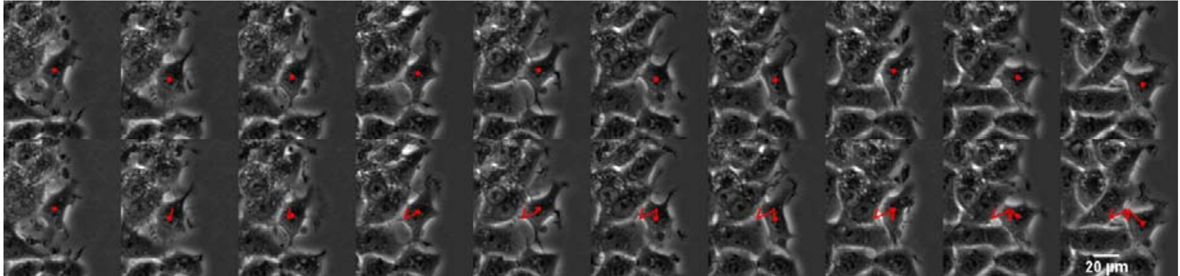


Figure 3.5 Manual Tracking method. Montage of a single MCF-7 cell tracked over 10 frames with point and track overlay.

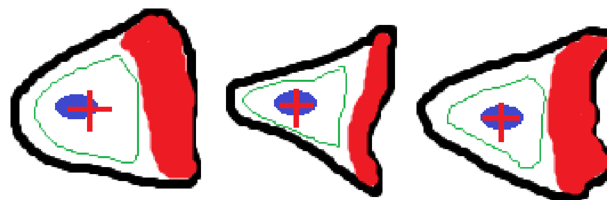


Figure 3.6 Conventions for Individual Cell Tracking. The green outlines represent the lamellar portions of the cells which are relatively stable and slow to change shape and are responsible for contractility. The red shades represent the dynamic region of lamellipods with constantly changing areas, which can give rise to false movement of track points even though no cell movements are recorded, especially if the cells in question have high lamellipodial activity. Cell track points (red crosses) are placed within the approximate centers of the lamellar regions, disregarding the shape change of the cell induced by the lamellipods. In this way, only actual cell movements are recorded.

3.4.2 2D Migration Parameters

Cell migration parameters that have been commonly used in literature (Beltman et al. 2009) were calculated and analyzed from the data obtained through cell tracking. The following are the definitions of the parameters used in this thesis.

Displacements and cell migration speeds:

To quantitatively study the 2D collective cell migration, we used several cell displacement parameters (Figure 3.7). The Accumulated distance (AD) defined by Eqn (1) refers to the total path distance covered by a migrating cell for the entire duration monitored, while the Euclidean distance (ED) is the displacement between the current position of the cell and its starting position (Figure 3.7A). For evaluation purposes, we used the mean, the maximum and the final values of the ED to estimate the straightness index (refer to section on Confinement Ratio). The Monolayer Edge Distance (MED) defined by Eqn (2) is the displacement between the initial and final positions of the migrating edge of the monolayer (Figure 3.7B).

$$AD = \sum_{i=0}^{n} AD_i \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(3.1)$$

$$MED = \frac{\sum_{i=0}^{n} MED_i}{n} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(3.2)$$

Here, n is the number of measurements made per video.

Cell speeds were calculated from the accumulated distances over the period of monitoring.

Confinement ratios:

The ratio of the Euclidean displacements of the cells to the total distance traveled by them has been used as an indicator of the straightness of the path track of a cell. In our measurements, we used the various ED variants to obtain the confinement ratios (CR) using the following relations:

$$CR = \frac{ED_{final}}{AD} \quad \text{.......................................} (3.3)$$

$$CR_{EDmean} = \frac{\sum_{i=0}^{n} \mathrm{ED}_i}{n.AD} \quad \text{.......................................} (3.4)$$

$$CR_{evol} = CR_i = \frac{\mathrm{ED}_i}{\sum_{j=0}^{i} AD_j}, \; i = 0,1,2,3,\ldots n \quad \text{..............................} (3.5)$$

where CR is the confinement ratio obtained from the Euclidean distance at the final position of the cell, CR_{EDmean} is the mean of the Euclidean distances for individual cell tracks and CR_{evol} is the progression of the ratio as the cells migrate over 24 hours.

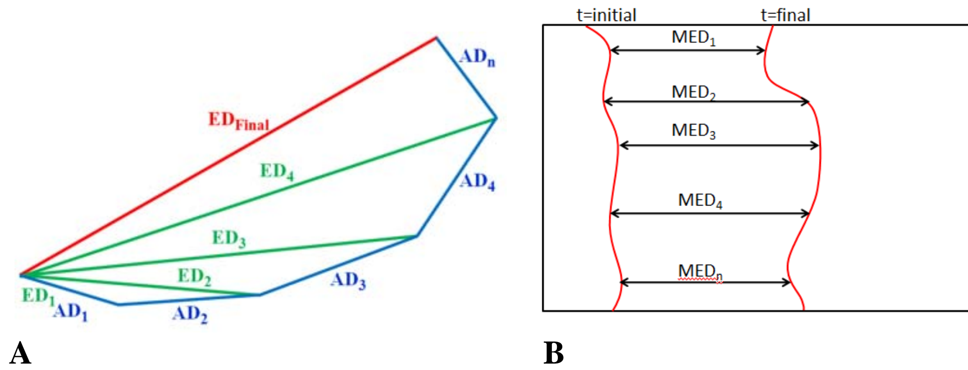


Figure 3.7 Basis for migration parameter estimation. (A) Blue track represents the path traced by a migrating cell. Red track represents the beeline distance between the initial and final position of a cell. (AD=Accumulated Distance, ED=Euclidean Distance) (B) Red curves represent the initial and final positions of the front edge of the migrating cell monolayer. (MED=Monolayer Edge Distance)

3.5 Individual Cell Motility Analysis

Individual cell motility was studied using kymographic analysis and measurements on single cell protrusions. Kymographs (Figure 3.8) are images which are obtained by arranging a pixel wide region of interest, from each frame of the entire length of the video, adjacent to each other in the order of the frame sequence. The result is a single image that shows the moving edge of the cell in the region of interest, allowing for instant observation of the movement that has occurred over the entire length of the

video. For kymographic analysis, video sequences of single cells were obtained were obtained from the edge of the monolayer. The video clips were chosen such that the time length was 2.5 hours and the frame intervals were of 1-2 minute duration. The kymograph sections were taken at multiple positions of single cells. The following section describes the parameters that were used to quantify and differentiate the lamellipod formation in the three cell lines.

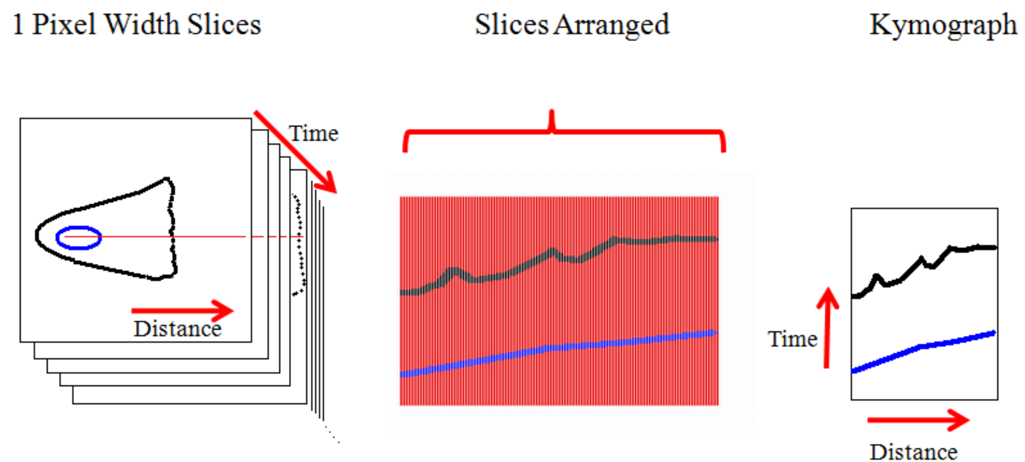


Figure 3.8 Method of Kymography. Single pixel width slices, in the region of interest, are obtained from videos of migrating individual cells captured at 1 minute intervals. The slices are arranged parallelly to give a static image of the motion of lamellipods at the leading edge of the cells.

3.5.1 Individual Cell Motility Parameters

The parameters for assessing individual cell motility mechanisms (Figure 3.9) in the three breast epithelial cell lines were developed to attain two immediate objectives: to correlate with the collective cell migration behavior pattern observed by tracking analysis and to differentiate between the behaviors of these cells. The latter was necessary because of the similar origins of these cell lines, i.e., breast epithelium. All these measurements were performed using the open source software Image J.

Thickness of ruffles (TR):

The RT is measured as the maximum thickness of the ruffles or dark border seen at active migrating edge of the lamellipods.

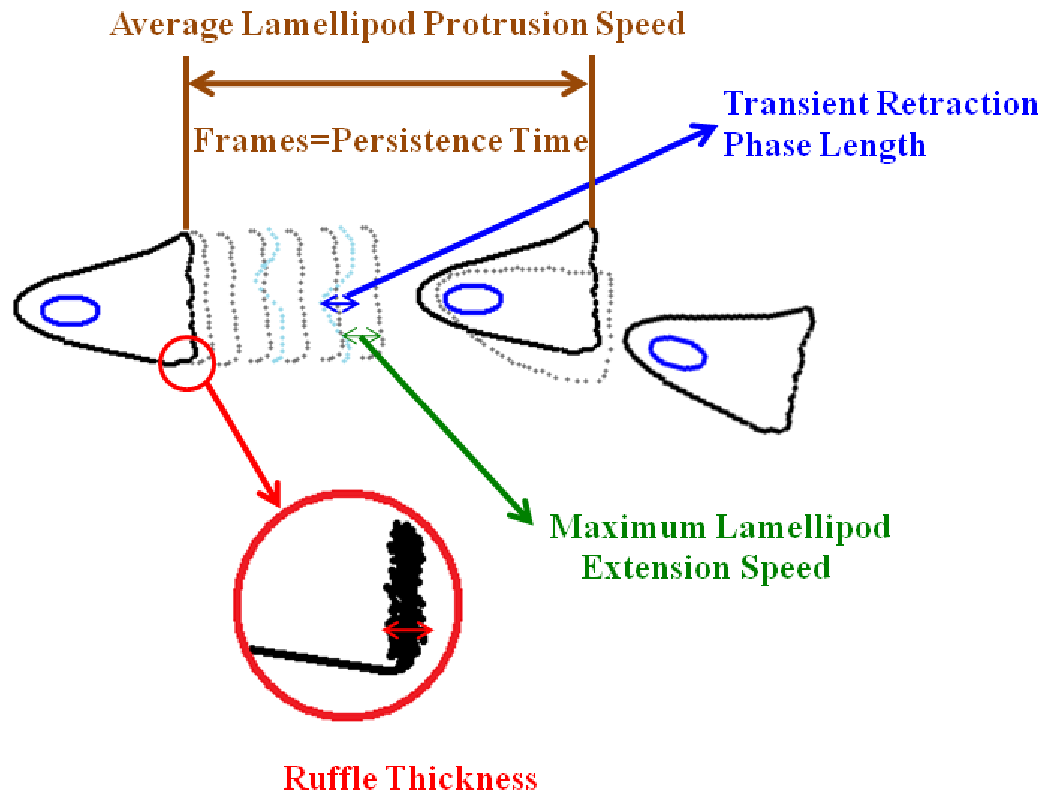


Figure 3.9 Individual Cell Motility Parameters. (See text for definitions)

Retraction Phase Length (RPL) of Lamellipods:

The RPL is the maximum retraction in lamellipods occurring during the protrusive phase of the lamellipod formation. The cell must have a protraction phase immediately following the retraction. Else the cell could be retracting due to several reasons (lamellipods on other side, general cell contraction etc).

Lamellipod Extension Speed (RES):

The RES is the maximum length of lamellipod edge extension that occurs in a single stretch (per minute or adjacent frames) in a particular direction. This indicates the maximum protrusion extension that is possible in short bursts for a cell line.

Average Lamellipod Protrusion Speed (LPS):

The LPS is the maximum speed of sustained lamellipod protrusion occurring in a particular direction in a migrating cell. The number of frames across which it is measured (Lamellipod Protrusion Persistence Time, LPPT) might differ for different cells. (For example although the drag on the cell body is uniform, the MCF-10A cells tend to change their directions after a few frames due to strong intercellular adhesion and temporary splits in the lamellipodial border, that is better visualized in video sequences. The dominance of one of the divisions leads to smooth deviation in the migratory tracks of the cells. The MDA-MB-231 cells, on the other hand, tend to have a sustained protrusion in a particular direction for longer times).

Maximum Lateral Displacement (MLD) of the cell bodies:

The maximum lateral displacement that a cell body undergoes provided it returns to its base position at least once before it changes direction. The cell body must not have moved to more than its thickness in the forward direction during the cell body displacement. This parameter was specifically introduced to differentiate the defective motility of MCF-7 cells from the other cell lines (Figure 3.10).

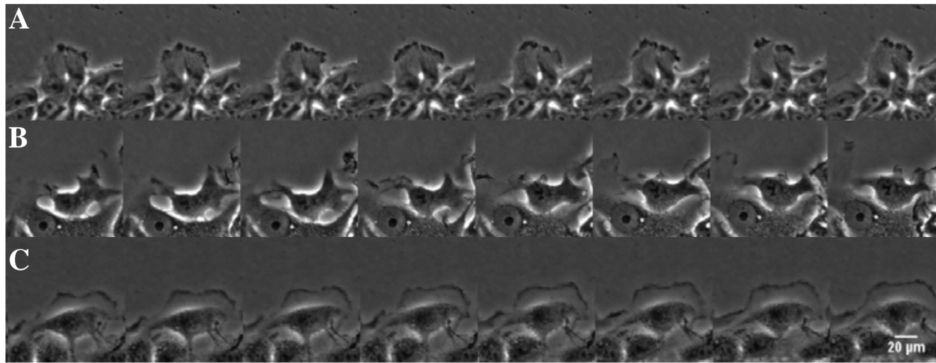


Figure 3.10 Montage of cell lamellipod formation on culture coated surfaces captured at 4min intervals for MCF-10A (A), MCF-7 (B) and MDA-MB-231 (C). MCF-7 lamellipods are non-uniform compared to the other two cell lines, leading to irregular displacements of the cell body, the lateral ones contributing to the inefficiency of their overall migration.

3.5 Statistical Analysis

Statistical tests were performed using Origin Pro 8.1 to analyze all measured data based on the type of distributions. The distributions were tested by the Kolmogorov-Smirnov normality test. ANOVA and two sample t tests were used to compare the means between the tested conditions. Most of the data followed normal distribution. Some of the data which had non-normal distribution were evaluated with non-parametric statistical tests. The population size of most of the measured parameters such as cell migration speed and displacements of individual cells was large, and hence the resulting data had a wide range of standard deviation. Hence a confidence level of 95% was used as the significant level in the statistical tests. For cell migration parameters, this confidence limit has been widely accepted as a standard. The results are reported as frequency plots or box charts.

Chapter 4
Collective Migration of Breast Cancer Cells

4.1 Introduction

Collective cell migration is important in the growth and progression of cancers. Various modifications of cell migration assays have been used to evaluate cell migration with most of them utilising the wound healing strategy as the basic platform. As discussed in Chapter 2, wound healing assays have several limitations and non-wounding or a barrier approach which avoids cell damage could be more relevant in the physiological setting and in the progression of cancers. Modifications of migrational assays that utilise non-wounding techniques have been used in research since a long time (Decaestecker et al. 2007; Van Horssen and ten Hagen 2011). But a simple and reliable technique has still not been achieved. Moreover, techniques such as time lapse imaging and cell tracking have been used to analyse collective cell migration. But, the kinematics of collective migration has not been described or correlated well with disease of origin. As will be shown in the current and the next chapter, kinematics and individual cell motility analysis can be crucial in understanding collective cell migration. Hence, it is necessary to develop techniques and analytical methods to increase the accuracy and efficiency of assays. In this chapter, we will focus on two aspects of breast epithelial cell studies: the cytoskeletal organisation of breast cancer cells, and the analytical methods and results of collective migration analysis.

Since cell motility is invariably influenced by cytoskeletal organisation, a study of important cytoskeletal structures is a necessary adjunct to evaluation of cell migration. As reviewed in Chapter 2, cytoskeletal structures derived from Actin are responsible

for the dynamics of changing cell shape and inducing motility of the cell body in normal as well as cancerous cells. Many studies in breast cancer research involve comparisons between the benign and malignant cells to highlight the differences between them. However, a comparative study of native configuration of the cytoskeletal structures in breast cancer cells has not been reported. Therefore, a study of cytoskeletal structures such as stress fibers, in benign and malignant breast epithelial cells, can be important in understanding the basic mechanisms that are involved in the dynamics of cytoskeletal organisation and explaining the observed differences in cell motility.

Several studies are focused on cell migration and evaluation of anticancer agents, in various cancers including breast cancers, with time lapse imaging and cell tracking analyses (Mathew et al. 1997; Rajah et al. 1998; Garib et al. 2002). But detailed descriptions of the collective cell migration and the role of individual cell mechanisms have largely been ignored. Also studies have analysed breast cancer cell migration but collective migration in depth has not been reported. Therefore, for a better understanding of collective behaviour in breast cancer cell populations, and to develop efficient methods of therapy, a detailed and quantitative study of collective cell migration including kinematic analysis is necessary.

This chapter reports the Actin cytoskeletal structure and the results of quantitative and kinematic analysis of collective cell migration in the benign (MCF-10A) and malignant (MCF-7 and MDA-MB-231) breast epithelial cell derivatives. The modified non-wounding cell migration assays described in the previous chapter were performed under tissue culture coated and collagen IV coated conditions, and the migrating cells of the monolayers were time lapse imaged using phase contrast

microscopy to study collective migration. Individual cells from the video sequences in each condition were manually tracked and the resulting data was analyzed for various migrational parameters. The cytoskeletal organization was examined in the form of stress fibre formation at the leading edge and the confluent centres of the monolayers.

4.2 Stress Fiber Organization in Breast Epithelial Derivatives

Stress fibers were observed in spreading cells seeded in low density as well as in cells at different locations within the monolayer. Cells used for spread imaging were seeded to about 30-40% confluence while the cells at migrating edge and centers of monolayers were obtained from the migration experiments.

The stress fibers were thicker and well formed in the MCF-10A and the MDA-MB-231 cell lines. More efficient bundling and orderly arrangement of stress fibers were observed in the highly invasive cancer cell lines (Figure 4.1). The larger cells among the MDA-MB-231 cells exhibited a dense distribution of organized stress fibers indicating that the process of stress fiber formation is exaggerated when sufficiently facilitated by immobility of the cells. Furthermore, only MDA-MB-231 cells showed overlapping of cells over each other (Figure 4.2) that corroborates their mesenchymal migration behavior that was observed in collective migration track analysis. This also explains the lack of contact inhibition in these cells that contributes to the haphazard collective migratory pattern. The MCF-7 cells showed very poor stress fiber formation with more of thin and less organized filaments and multiple brightly stained spots that indicated polymerizing or depolymerized ends of Actin filaments. The stress fiber formation was not affected by the position of the cells in the monolayer, although visibility was clear at the edges due to availability of free space for cell spreading.

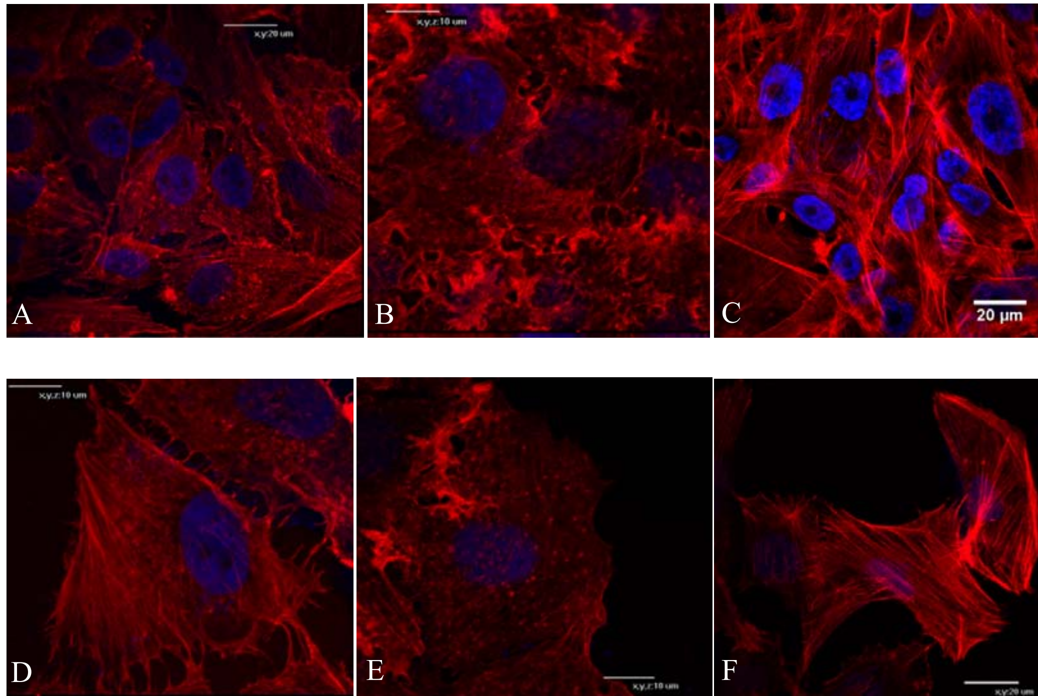


Figure 4.1 Confocal microscope images of stress fiber formation in MCF-10A (A, D), MCF-7 (B, E) and MDA-MB-231(C, F) breast epithelial cell lines. Cells were stained for filamentous Actin (Phalloidin-TRITC) and nucleus (DAPI). Top panel: cells at confluent center. Bottom panel: cells at monolayer edge.

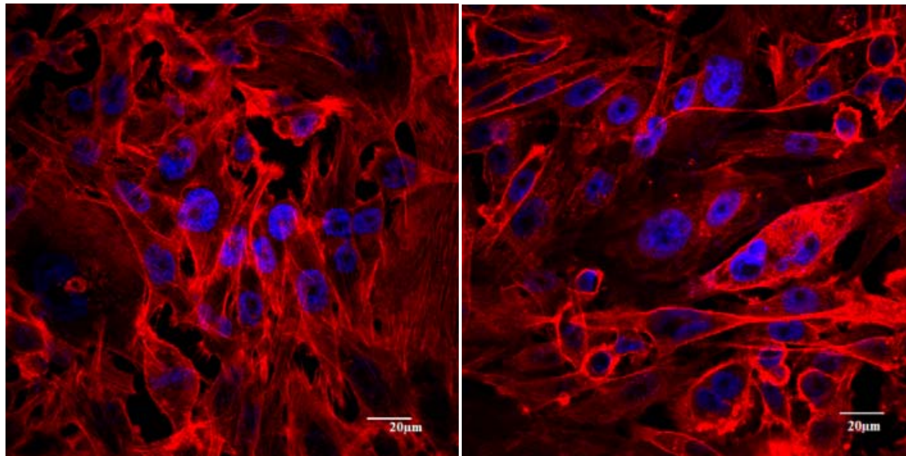


Figure 4.2 Confocal microscope image of MDA-MB-231 monolayer stained for Actin (Phalloidin-TRITC) and nucleus (DAPI). Overlapping of cells is visible in both the images.

4.3 Collective migration Tracking Results

The collective cell migration of breast epithelial derivatives was evaluated under haptokinetic conditions that involved culture-coated (N=2 for MCF-7 and MCF-10A, 1 for MDAMB-231) and human collagen IV coated surfaces (N=1) through time lapse phase contrast microscopy. All the cells physically located within the frame, for the entire length of the video, were manually tracked using the Image J plugin as described in Chapter 3. However, due to the haphazard nature of migration of the highly invasive cell line MDA-MB-231, fewer cells could be tracked, especially on the collagen coated samples. The data obtained by tracking individual cells were analyzed using two different techniques. The first technique utilized cell track path overlays on actual phase contrast images and provided information on the collective migration patterns such as finger like projections during cell sheet migration and individual cell properties such as intercellular adhesion. The second technique, which involved determining quantitative parameters of migration from the recorded track coordinates, was useful in estimating individual cell migration as well as in statistical quantitation of cell population behavior.

4.3.1 Collective Migration Behavior by Track patterns

Figures 4.3-4.5 show the complete track paths of the individual cells overlapped on the final frames of the 24-hr videos of the three different cell lines. Clustering of tracks was performed based on the proximity of the final positions of the cells. Although this clustering is arbitrary it facilitates the investigation of leader cell status, intercellular adhesion and migratory track patterns. Figures 4.3-4.5 also show 2D plots of cell tracks, with the starting positions shifted to a single point of origin. These graphs show the degree of lateral dispersion exhibited by the migrating cells. A high

degree of lateral dispersion will be observed in cells that have freedom of movement in all directions, such as in sparsely seeded cultures. In a monolayer of cells, the amount of lateral dispersion for each cell depends on their interactions with adjacent cells.

The benign MCF-10A cells were found to migrate in cohorts as evidenced by prominent clustering of groups of cells. This also means that these cells have strong intercellular adhesion that affects their coordination. The track patterns have a smooth evolution affirming the steady coordinated directional movement of the cells. The MCF-7 cells appeared to be well clustered, although the phase contrast images showed disturbed intercellular adhesions that appeared as gaps between the cells. The track patterns of MCF-7 cells were highly tortuous and therefore inefficient. This was due to repeated movement of cells over already traversed paths and could be attributed to excessive cell body movements (Figure 4.10 and Chapter 6). The MDA-MB-231 cells typically showed absence of cell clusters during migration indicating that intercellular adhesion was affected. The track paths of MDA-MB-231 cells also showed extensive overlapping, with a spaghetti-like appearance, representative of a swarming pattern of migration that is observed in mesenchymal modes of migration.

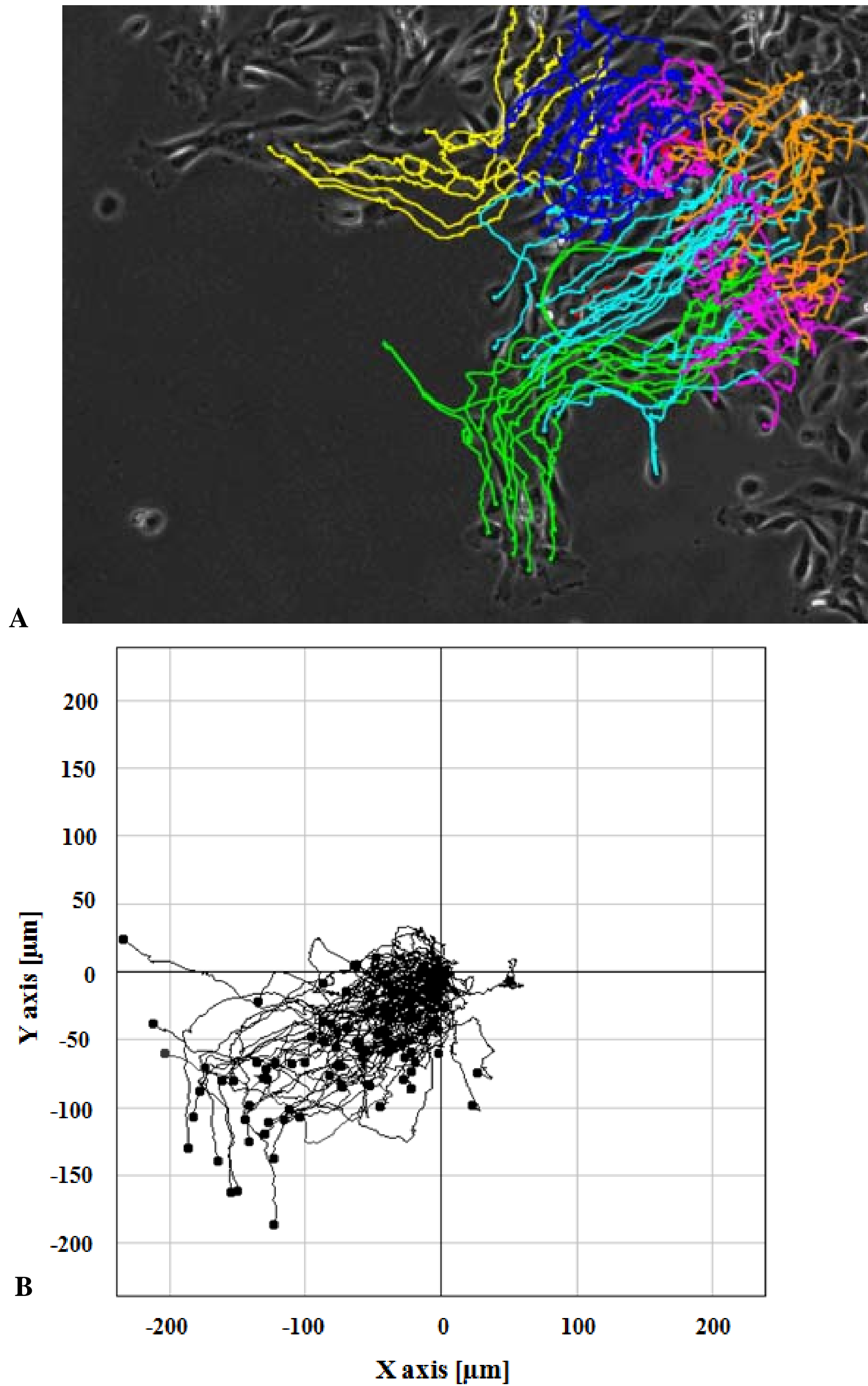


Figure 4.3 Tracks of individual MCF-10A cells. (A) Cell tracks overlapped on final frame of the 24-hr monitored video. Color coding shows the proximity of cell clusters over progression of time. (B) 2D plot of cell tracks with the starting points shifted to the origin. Lateral dispersion is minimal in the MCF-10A cells. (Scale in µm)

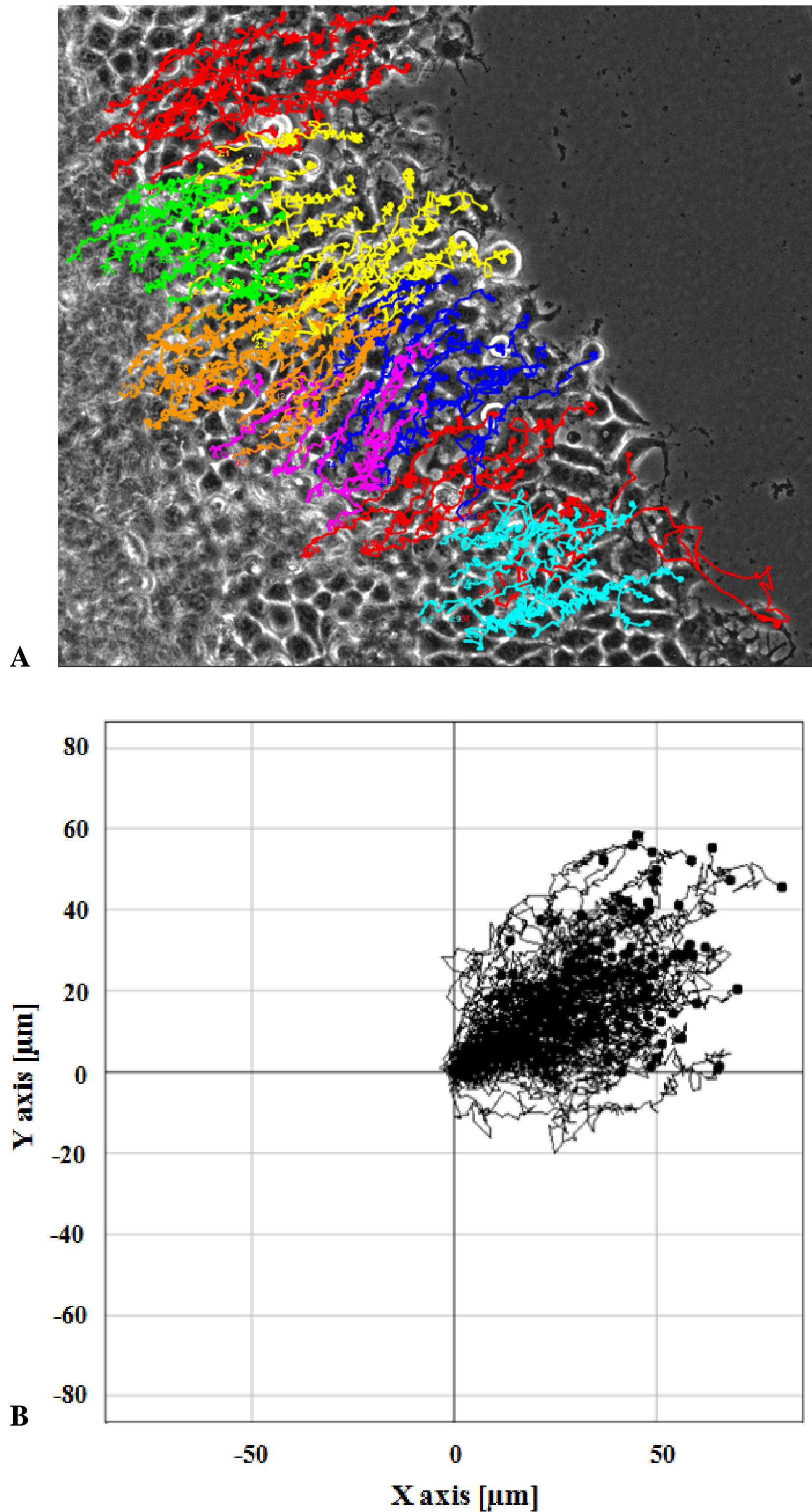


Figure 4.4 Tracks of individual MCF-7 cells. (A) Cell tracks overlapped on final frame of the 24-hr monitored video for tissue culture-coated condition. Color coding shows the pseudo-clustering of MCF-7 cells over progression of time. (B) 2D plot of cell tracks with the starting points shifted to the origin. (Scale in µm)

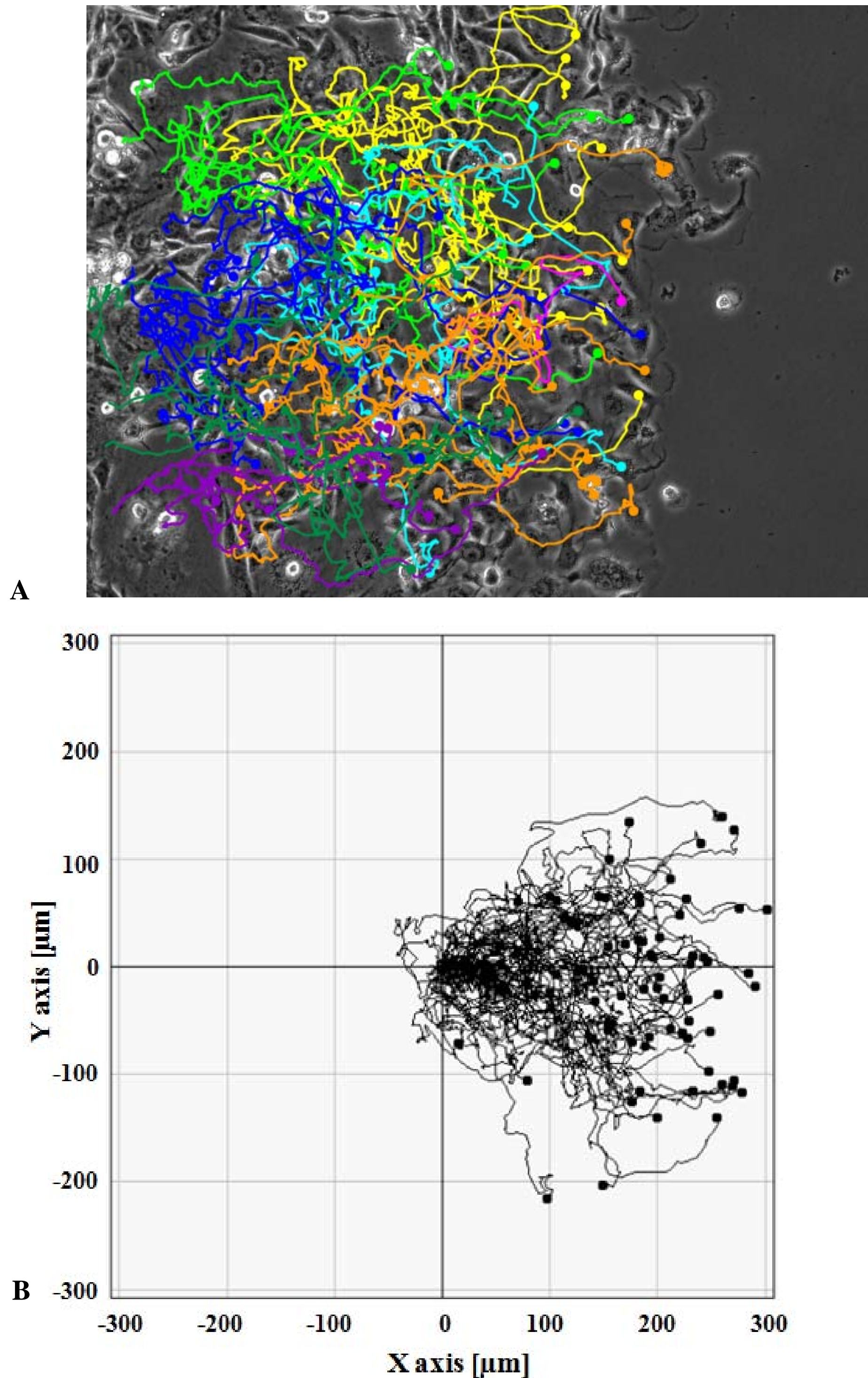


Figure 4.5 Tracks of individual MDA-MB-231cells. (A) Cell tracks overlapped on final frame of the 24-hr monitored video for tissue culture-coated condition. Color coding shows the proximity of the cell clusters over progression of time. (B) 2D plot of cell tracks with the starting points shifted to the origin. A higher degree of lateral dispersion is noted in the highly invasive cell lines. (Scale in µm)

4.3.2 Cell Migration parameters

The cell migration parameters estimated from cell tracks include migration distances (AD and ED), cell speed, confinement ratios and migrational angles of deviation. A comparative report of the results follows.

Cell Migration Distances

The highly invasive cells migrated to larger distances as evidenced by monolayer edge distances (MED) as well as individual cell track distances (AD and ED) (Figure 4.6). Collagen IV coating had a positive effect on all the types of migratory distances in all the three cell lines . However, the increase was statistically significant in the highly invasive cells, while the benign and non-invasive cells showed slight and consistent increases in the collagen coated conditions which were not statistically significant. These results are consistent with the previous studies on these cells (Rajah et al. 1998; Castro-Sanchez et al. 2010).

Cell Migration Speed

The MDA-MB-231 cells had higher cell migratory speeds compared to the benign and the non-invasive cells (Figure 4.7). The MCF-7 cells showed migration speeds similar to the MCF-10A cells. Since movement of all the cells in a frame contribute to the collective migration of the monolayer, the cell speed has to correlate with the various types of displacements measured. However, this was not the case since the cell migration speeds did not correlate well with the observations on monolayer edge distance as well as the Euclidean displacements. (Note that since cell migration speeds were calculated from AD's, they would correlate). The ED's only account for beeline displacements of the individual cell while the MED incorporates both the

individual cell displacements and outward growth of the monolayer contributed by cell proliferation. Therefore cell speeds and ED were similar for the MCF-7 and MCF-10A, but MED was clearly higher for the benign cells. This emphasizes the inefficiency of the migratory movements of MCF-7 in producing effective cell body displacement thus leading to insufficient collective migration. This was also evident through the cell migration track patterns as described in the preceding section.

4.3.3 Confinement Ratios

Confinement ratio or the straightness index (Benhamou 2004) has been used in research as an indicator of directional persistence during analysis of migration (Bogle and Dunbar 2008; Beltman et al. 2009). This simple index calculated as a ratio of Euclidean distance to Accumulated distance can have values from a maximum of 1, indicating a straight path (ED = AD) and a minimum of 0 (ED <<AD or ED = 0) representing very less or no effective movement (Beltman et al. 2009). Various confinement ratios were measured based on different values of ED possible, as discussed in Chapter 3. For a recapitulation, ED's can be calculated when a minimum of 3 frames in the video have elapsed, for the total number of frames and for the farthest position reached by the cell during its 24 hour migratory path. Theoretically these could vary with different conditions for a single cell line, and had to be measured for confirmation.

All the confinement ratios were consistent with the findings that the benign cells had more directional tracks followed by the highly invasive cells (Figure 4.8). An interesting finding was that the MDA-MB-231 showed a decrease in the CR under collagen IV coated condition. The reason for this is not clear, but it is possible that collagen coating increases the lateral degree of dispersion which is observed in the

individual cells of cell lines with weaker or defective intercellular adhesion or those in mesenchymal modes of migration. This explanation would be consistent with the drastic increase in MDA-MB-231 cell migration noticed on Collagen IV coated surfaces. However, it is also possible that the reduced number of cells that were tracked for collagen coated conditions was responsible for this variation.

4.3.4 Migratory Angles of Deviation

The migratory angle of deviation is the angle by which a cell changes its movement during its migration. Starting from the first frame, coordinates of successive 3-frame sequences were used to measure this entity. The results were pooled for all the cells measured for the entire 24 hour period. This provides an estimate of the number of deviations performed by the cells during their entire migratory course. Cells which have directional persistence would ideally show a peak at lower angle values (Figure 4.9). Most of the benign cell movement had lesser track deviations as observed from a predominant peak at lower values of angles. The MDA-MB-231 cells also showed slight peaks at lower angles, but the effect was not prominent. This can be explained based on the track patterns which showed their potential for frequent changes in migrational direction, although it could also be due to the less number of cells that could be tracked for these cell lines. Collagen IV induced a significant decrease in the mean migratory angles for the benign cells while a slight increase was noted for highly invasive cells. The graphs for MCF-7 cells showed plateauing over the entire range of angles as can be expected from the track patterns. This also signifies the multidirectional deviations in these cells, which are much more apparent in the single cell analyses.

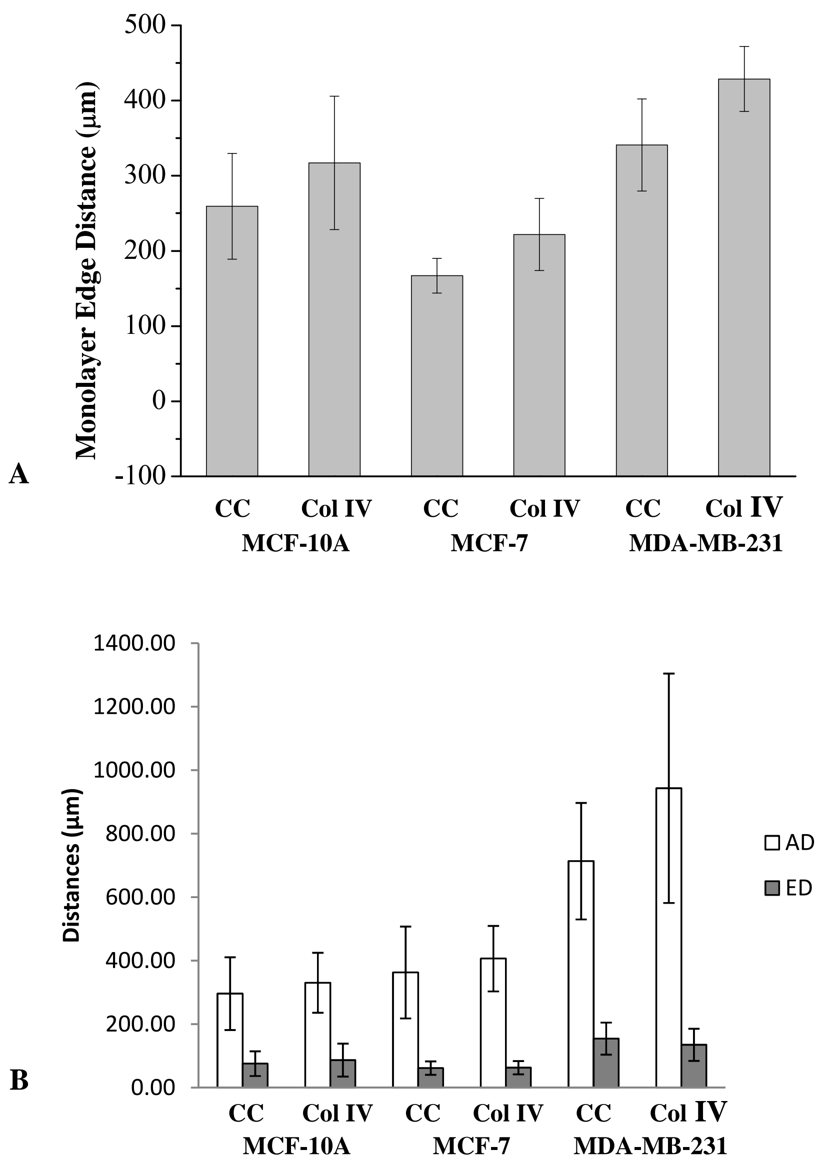


Figure 4.6 Cell Migration Distances. (A) Comparing Monolayer Edge Displacements of cell lines for both cultured coated and collagen IV coated surfaces. (B) Accumulated Distance (AD) and Euclidean distance (ED) of cell line cells for both cultured coated and collagen IV coated surfaces. (CC = Culture coated surface, Col-IV = Human Collagen IV coated surface) Note the comparable values of AD for both MCF-7 and MCF-10A (See text for explanation). Error bars: Standard deviations of mean values.

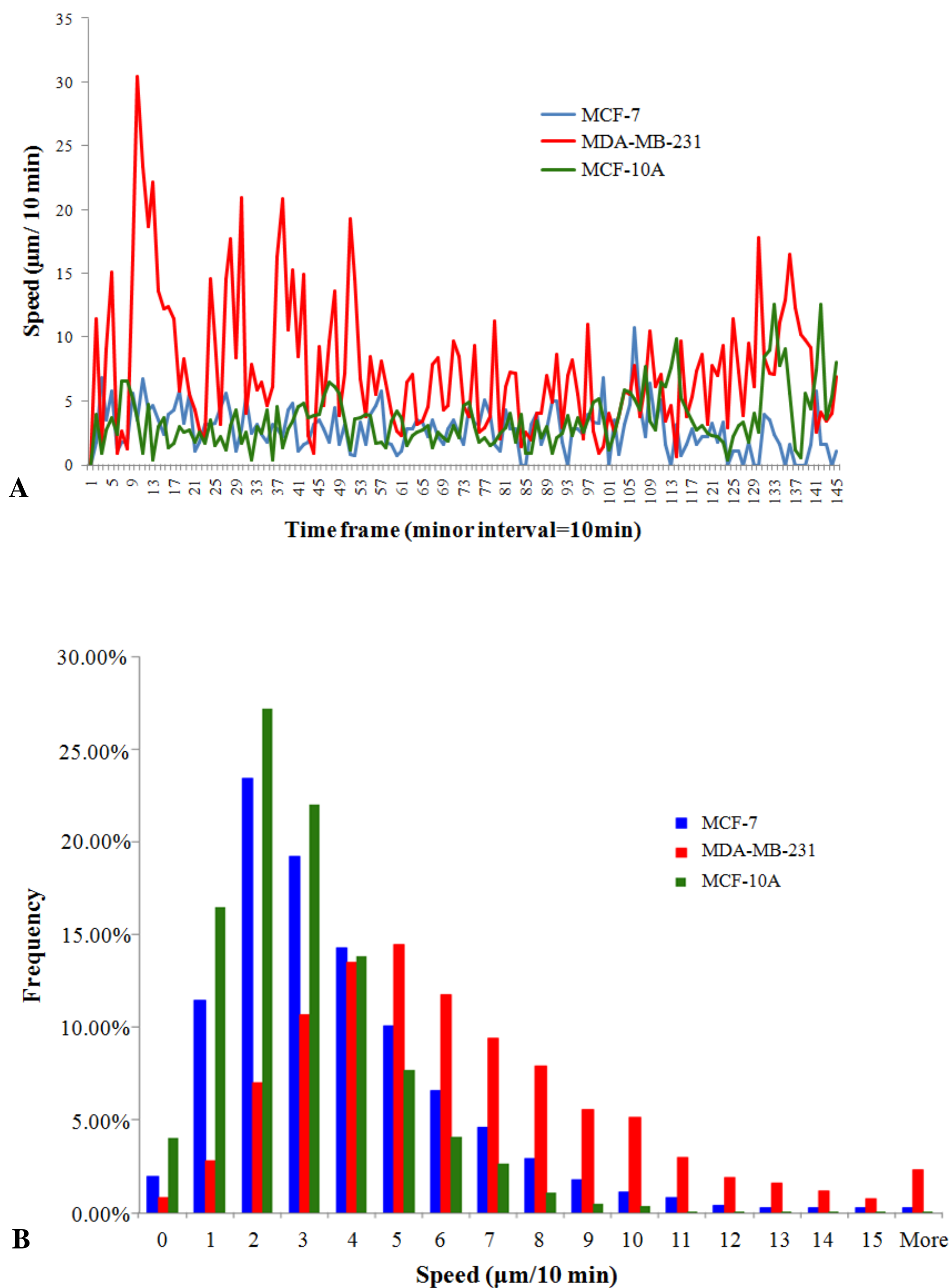


Figure 4.7 Cell Migration Speed. (A) Typical 24 hour cell migration speeds of individual cells for the three breast epithelial cell lines. The wide oscillation of MDA-MB-231 cells reflect their capability for higher cell speeds. The lower values in the curves, which appear to be of similar value for all the cell types, either represent the rest phases (minimal movement) of the cells or the pauses during change of directionality. (B) Frequency distribution of the cell speeds (Example=tissue culture-coated surface; Number of tracked cells, n=93 for MCF-10A, 121 for MCF-7, 82 for MDA-MB-231).

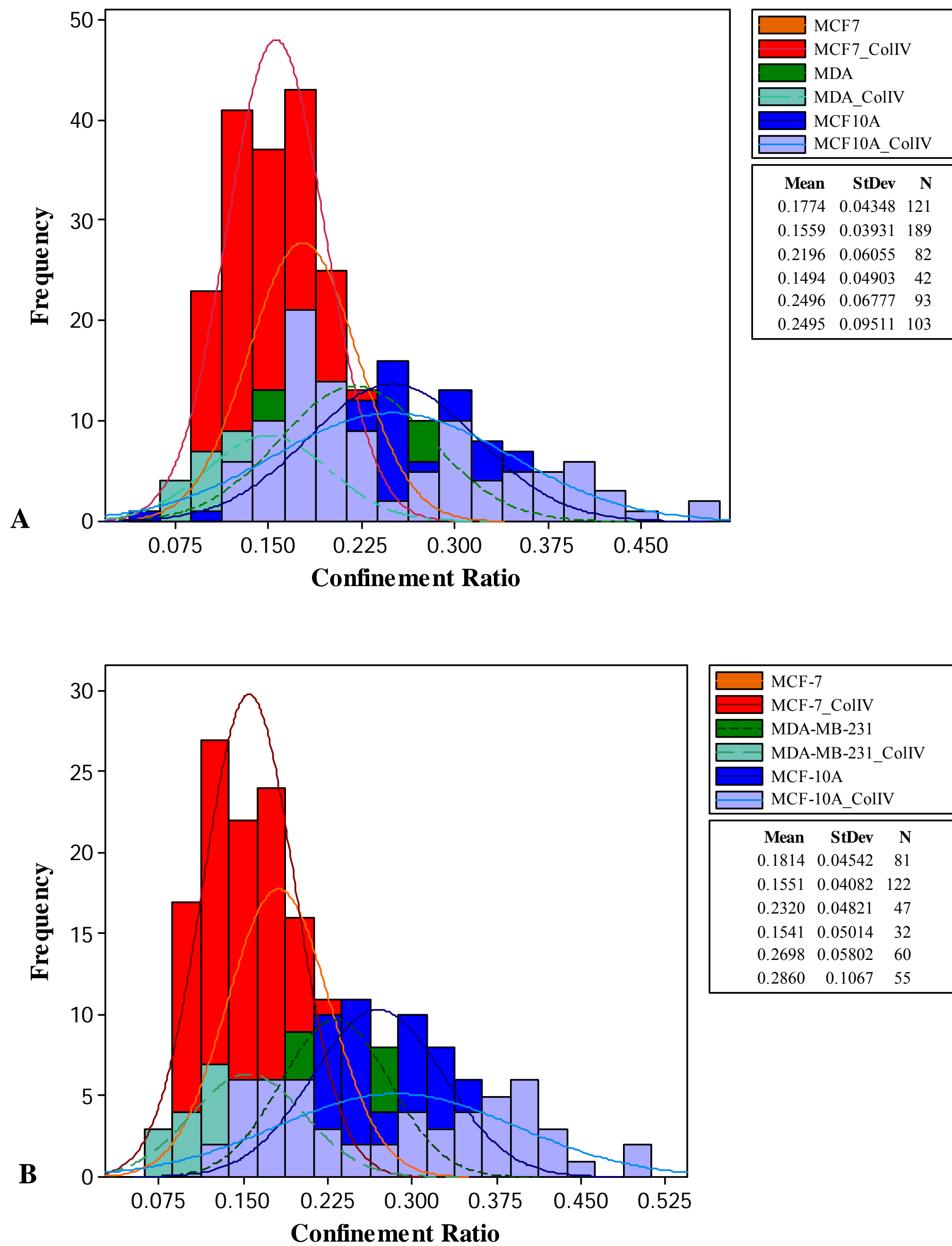
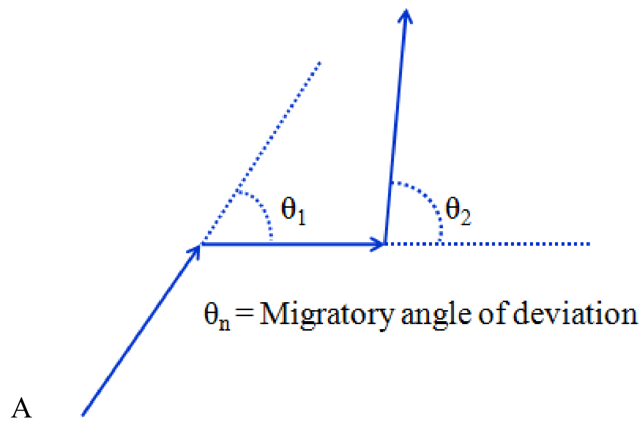
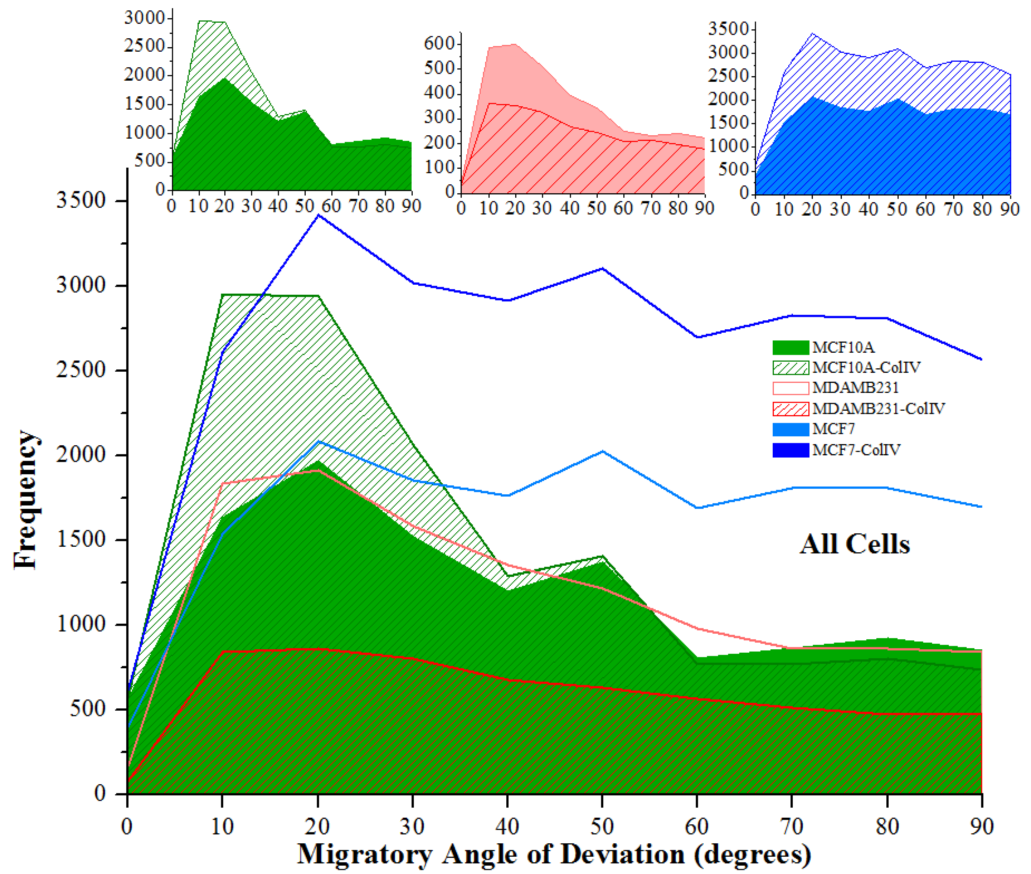


Figure 4.8 Frequency distribution of confinement ratios of cell tracks. The MCF-10A and MDA-MB-231 exhibit more directional cell migration tracks than the MCF-7 cells. Collagen IV substrate decreased the directionality among the highly invasive cancer cells (B) Confinement Ratio of leading cells of the front two rows. Final positions were used to identify the leader status of the MDA-MB-231 cells.


θ1
θ2
θn = Migratory angle of deviation
A


B
Frequency
Migratory Angle of Deviation (degrees)
MCF10A
MCF10A-ColIV
MDAMB231
MDAMB231-ColIV
MCF7
MCF7-ColIV
All Cells

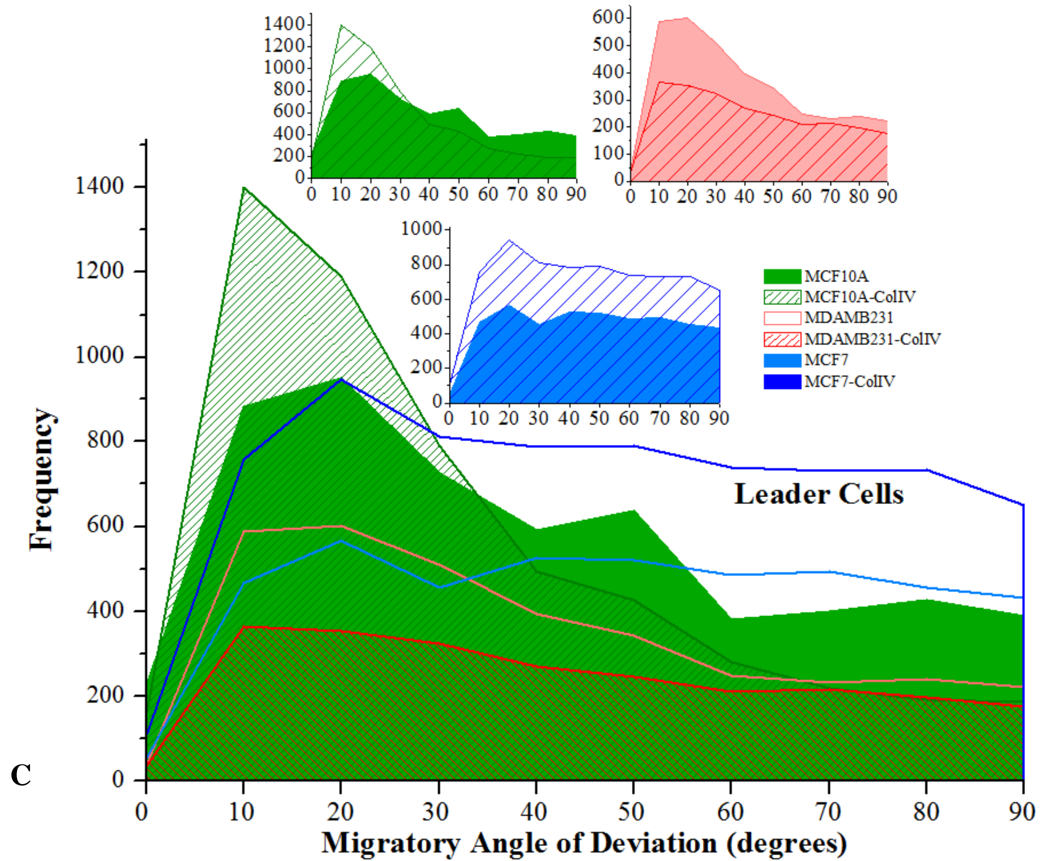


Figure 4.9 Migratory angle of deviation as denoted by θ_n(A). (B) Distribution of migratory angles of deviation of all the tracked cells for the three cell lines on both cultured coated and collagen IV coated surfaces. Majority of benign cells (green) show lesser track deviation (peaking towards the left) as compared to the malignant variants. The highly invasive MDA-MB-231 cells show lesser track deviation and hence have less tortuous tracks. Insets of individual cell lines, separated for clear view. (C) Distribution of migratory angle for leading row cells. Significant increase in directionality on collagen IV coated surface can be noticed for MCF-10A cells.

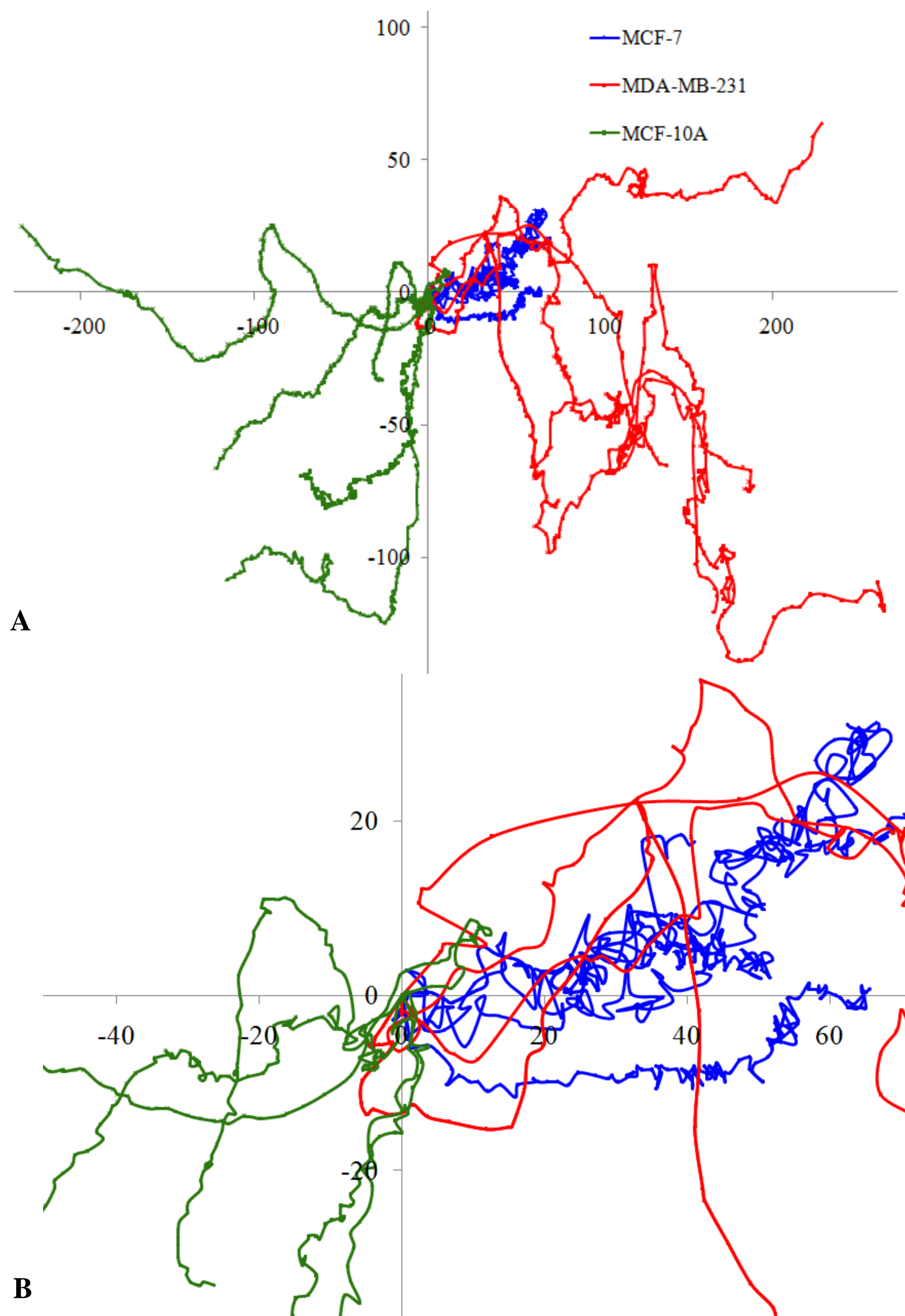
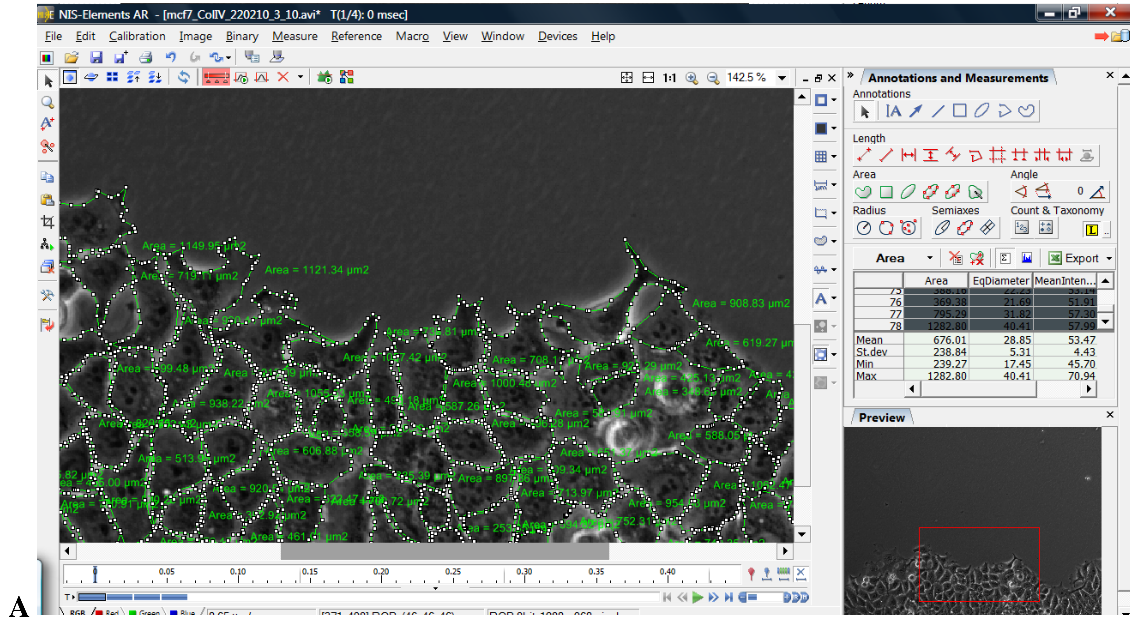
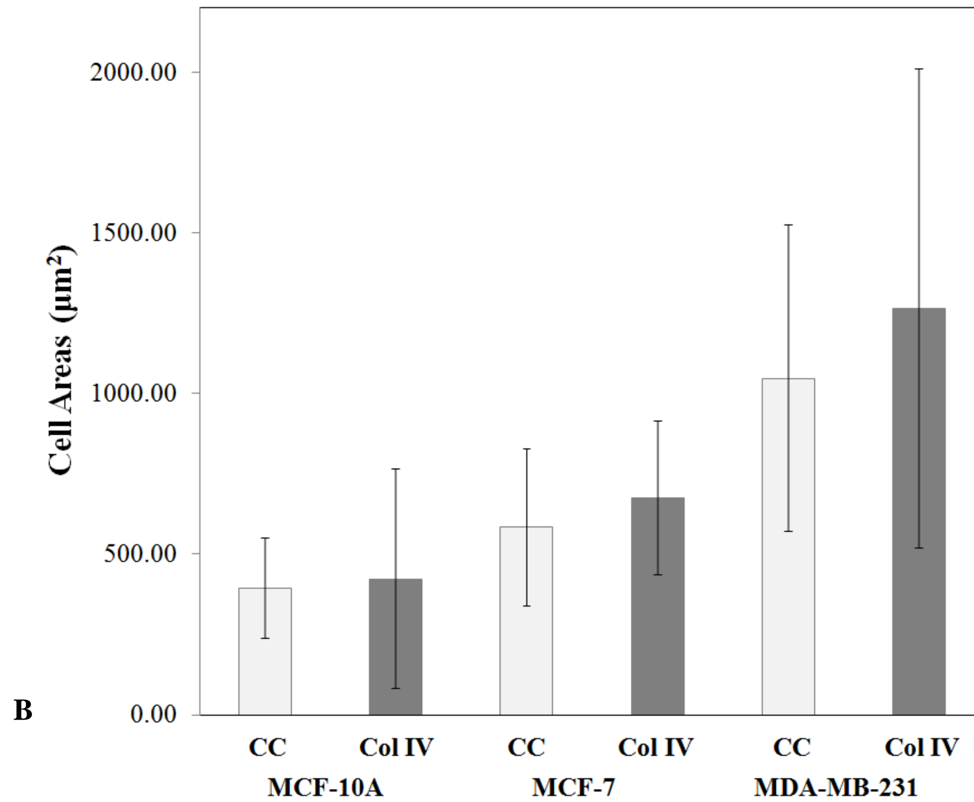


Figure 4.10 (A) 2D Plot of originized migration tracks overlapped for the three cell lines. (B) Tracks enlarged for clarity. The accentuated cell body movements of MCF-7 cells lead to frequently tortuous tracks resulting in inefficient total migratory displacement for these cell lines. The MCF-10A and MDA-MB-231 have similar smooth evolving tracks, with more abrupt angular deviations in the latter. (Scale in μm)



A



B

Figure 4.11 Cell areas of individual cells (A) Method of measurement using the software NISelements. (B) Collagen IV increased the cell spreading in the malignant cancer cell lines. The difference was not evident in the benign MCF-10A cells.

4.4 Discussion

4.4.1 Collective Migration Behaviour in Breast Cancer Cells

Collective migration analysis revealed the possible strategies that can be useful in the growth and spread of benign and malignant cells. In multicellular organisms, organized cell migration in epithelial sheets is crucial for the formation of functional structures and organs (Ewald et al. 2008; Watson and Khaled 2008; Vasilyev et al. 2009). The track paths of benign cell lines show a coordinated sheet-like movement with prominent clustering of cells. This can be likened to the organized behavior found in normal epithelial cell sheets. Furthermore, the ability to migrate in cohorts reflects the potential of these cells to form cystic structures, a characteristic of the fibrocystic disease from which they were obtained. On the other hand, the abnormal track patterns of individual malignant cells correlates well to their altered functional state, i.e. abnormally increased survival and proliferation coupled with tendencies to form masses and rudimentary, rather than organized and purposeful, structures. Although, the monolayers showed a clear and distinct migratory front during the time lapse imaging, the positional status of the cells at the leading edge was different in the three cell lines. The front rows of the benign cell monolayers had a tendency towards persistence of the leader cell status. This was due to the strong intercellular adhesion, as observed in the organized track patterns, which prevents reentry of individual cells into the monolayer and unrestricted lateral dispersion. The highly invasive cancer cells showed poor clustering, and no relation to the track patterns of the adjacent cells. Also, the cells showed truly individualized characteristics with cells even reentering the monolayers. The tracks showed that the leader cells in the final frame were different from those at the beginning of the imaging. The non-invasive MCF-7 cells

however maintained stable leader cell positions and showed apparently well clustered migration. This behavior required further analysis of single cell motility for clarification (Chapter 6) which revealed that altered individual cell lamellipod polarization and defective intercellular adhesion reinforced the pseudo-clustering of cells and prevented them from crossing paths as well as efficient migration.

For metastasis to occur, cells must possess the capability to reach lymph or blood vessels, breach its basement membrane and get into circulation. In this context, acquisition of swarming motility behaviour and highly invasive properties would bestow cancer cells like the MDA-MB-231 with a substantial potential to metastasize via circulation, in addition to local destruction of tissues. The track patterns of MDA-MB-231 cells had all the qualities of mesenchymal migration required for this potential. Furthermore Actin staining confirmed prominent stress fibre formation, and the ability of these cells to traverse above adjacent cells. This means that interactions such as intercellular adhesion and contact inhibition of locomotion are reduced, allowing for individualistic cell behaviour. The MCF-7 cells, on the other hand, were less efficient in collective migration on the 2D surfaces as seen on close observation of the track patterns. Further the MCF-7 cells have poor Actin filamentous structures, which might point to abnormal contractility mechanisms, as affirmed by low migratory distances but similar cell migration speeds when compared to the benign cells. Since MCF-7 were isolated from metastatic sites, these results corroborate the finding that cell migration might be as important as invasion in the spread of cancers.

4.4.2 Effect of Collagen IV coating

Collagen IV is a component of the basement membrane which forms a platform for cells to rest, migrate and rearrange in normal tissues. It is therefore among the first components to be encountered by migrating and invading cancer cells. Collagen IV has been implicated in regulation of migration of breast cancer cells (Castro-Sanchez et al. 2010) through pathways involving the receptor tyrosine kinase, DDR-1, and the surface receptor CD-9. In this study we have shown that Collagen IV coating increased cell spreading (Figure 4.11) for the malignant cells but not the benign cells. Collagen IV also drastically increased the cell migration speed (Figure 4.7) and the migratory distance (Figure 4.6) coverage in the highly invasive cells lines. The MCF-10A and the MCF-7 cells showed slight increase in these parameters, but the data was statistically insignificant. Collagen IV also decreased the mean migratory angles for the benign cells while slight increasing the deviations in the highly invasive cells. It is possible that collagen IV improves the directionality of benign cells because it doesn't significantly affect their migration. Since it positively affects the migration of the highly invasive cells, it might therefore increase the likelihood and frequency of cell protrusions in all directions leading to more frequent change of directions. However, this has to be confirmed with further studies since stages of no cell movement over 3 frames of the video sequence are disregarded in the measurement, and the number of invasive cells tracked for this condition was less in number. These results indicate that collagen IV might have a significant role in the increased migration of cancer cells especially those with invasive potentials.

4.4 Conclusions

To summarize, this chapter reported the use of a non-wounding cell migration assay in the quantitative as well as kinematic analysis of collective cell migration in breast epithelial derivatives. The results showed that individual MDA-MB-231 cells had high cell migration speeds and reduced intercellular adhesion as well as contact inhibition of locomotion that facilitated their mesenchymal type of migration. The MCF-7 cells had an apparent coordinated cell migration pattern as observed by track patterns, but the individual cell migration was riddled with inefficient and tortuous cell movements. This required further confirmation by single cell studies as will be described later. Finally, the coordinated and smoothly evolving track patterns of MCF-10A confirmed the similar characteristics of benign cells to normal epithelial cells.

Chapter 5
Two-Dimensional Non-Wounding Migrational Assays in Protein Knock-Down Systems

5.1 Introduction

The previous chapter introduced the modified 2D non-wounding migrational assay combined with cell tracking analysis which proved to be a valuable method in delineating the collective migratory behaviour of benign and malignant breast epithelial cells. However it was necessary to examine the reliability of the assay method in common research setups and to explore and compare the advantages of the system with ordinarily performed wound healing assays. In the following sections, this objective will be fulfilled by examining two different cell lines under conditions involving transfectional knock down of intracellular expressions of different proteins. The preliminary goal here is to show the effectiveness of the powerful combination of non-wounding migrational assay and the analytical approach followed in this work rather than the effect of the proteins. Adjunct assays for migration served as a confirmation of the results. The first cell line that we have used is the MDA-MB-231 modified by transiently knocking down the expression of DP103 protein, a DEAD-box protein involved in RNA metabolism of both eukaryotic and prokaryotic organisms (Heung and Del Poeta 2005). We also performed this method in the HCE-T cells stably transfected with shRNA’s against the enzyme transglutaminase-2 (TGM-2), a protein that is involved in cell adhesion and transmembrane signalling (Fesus and Piacentini 2002). Brief descriptions of the transfection methods and the results of analysis will now be presented.

5.2 DP103 Knock down in MDA-MB-231

5.2.1 Transfection Method

The transfected MDA-MB-231 cells were obtained from Dr. Alan Prem Kumar of Cancer Science Institute (CSI) as a part of the collaborative work. Transient transfection systems involving siRNAs were used to knock down DP103 protein in the MDA-MB-231 cells. Briefly, the cells were plated at 2×10^5 cells per 6-well plate or 3×10^6 per 100mm dish 24h prior to transfection. Lipofectamine™ RNAiMAX (Invitrogen) was used for transfecting the siRNAs into the cells according to the manufacturers' instructions. The following unique siRNAs (DPsi) that target the non-conserved coding regions of DP103 were used for the knock down:

#1: 5'-CCAGUGAUCCAAGUCUCAUAGCUUU-3';

#2: 5'-GCUGCCGCUUCUCAUUCAUAUUAUU-3' (Stealth RNAi™, Invitrogen). The control siRNA contained the inverted sequence for the luciferase gene:

5'-AGC UUC AUA AGG CGC AUG CTT (Qiagen, Valencia, CA).

The effect of the transfection was significant from 48 -72 hours after the procedure (Figure 5.1A). The 2D non-wounding assays with 24-hour time lapse imaging were performed within this time window. 50 cells were tracked for each condition and tracking analysis performed as outlined in Chapters 3 and 4.

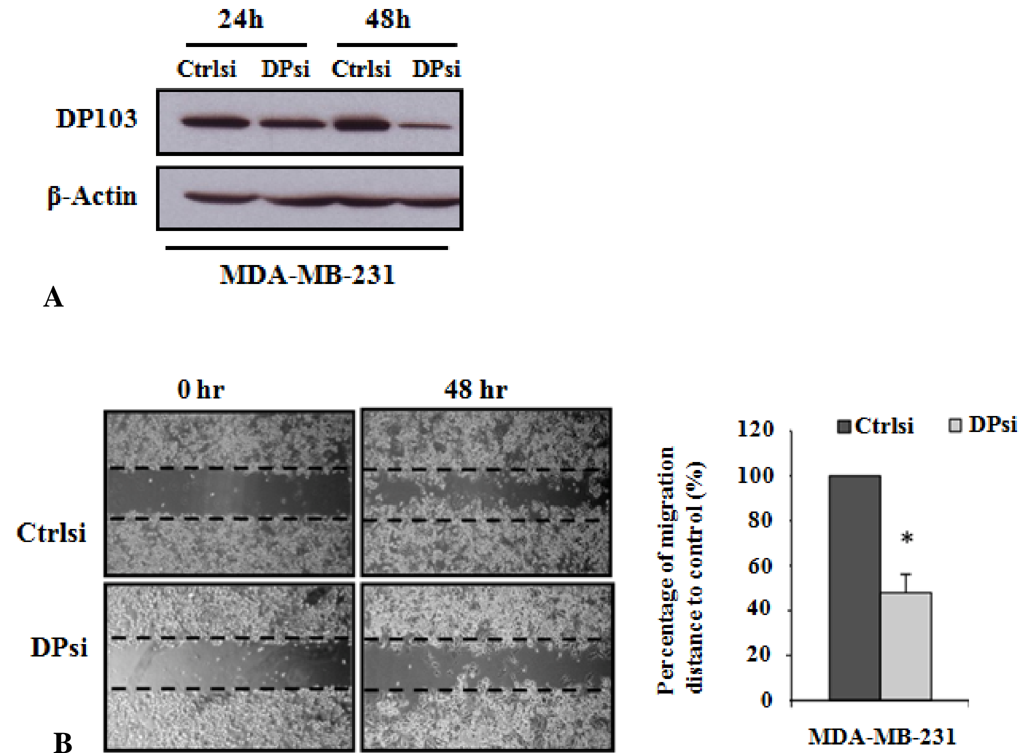


Figure 5.1. (A) Western Blot of control siRNA (Ctrlsi) and DP103 siRNA (DPsi) transfected MDA-MB-231 cells showing the knock down of DP103 expression at 24 and 48 hours after transfection. (B) Scratch Assay analysis showing reduced migration in DP103 knock down MDA-MB-231 cells.

5.2.2 Track Patterns and Cell Migration Parameters

As observed in the previous chapter, the MDA-MB-231 cells have a haphazard cell tracks with unhindered freedom for change in direction, giving the tracks a spaghetti-like appearance. DP103 knockdown did not affect this pattern of track development (Figure 5.2) since lateral dispersion of cells is similar in both the control and the experimental condition as can be observed in the 2D plots (Figure 5.3 A, B). Since the track patterns the distances traversed by the cells in both the conditions appear to be similar on the 2D plots and the overlapped patterns, we have plotted the endpoints of individual cells to show the actual effect on cell migration (Figure 5.3 C, D). The

center of mass (positive symbol in Figure 5.3 C, D) is the average distance from the origin that a single cell would be displaced by, if it were to represent the displacements of all the tracked cells taken together. Center of mass (CM) was measured using the image J plugin MTrackJ, from the coordinates of all endpoints (final positions) of the cell tracks as follows.

$$x = 1/n \cdot \sum \text{Endpoint x value} \qquad (5.1)$$

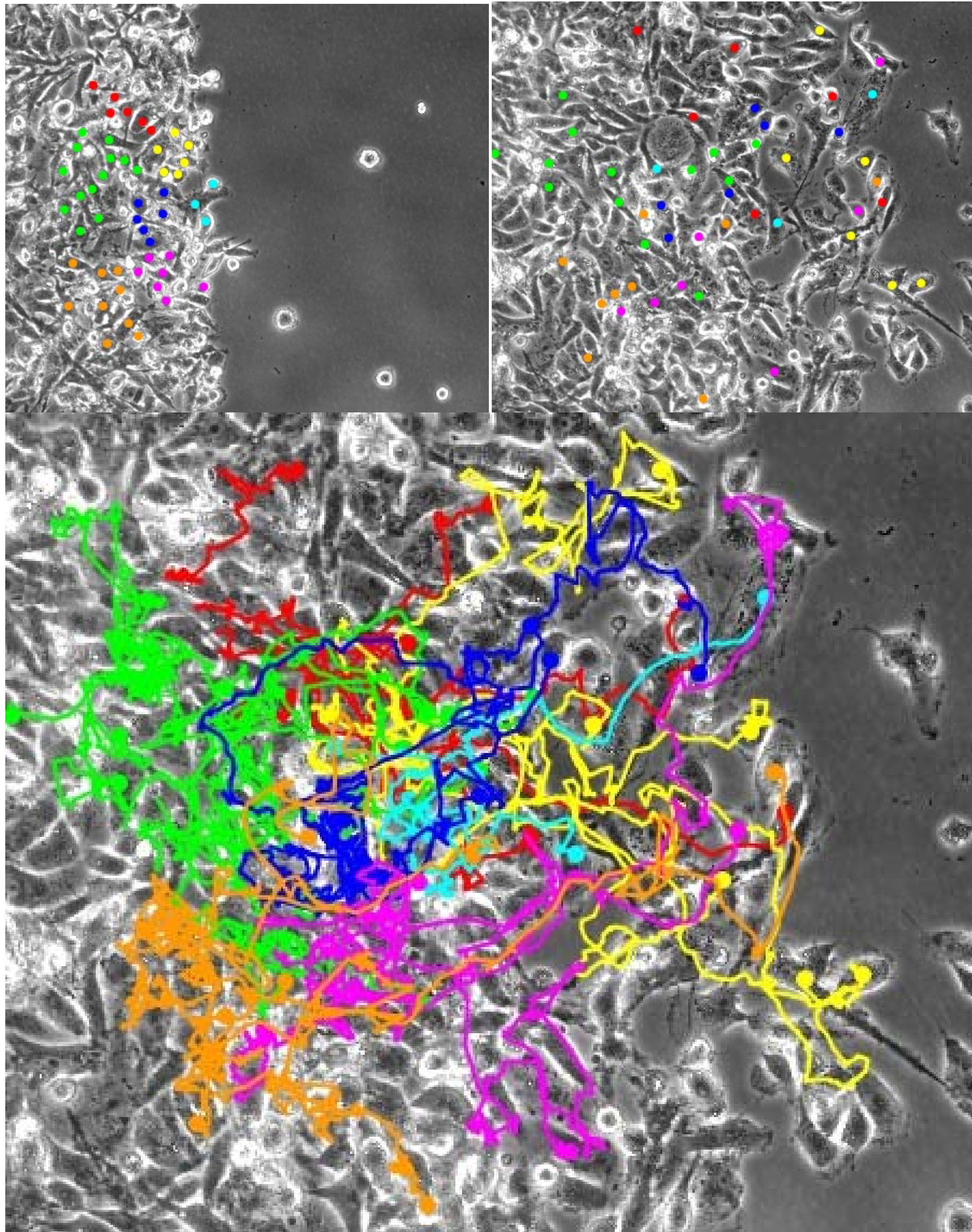
$$y = 1/n \cdot \sum \text{Endpoint y value} \qquad (5.2)$$

where x and y represent the coordinates of the endpoints of a particular cell track.

$$\text{CM Length} = \sqrt{x^2 + y^2} \qquad (5.3)$$

where CM Length represents the distance from the origin to the center of mass. A circle of this diameter would allow an estimate of the number of cells that have actually displaced to larger distances than the average displacement for that experiment. Analysis showed that a higher percentage (72%) of control siRNA transfected MDA-MB-231 cells traversed to distances more than center of mass compared to 44% for the DP103 knock down variants. These results prove that cell migration and displacements were significantly reduced when DP103 was knocked down in the MDA-MB-231 cells as compared to the control siRNA transfected cells.

Quantitative measurements of cell migration tracks were performed to obtain migration parameters as mentioned in Chapter 3. The analysis revealed that the DP103 KD variants had a statistically significant ($p=0.05$) decrease in the cell migration distances (AD and ED) and the cell migration speeds (Figure 5.4 A-C). These results were consistent with the results of scratch assays (Figure 5.1 B).


A

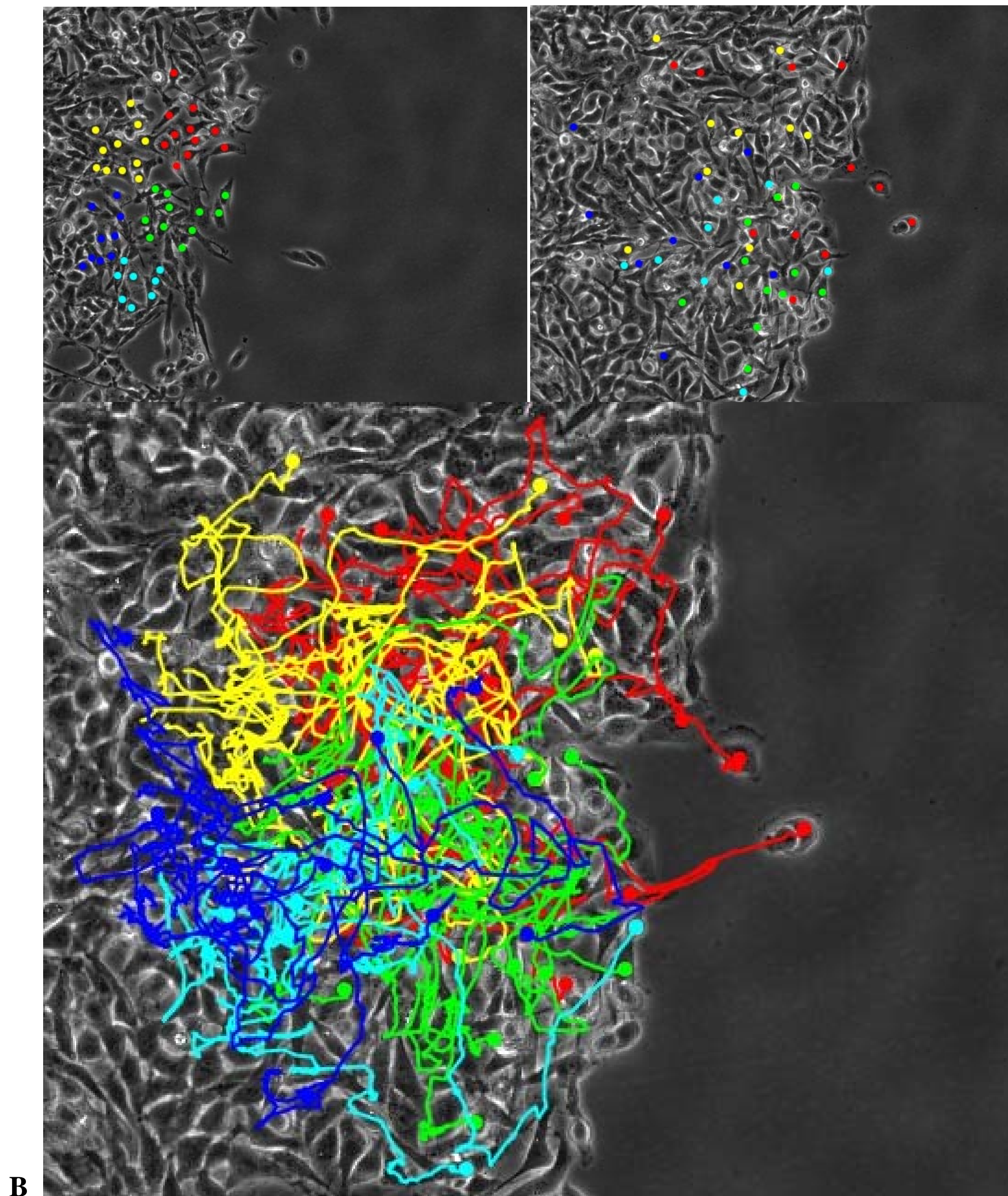
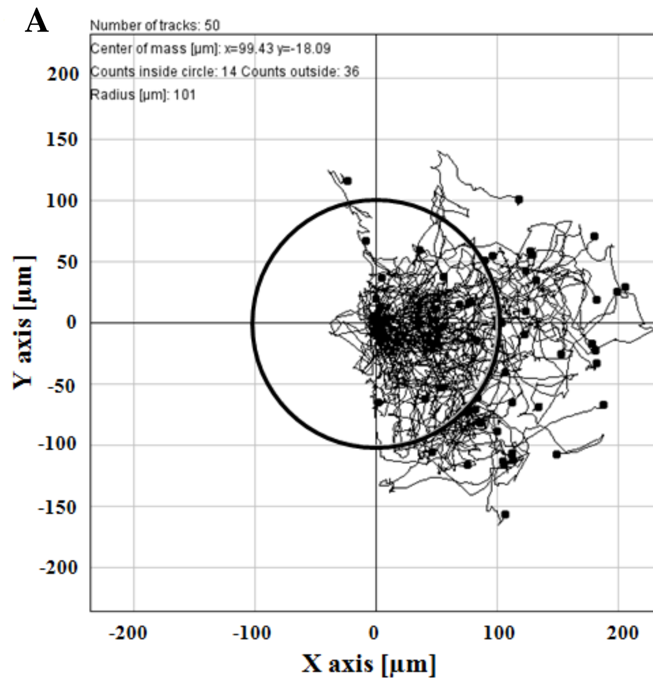
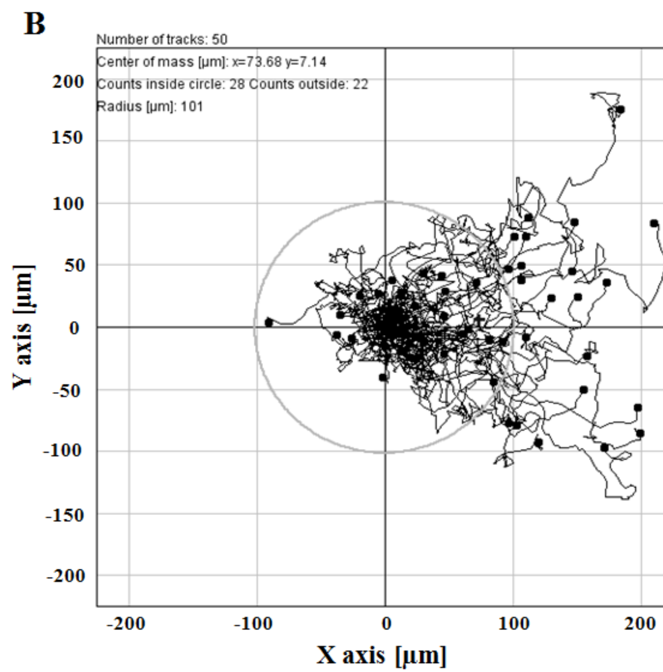


Figure 5.2 Collective migration by track patterns. The images show the initial and final frames with end points overlay (top panel) and the final frame with track overlay (bottom panel) of the tracked videos for control siRNA (A) and DP103 knock down siRNA(B) treated MDA-MB-231 cells. Cluster code shows the dispersal of cells in the top panel. The final frame with tracks reveals the characteristic haphazard and overlapping paths taken by the migrating MDA-MB-231 cells in the course of a 24 hr live time lapse video monitoring.

A



B



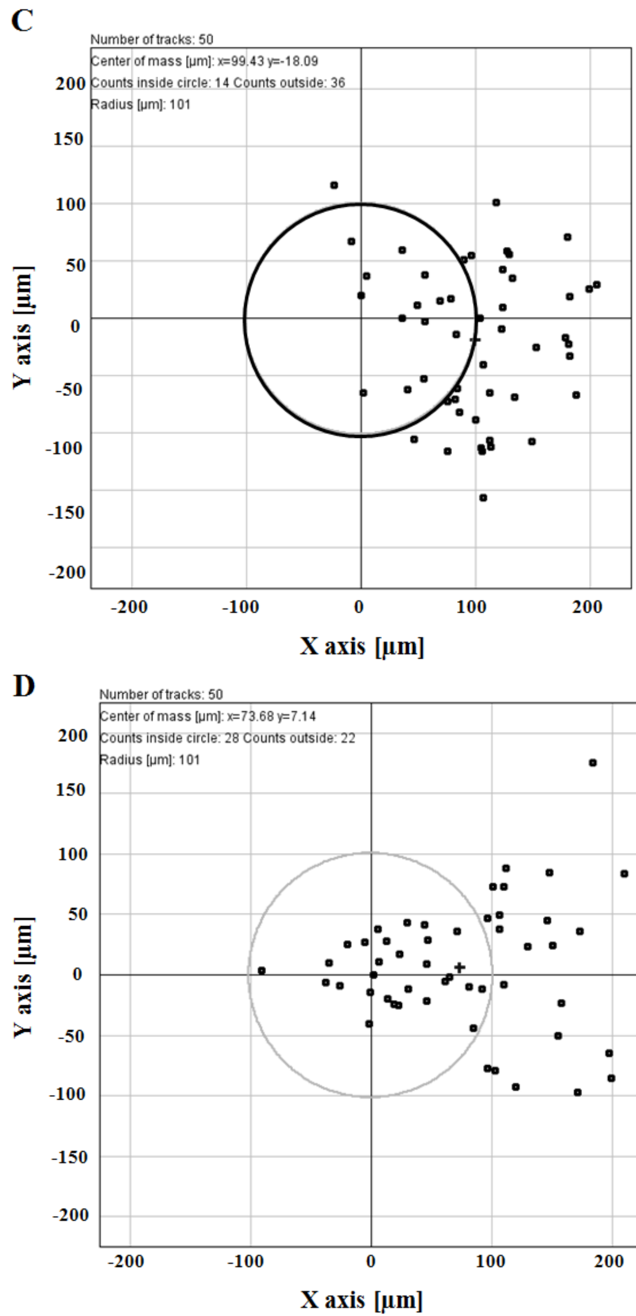


Figure 5.3 2D plots of MDA-MB-231 cell collective migration. Graphs show cell track plots of Control siRNA (A) and (C), and DP103 Knock down siRNA (B) and (D) treated MDA-MB-231 cells, with starting points of cell tracks transposed to the origin. Control siRNA treated MDA-MB-231 cells show increased migratory distances with a majority of the cells (72%) outside a radius of 101 µm that coincides with the center of mass (positive sign) of all the cell coordinates, correlating with the higher monolayer edge distance covered by these cells. B,D. DP103 knock down siRNA treated cells show decreased migratory distances with 46% of the cells inside a radius of 74 µm that coincides with the center of mass of all the cell coordinates (circle not shown) , and 56% of the cells inside a radius of 101 µm in comparison to the control siRNA treated cells.

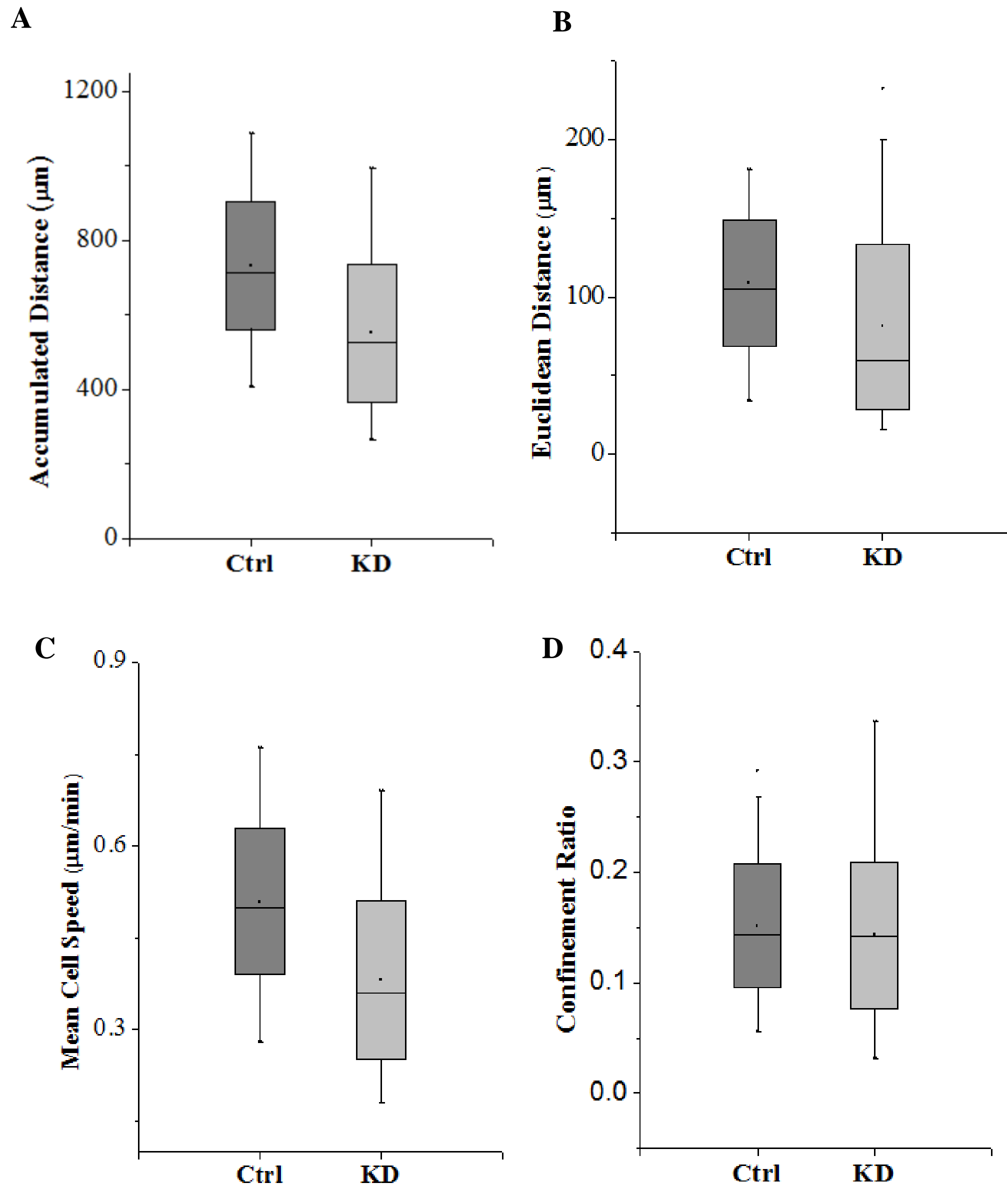


Figure 5.4 Cell Migration Parameters for Control siRNA (Ctrl) and DP103 knock down siRNA (KD) transfected cells. Control cells show a significant increase in the AD (A), ED (B), and mean cell speeds (C) in comparison with the DP103 knock down (KD) variants. The confinement ratios (D) were however not significantly different, indicating that knock down of DP103 expression affects the motility of individual cells and therefore collective migration but not directionality. Box edges represent standard deviation; the small square within the box represents the mean and the divider the 50% value. Outliers are represented by x.

5.2.3 Confinement Ratios and Migratory Angle of Deviation

As described earlier, the confinement ratios are used as an indicator of the straightness of the track paths followed by migrating cells. The migrational angle is also a measure of deviation of cell tracks, but in contrast to CR they provide a quantitative estimate of the cell deviations. Confinement ratios were essentially unaltered among the two variants (Figure 5.4 D) essentially affirming the observations that the track patterns in both conditions were similar. However, the mean migrational angle of deviation for the DP103 knockdown (37.41±0.31SEM) cells was significantly higher than the control siRNA (33.45±0.29SEM) treated cells. The graph (Figure 5.5) also shows a redistribution of the values towards higher values for the knock-down variants.

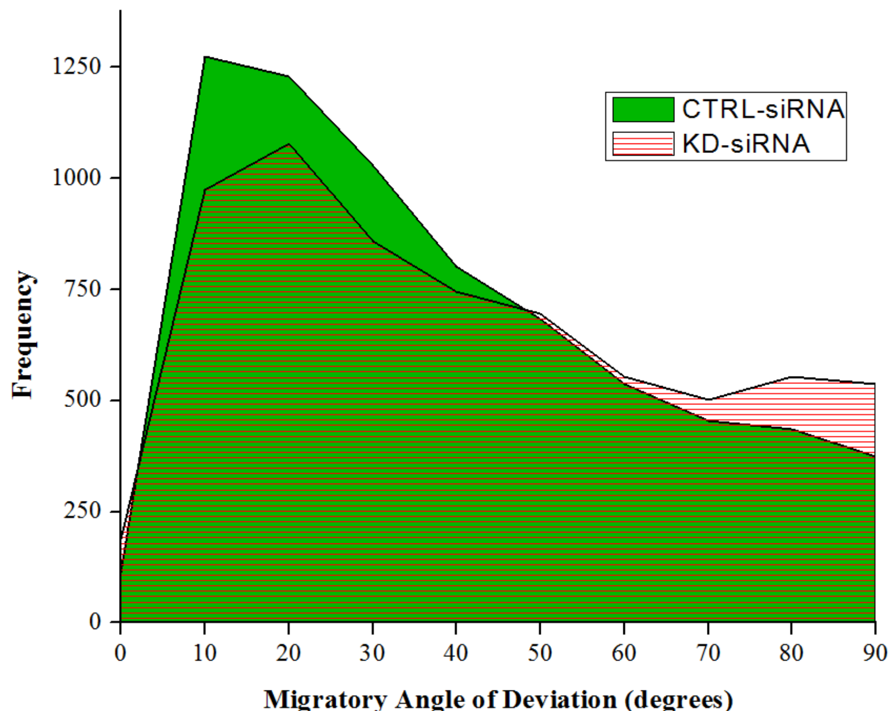


Figure 5.5 Migrational Angles of cell track deviations. Frequency distribution of migrational angles in Control siRNA and DP103 KD siRNA cells.

5.3 TG2 knockdown in HCE-T

5.3.1 Transfection Method and Experiment

The transfected control and TGM-2 knock-down variants of HCE-T cells were obtained from Dr. Louis Tong of Singapore Eye Research Institute (S.E.R.I) as a part of ongoing collaboration. Samples were prepared for monitoring collective cell migration as described in Chapter 3. Time lapse imaging was performed for 24 hours duration with 10 minute intervals between the frames. A brief description of the transfection method that was used to produce these cell lines is included below followed by the cell track analysis.

Human SV-40 immortalised corneal epithelial cell line at passages 88 to 98 was used for experiments. The cell lines were cultured in DMEM-F12 supplemented with 5% FBS. All cells were maintained in a humidified 5 % CO2 incubator at 37 ºC and fresh medium replaced every 2 days. Briefly, the transfection method (Png et al. 2011) was as follows.

pSM2 vectors subcloned with non-specific scrambled and TGM-2 silencing shRNA sequences were transfected into the retroviral packaging cell line PA317 using Lipofectamine Plus reagent. After six hours, the medium was replenished with fresh DMEM supplemented with 10% fetal bovine serum, penicillin (100 Units/ml), streptomycin (100 g/ml) and 2 mM L-glutamine and cultured at 37°C and 5% CO2. The viral supernatants were filtered with 0.45µm syringe- mounted filters and stored at -80°C. HCE-T cells were seeded and allowed to adhere. Twenty four hours later, medium was replaced with the viral supernatants and the cells cultured with 8g/ml Polybrene for 3 consecutive days. HCE-T cells stably transfected with non-specific

scrambled shRNA (referred to as shRNA) and TGM-2 silencing shRNA (shTG) (Figure 5.6 A) were subsequently isolated in the presence of 0.15 µg/ml of puromycin. The 2D non-wounding migrational assays with time lapse imaging were performed for shRNA and shTG cells as described in Chapter 3. 50 Cells were manually tracked for each condition from the video sequences followed by quantitative and kinematic analysis.

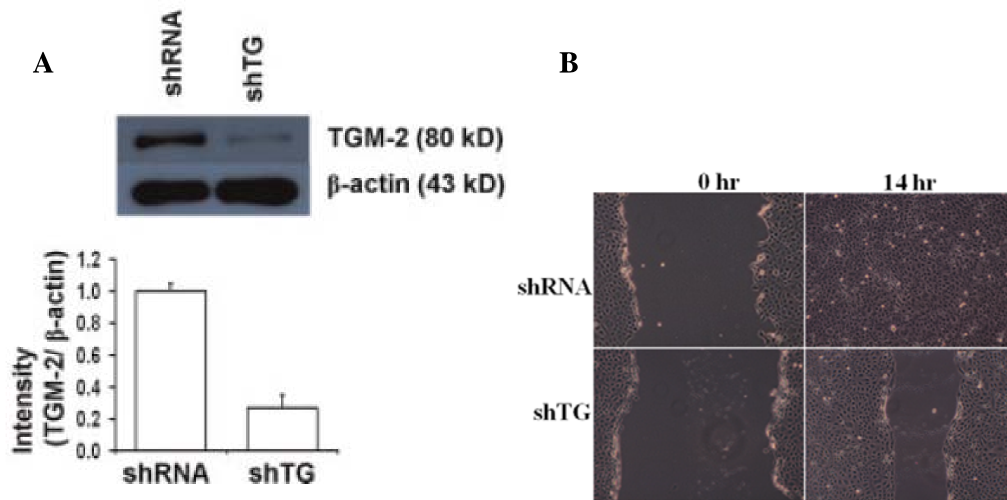


Figure 5.6 (A) Western blot showing the expression of TGM-2 protein in shRNA and shTG cells. Bottom: Bar chart showing densitometry for the Western blot. (B) Wound healing assays showing reduced migration in TGM-2 knockdown cells (Figure 39A and Legend reproduced from the reference (Png et al. 2011)

5.3.2 Track Patterns and Cell Migration Parameters

HCE-T cells have epithelial cell morphology. The collective migration patterns observed in these cells had directional quality with high positional fidelity among the cells as observed by color coded clustering of cell tracks (Figure 5.7). The track pattern evolution and clustering of cells indicated strong intercellular adhesion among both the variants of HCE-T cells. Furthermore, almost all of the leader cells

maintained their positions, indicating intact intercellular adhesion and directional persistence of cells. The knock down of TGM-2 did not affect the collective behavioural pattern as well as intercellular adhesion of cells. However quantitative analysis of cell track data showed reduced cell migration distances (AD and ED) as well as cell migration speeds (Figure 5.8 A-C) in the shTG cells.

5.3.3 Confinement Ratios and Migratory Angle of Deviation

The confinement ratios (Figure 5.8 D) and the migratory angles of deviation (Figure 5.9) were essentially unaffected in both the transfected HCE-T cell variants that were monitored. However, as expected in epithelial cells with strong intercellular adhesion, both the conditions exhibited lower migratory angles of deviation.

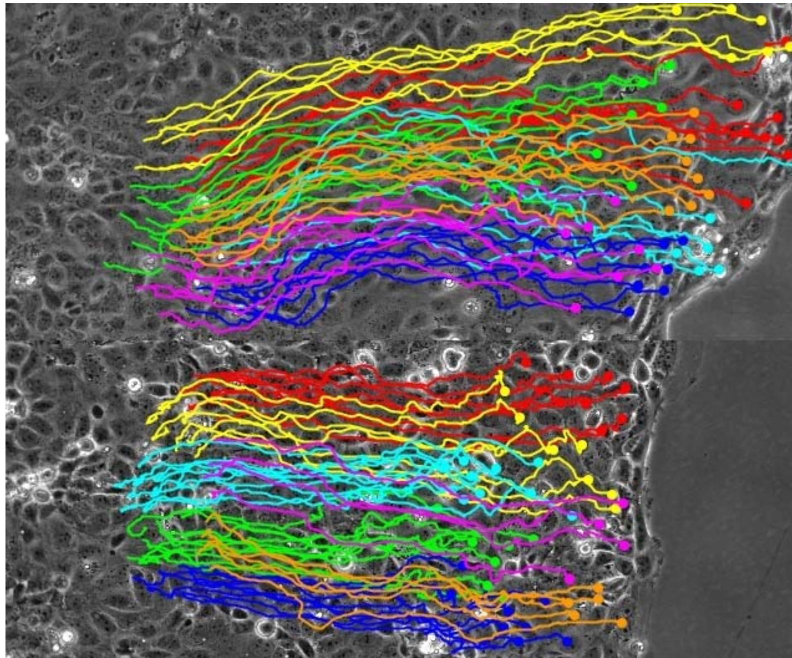


Figure 5.7 Collective migration in HCE-T cells. The image is vertically combined composite showing the final frame of a 24 hour video with overlapped cell tracks of scrambled shRNA transfected control cells at the top and TGM-2 silencing shRNA transfected cells at the bottom. Track patterns reveal that the both cell variants retain their collective migration pattern probably due to strong intercellular adhesion. Cell migration, however, is affected in the knock down condition.

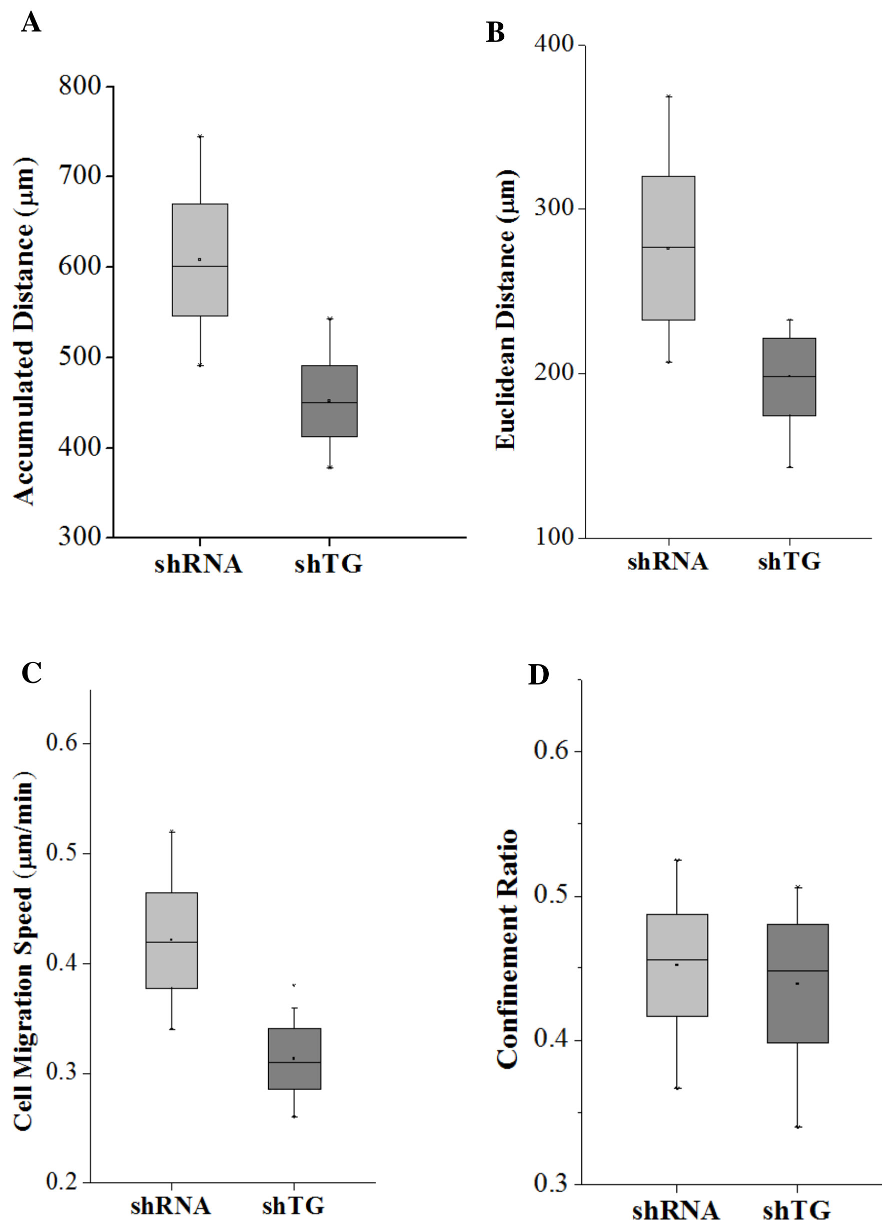


Figure 5.8 Collective Cell Migration Parameters for HCE-T cells transfected with non-specific scrambled sequence shRNA (shRNA) and TGM-2 silencing shRNA (shTG). shTG cells show a significant decrease in the AD (A), ED (B), and mean cell migration speeds (C) in contrast to the shRNA cells. The confinement ratios (D) were unaffected by the knockdown of TGM-2. Box edges represent standard deviation; the small square within the box represents the mean and the divider the 50% value. Outliers are represented by x.

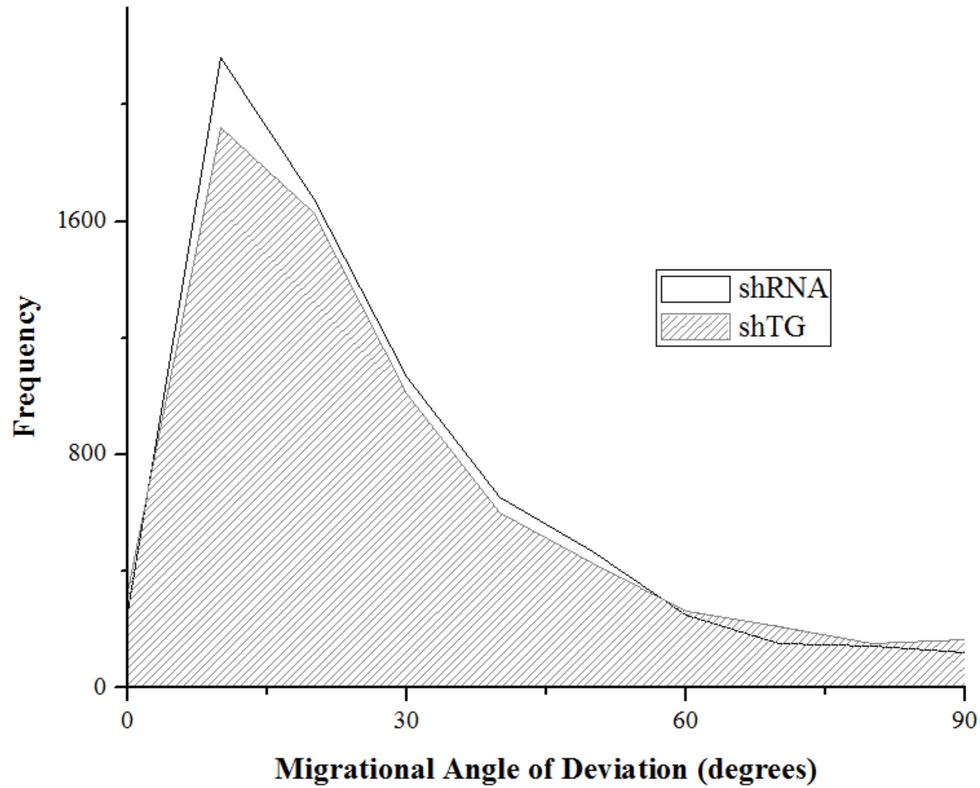


Figure 5.9 Frequency distribution of Migrational angle of deviation. TGM-2 knockdown did not have any effect on the migrational angle of HCE-T cells.

5.4 Discussion

5.4.1 Effect of DP103 Knock Down on Collective Cell Migration in MDA-MB-231 cells

Collective migration in the MDA-MB-231 breast cancer cells mainly involves movement of cells in an individualistic fashion, a character attributed to mesenchymal modes of migration, as described in the previous chapter. This type of movement, along with defective intercellular adhesion and contact inhibition in these cell lines, gave rise to the characteristic “spaghetti-like” appearance to the track patterns. In these sections, collective migration kinematic analysis revealed that both the control

and DP103 knock down MDA-MB-231 cells retained their individualistic migratory behaviour as seen by the high degree of lateral dispersion and loss of leader cell status. Quantitative estimates further showed a statistically significant decrease in cell migration distances (AD and ED) and mean cell migration speed. This means that DP103 is involved in the regulation of migration in MDA-MB-231 cells.

The confinement ratios, which are gross indicators of straightness of cell tracks, remained unaltered. This suggested no involvement of DP103 in affecting the directionality of the cells. However, results of migratory angles of deviation, which are more sturdy indicators of deviations in cell tracks, revealed a redistribution of the frequency of deviation in DP103 knock down MDA-MB-231 cells. The mean migratory angle of deviation was significantly increased in the knock down variants. This indicates that DP103 is probably involved in the regulation of directional migration of MDA-MB-231 cells, although the mechanism by which it affects and the significance of this effect still cannot be commented unless evaluated by further experiments. Previous results have shown that mesenchymal type of migration in cells is regulated by Rac1and directionality is controlled by cdc42, both being Rho GTPase proteins (See review by (Parri and Chiarugi 2010)). Therefore it is possible that DP103 might act through signalling pathways involving these proteins.

In conclusion, the results of these analyses thus show that the DEAD-box protein, DP103, is required for the migration of MDA-MB-231 cells with regulatory effects on their directional persistence. Development of anticancer drugs against this molecule might be beneficial in the treatment of breast cancers and in restricting metastastic spread.

5.4.2 Effect of TG2 Knock Down on Collective Cell Migration in HCE-T cells

The collective migration of epithelial cell sheets follows an organised pattern (Angelini et al. 2011). The cell track analysis of time-lapse images revealed highly coordinated collective migration of HCE-T cells that were transfected with the control siRNA (scrambled shRNA). The track patterns showed that the cells possessed strong intercellular adhesions as revealed by the color coding of clusters of cells. The intercellular adhesion and the directional persistence of the cells were also revealed by most of the leader cells, which maintained their positional status and forward motion throughout the monitoring. The kinematic analysis thus showed that the scrambled shRNA HCE-T cells follow strictly organized collective migration behaviour.

The cell track patterns of shTG cells revealed similar behaviour with unaffected intercellular adhesion and leader cell status, indicating that TGM-2 was not involved in the control of these components. The quantitative analysis showed decrease in both cell migration distances (AD and ED) and mean cell migration speed. However confinement ratios in both the conditions were unaffected. The results of migratory angles also corroborated with those of CR measurements. This indicates that TGM-2 is involved in the regulation of cell migration of HCE-T cells but not the directionality of the cells.

As mentioned in the literature review the Rho GTPase protein, Rac is involved in the formation of lamellipodial protrusions and is thus responsible for distribution of focal adhesions. Since TGM-2 is involved in cell adhesion (Fesus and Piacentini 2002), it is possible that its actions on migration are mediated through proteins involved in lamellipodial protrusion and focal adhesion formation. This study proves that TGM-2 regulates the rate of cell migration in HCE-T cells, but due to lack of control on other

parameters such as intercellular adhesion or directionality it does not affect the collective migration behaviour.

5.5 Conclusions

In this chapter we have performed the 2D non-wounding migrational assays in combination with quantitative and kinematic analysis of collective cell migration. The experimental setups that were examined included two different protein knock down systems involving breast cancer cells and human corneal epithelial cells. The results have helped in establishing not only that cell migration was affected due to knock down of proteins, but also the changes in dynamics involved in collective migration behaviour. This was achieved through a systematic quantitative approach combined with careful kinematic analysis of the data from cell tracks. The results of the experiments also showed that proteins that affect migration of cells may not have any effect on the collective migration behaviour. This finding is significant since it separates the collective behavioural patterns from migration as an entity, and emphasizes the importance of the collective migratory patterns in cancers that might provide them with serious advantage to spread and wreak destruction of adjacent structures.

Thus, whereas ordinary analysis with scratch assays would reveal only a difference in migration, the currently employed method allows for strong correlation of the adhesive interactions and migratory properties of cells that are important during collective migration. In the next chapter, we will show how this analysis method can be enhanced by including single cell motility analysis in the repertoire.

Chapter 6
Contributions of Individual Cell Mechanisms to Collective Migration in Breast Cancer Cells

6.1 Introduction

The previous two chapters showed the capabilities of the 2D non-wounding migrational assays in estimating collective cell migration. Also, the kinematic analysis was found to supplement the quantitative results from cell migration track data. These methods were useful in showing the details of collective migration in breast cancer cell migration and normal epithelial cells as well as delineate the functional roles of proteins in collective cell migration. Although this method helps in understanding the collective migration of cells, it does not provide much information on the mechanisms in individual cells that are primarily responsible for the collective behaviour. Furthermore, various types of cells employ different strategies for movement. For example, different types of cell migration modes have been described such as amoeboid migration (Sanz-Moreno et al. 2008) and mesenchymal migration (Sahai and Marshall 2003) . Cells migrating in these modes can apparently produce similar results of migrational parameters, such as cell speed and migratory distances on ordinary migrational assays, but the underlying mechanisms in these cases would be different. Therefore, reports of collective migration as available by common migration assays will neither be accurate in providing sufficient information on the basic operating mechanisms, nor helpful in understanding the relevance of collective migration in biological environment. In the light of these circumstances, it is thus necessary to combine the analysis with detailed examination of individual cells.

In this chapter we have used kymographic analysis of lamellipodial protrusions to study the migrational mechanisms of individual cells. Kymographic sections, for the unit pixel wide region of interest in migrating cells, produce a single image representing the movement of the structure in the selected region, for the entire image sequence. This allows for studying the dynamics of motion of protrusive structures using single images. Kymographic analysis has been used to study dynamics of lamellipod extension and focal adhesion formation (Giannone et al. 2004; Giannone et al. 2007), protrusive forces resulting from dynamics of tropomyosin based Actin motility in lamellipodia ((Bugyi et al. 2010)), and effects of protein inhibitors (Totsukawa et al. 2004) and growth factors (Harms et al. 2005) on lamellipodial protrusions to mention a few. Some of these have also studied cell migration by tracking individual cells. Others have studied motility in isolated diseased cells to correlate to the overall pathogenesis. For example Partin et al have used fourier analysis to study single cell motility of cancer cells by quantifying cell membrane and area changes and associate it to metastatic potential (Partin et al. 1989). But a correlation of protrusion dynamics to collective migration has not been reported, to the best of our knowledge. In the following sections we will describe our results with kymographic analysis of individual cells of the three breast cancer cells considered before, and discuss the implications in conjunction with cell track analysis of collective migration.

6.2 Kymographic Analysis of Lamellipod Protrusions

As described in Chapter 3, the videos for kymographic analysis were of 2.5 hours duration with 1-2 minute frame intervals. The kymographic sections were taken at multiple levels of single cells. 20 cells were analysed for each cell line using multiple

sections for each cell. The typical kymographs of the three breast epithelial cell lines are shown in the Figure 6.1. The MCF-10A cells (Figure 6.1A) mostly show steady progression of protrusions, with frequent retractions at the edge of the lamellipods.

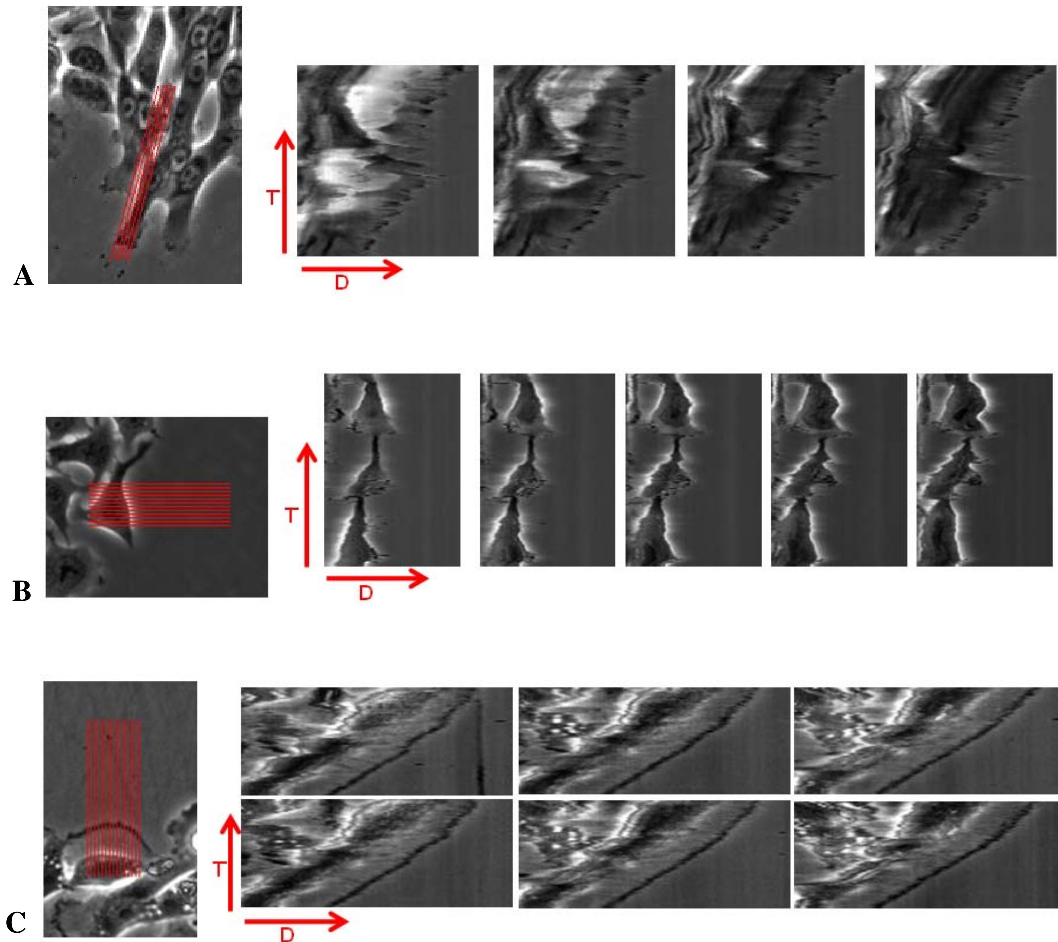


Figure 6.1 Kymography of Lamellipod protrusion. (A) MCF-10A cell (B) MCF-7 cell and (C) MDA-MB-231cell. Red lines on the initial images represent the regions of interest that were selected for kymographic analysis. T = time, D = Distance

The MDA-MB-231 cells (Figure 6.1 C) have similar protrusions but surprisingly have very minute or no retractions at the lamellipodial edges. The MCF-7 cell (Figure 6.1 B) lamellipods were hard to study with the kymographs since the direction of movement of the cell bodies were frequently changing. The cell body movements were clearly visible as separate cell bodies in the kymographs, with the constrictions

indicating the time when the cell had entirely moved out of the region of interest. These findings provoked us to quantitate some of these phenomena, which led to the development of the subsequently mentioned parameters. Some of these parameters have definitely been used in previous literature, but our contribution here is to link the findings to the individual cell motility that ultimately is responsible for collective cell migration behavior.

6.3 Lamellipod Protrusion parameters

The parameters concerned with the lamellipod protrusion include the average lamellipod protrusion speed (LPS), maximum lamellipod extension speed (LES), the lamellipod persistence times (LPT) and the lengths of transient retraction phases in lamellipod protrusions (RPL), which were described in detail in Chapter 3. For a brief recapitulation, the LPS is an average of the speeds recorded for the lamellipodial edge of a migrating cell, for as long as it migrates in a uniform and persistently forward direction. The number of frames over which this parameter was measured is referred to as the LPT. Since LPS is averaged over different number of frames, the LES was introduced, which measured lamellipod protrusion within a single frame. This allowed us to estimate which of these is more important in cell migration. Transient RPL, as the name suggests, is the length of the retraction phases in the lamellipods, provided the retraction is followed by lamellipod protrusion and uniform cell body movement in the forward direction.

The LPS's were slightly higher for MCF-10A when compared to MCF-7 although the difference was statistically insignificant at $p<0.05$ (Figure 6.2), but the MDA-MB-231 cells showed the highest values for these parameters. However the RPL was significantly different across all the cell lines with the MCF-10A cells showing the

highest retraction phases and the highly invasive cell lines showing almost no retraction phases. The LPPT were significantly higher for MDA-MB-231 (Figure 6.3) than the other two cell lines. These results correlate with the cell migration speeds reported in Chapter 4 but not with the CR and migratory angles, and will be discussed later. The LES were higher in the MCF-7 cells and the lowest for MCF-10A cells which does not correlate with the migration speeds by tracking. Therefore, LES is probably not a good indicator for sustained cell migration. It is to be noted here that the lamellipodial protrusion parameters for MCF-7 were measured for the dominant lamellipods i.e., those producing movement of cell body in a particular direction in the sequence. This is important because lamellipods are ill formed in MCF-7 and occur in multiple directions simultaneously. Thus all these results indicate that LPS and the RPL are important parameters in the migration of individual cells and are sufficient to explain the variations observed in the migratory distances covered.

6.3 Ruffle Thickness

Ruffle thickness was measured as an additional parameter since higher ruffling rates have been considered to indicate lower lamellipod adhesion and inefficient migratory rates (Borm et al. 2005). Our results show that ruffle activity was higher in the MCF-7 than the MCF-10A and lowest in the MDA-MB-231 cells (Figure 6.4). These results agree with the lamellipod protrusion parameters, and thus ruffle formation could be a critical factor in determining the efficiency of unidirectional lamellipods.

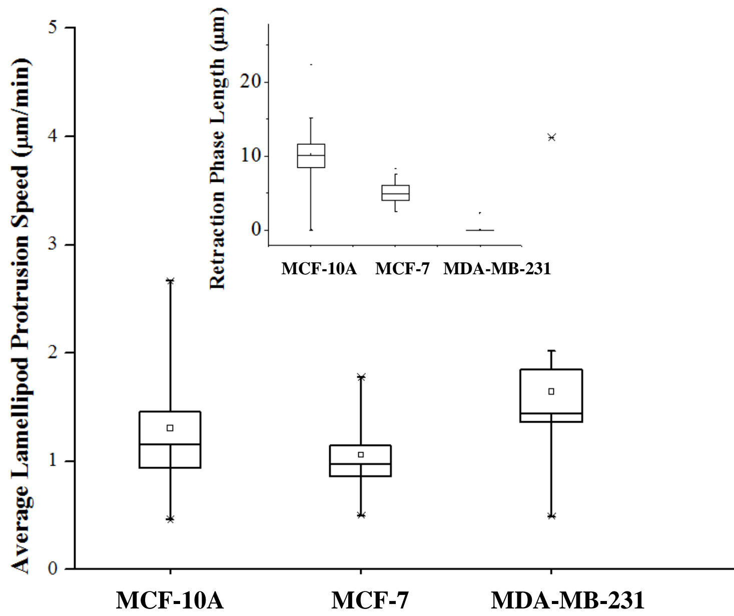


Figure 6.2 Lamellipod Protrusion speed and Retraction Phase Length. Box edges represent standard deviation; the small square within the box represents the mean and the divider the 50% value. Outliers are represented by x.

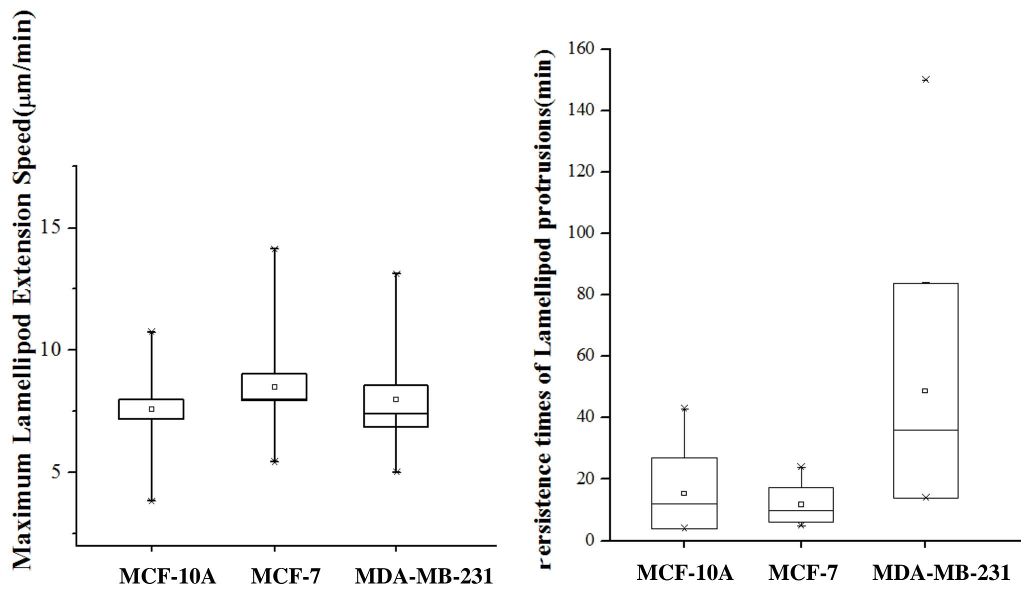


Figure 6.3 Lamellipod Extension Speed and Persistence Times. Box edges represent standard deviation; the small square within the box represents the mean and the divider the 50% value. Outliers are represented by x.

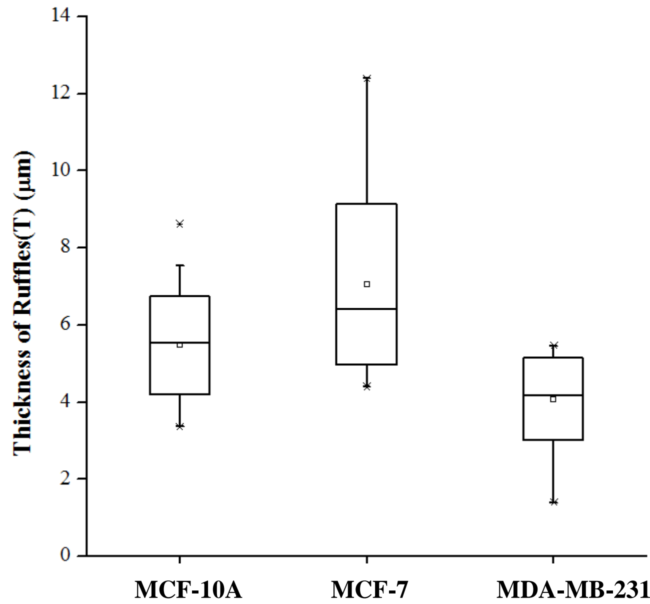


Figure 6.4 Ruffle Thickness. Box edges represent standard deviation; the small square within the box represents the mean and the divider the 50% value. Outliers are represented by x.

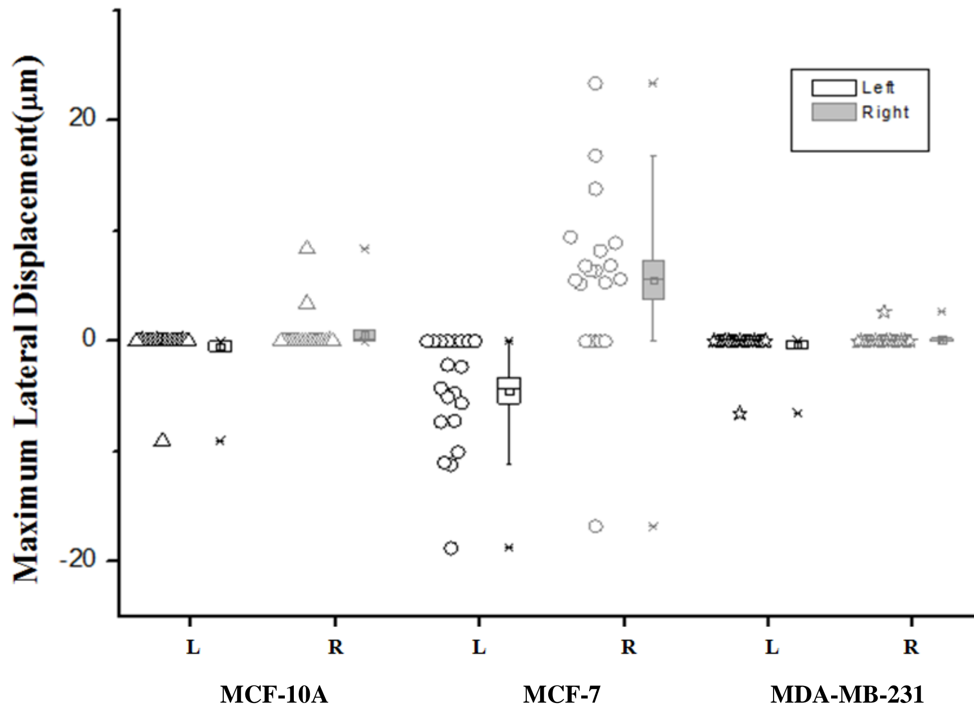


Figure 6.5 Maximum Lateral Displacement of cell bodies. Data plot, for MCF-10A (triangles), MCF-7 (circles) and MDA-MB-231 (stars), beside box plots showing the maximum lateral displacement of cell bodies during 2.5 hour duration. This abnormal movement is seen only in MCF-7 cells as a result of defective lamellipod polarization. Box edges represent standard deviation; the small square within the box represents the mean and the divider the 50% value. Outliers are represented by x.

6.4 Maximum Lateral Displacement

This parameter (MLD) was developed circumstantially, to demonstrate the reason for inefficient movements in the MCF-7 cell motility. The tortuous displacements of MCF-7 cell bodies stand out in contrast to the smoothly evolved tracks of the other cell lines. This clearly explains the tortuous cell tracks reported in Chapter 4 (Figure 6.5) and helps in establishing defective but multiple lamellipodial polarizations as the cause (see discussions later).

6.5 Discussion

This Chapter reports the important method and results that were useful in correlating the individual cell movements with the development of collective migration. Individual cell motility was estimated by measuring parameters for lamellipodial protrusion. The results were correlated with the collective cell migration analysis performed in Chapter 4.

6.5.1 Lamellipodial Protrusion Speeds and Transient Retraction Phases are Important Determinants of Individual Cell Motility

Characteristics of lamellipod formation are specific to particular cell types. Our results showed that the lamellipod protrusions in the benign cells were comparable to those of the highly invasive cell line with the notable difference being the higher ruffling activity (as revealed by RT) and a more dynamic exploratory function compared to the lamellar portion of the cell body with larger retraction phases (RPL). The protrusion formation in the MCF-7 cells differs from the other cells by the

presence of multiple locations of lamellipod formation (multiple lamellipodial polarizations).

Lamellipodial protrusions have been linked to the formation of focal complexes which are responsible for adhesion and contractility of the cells that results in cell movement (Galbraith and Sheetz 1998; Gupton and Waterman-Storer 2006; Giannone et al. 2007). Increased lamellipod protrusion speeds are the results of extension of larger portions of membranes in a sustained fashion. Also, as noticed from our results, lamellipod extension speeds do not contribute to the speed of the cell. Rather, the steady rate of lamellipod protrusion as measured by the LPS's are more sturdy pointers of cell motility. Thus, LPS's in conjunction with LPT and RPL are reliable indicators of individual cell motility.

6.5.2 Lamellipodial Polarisation Determines Directional Persistence/Efficiency of Cell Movement and Collective Cell Migration

From our results, we showed that although MCF-7 cells had higher LES, their sustained directional movements suffered because of frequent cell body movements, as delineated by MLD (Figure 6.5). Also, this correlated with the less efficient collective migration of these cells on the 2D surfaces as revealed by the monolayer edge distances (MED), despite them having comparable cell migration speeds, LPS and LPT and higher LES with respect to MCF-10A cells. However, these cells had higher RPL and larger values of RT. These results along with the MLD collectively suggest that the main defect in these cells lies in their lamellipodial polarisation. Since studies have shown that proteins affecting cell motility such as Rho proteins and ROCK are upregulated in MCF-7 (Nagaraja et al. 2006; Lai et al. 2010), altered contractility mechanisms are also significant in this type of cell migration.

Importantly, simultaneous lamellipodial formations occurring in multiple locations in the MCF-7 cells probably provide multiple focal adhesional attachments resulting in the characteristic cell body movements (Figure 6.5 B). For example, it has been shown that new focal adhesions in fibroblasts are formed within and at the base of lamellipodium ((Izzard and Lochner 1980)). Cell membrane protrusions such as lamellipodia and filopodia initiate and are stabilized by adhesion to the extracellular matrix which involves the aggregation of integrins and other proteins to form specialized spots called the focal complexes, under the regulation of Rac and cdc42 (Nobes and Hall 1995; Allen et al. 1997; Hall 1998; Rottner et al. 1999; Small and Vignal 2004). This means that the distributions of focal adhesion sites are determined by the lamellipodial polarization in a cell. For example, in a migrating cell like keratocytes, and the MDA-MB-231 cells in our experiments, persistence of direction is observed because lamellipodia are well polarized in a particular direction which ensures uniform contractility and focal adhesion formation.

Thus, the results of our analysis prove the significance of lamellipod polarization in the directional motility and hence efficient migration of cell lines.

6.5.3 Correlation of Individual Cell Motility Parameters with Collective Migration Analysis

The tracks of migrating cells reflect the basic motility mechanisms that are responsible for the movement pattern of the cells. In Chapter 4, we have shown that the MCF-10A cells exhibited straight (directional) and smooth tracks with change of directions that involved clusters of cells rather than a single cell, indicating the play of several local environmental factors as well as strong intercellular adhesion. In contrast the migration tracks of the MDA-MB-231 cells showed predominantly smooth

(directional) curves although with frequent changes in direction of the tracks. The MCF-7 cells showed highly tortuous cell tracks due to the cell body displacements. A comparison of the individual cell motility mechanisms as elucidated by the kymographic analysis, with the results of collective migration, provides an explanation for the patterns observed in these cell tracks.

The benign MCF-10A cell lines chiefly expressed unidirectional (polarized) lamellipods (flat, broad, bulging perimeter with few divisions in the continuity of a single cell lamellipod) which essentially persisted in a particular direction throughout their migration as evidenced by the uniform drag on the cell bodies and strong intercellular adhesion as shown by lack of the MLD. This is responsible for the smooth evolution of their cell tracks. Results of RPL and RT are able to explain the strong intercellular adhesion and the decreased migration speeds when compared to isolated MCF-10A cells and the highly invasive cells.

The MDA-MB-231 cells differed from the MCF-10A having uniform but multidirectional (non-polarized) lamellipods (flat, broad, clean bulging perimeter). The sustained formation of protrusions was reflected by the higher values of LPS, LPT and almost absent RPL. The RT was also low and therefore these cells had superior lamellipod polarization dynamics compared to the other cell lines. These results explain the haphazard quality of the tracks that is characteristic of “swarming” motility seen in mesenchymal migration.

In contrast to the above cell types, the MCF-7 cells exhibited altered lamellipod polarization that were formed simultaneously and characterized by triangular projections at multiple locations (three or more) of the cell periphery. The dominant of these protrusions hauled the cell body in their directions giving rise to the lateral

displacements (evident on MLD) that was responsible for the tortuosity of the cell tracks. The measurements of dominant lamellipods in these cells revealed a high LES and RT but low LPS and LPT, which were consistent with the cell speeds and the quality of the tracks reported earlier the earlier chapters.

However, the results of kymographs did not entirely agree with the confinement ratios (CR) and migratory angles of deviation. For example, the CRs were higher, and the migration angles showed a prominent peak at lower values, for the benign cells in comparison with the malignant cell lines. This is because the CRs and the migration angles were obtained from cell tracks over a period of 24 hours duration. The changes in the directions of the malignant cells are drastic because they can form lamellipodial protrusions in any direction. But when these lamellipodial protrusions gain dominance, they persist for a long time, leading to constant directionality of cell migration for that period. Further, the kymographs were performed for duration of 2.5 hours while the LPS represented the averaged protrusion speed over the number of frames during which the cell persisted in a particular direction. Also, the benign cells have splits in lamellipod formation, although the polarization is unidirectional. This causes frequent but small changes in directions of these cells which resulted in the low LPT values in comparison to the highly invasive cell lines. Altogether, this explains the discrepancy found between these sets of parameters, and thus the relevant interpretation still holds good.

Thus our analyses have shown that lamellipod protrusion rates correlates with the cell migration speeds observed in collective migration analysis. Also, RPL's correlated well with the status of intercellular adhesion of the cells during collective migration with the cells having stronger intercellular adhesion (MCF-10A) exhibiting frequent

retraction phases in the lamellipod formations while those with defective (MCF-7) or almost ineffective (MDA-MB-231) intercellular adhesion showing minimal or absent retraction phases, respectively.

With the results from collective migration and individual cell motility analysis, we venture to explain the significance of lamellipod polarization and other molecular events that affect this parameter (Figure 6.6). These results might also be significant in the understanding of motility and collective migration parameters of other types of cells. It is known that lamellipodial polarization determines the sites of focal adhesion formation and these, in turn, determine the contractility and directional motion of the cells (Parker et al. 2002; Wang et al. 2002; Brock et al. 2003). Studies of cell migration on surfaces of varying rigidity have demonstrated the motility of cells in the direction where they exert greatest traction forces (Lo et al. 2000). Uniform and directional lamellipodial formation therefore favor groups of focal adhesions that help in the subsequent development of sustained contractility of the cell bodies, leading to smoothly evolved migratory track patterns.

The individual cells of MCF-10A revealed uniform lamellipod polarizations which were accentuated in directionality by strong intercellular. This resulted in directionally distributed focal adhesion spots facilitating the directional contractility that was observed in the cells adhesions (Figure 6.6A). The effect was similar in the MDA-MB-231 cells (Figure 6.6C) which, however, lacked the restricting influence of intercellular adhesion and contact inhibition by adjacent cells. The result was sustained contractility similar to the benign cells, but with enhanced migratory speeds. The MCF-7 cells however had multiple lamellipodial polarization resulting in cell

contractility in different directions (Figure 6.6B), and therefore the alternating cell body displacements.

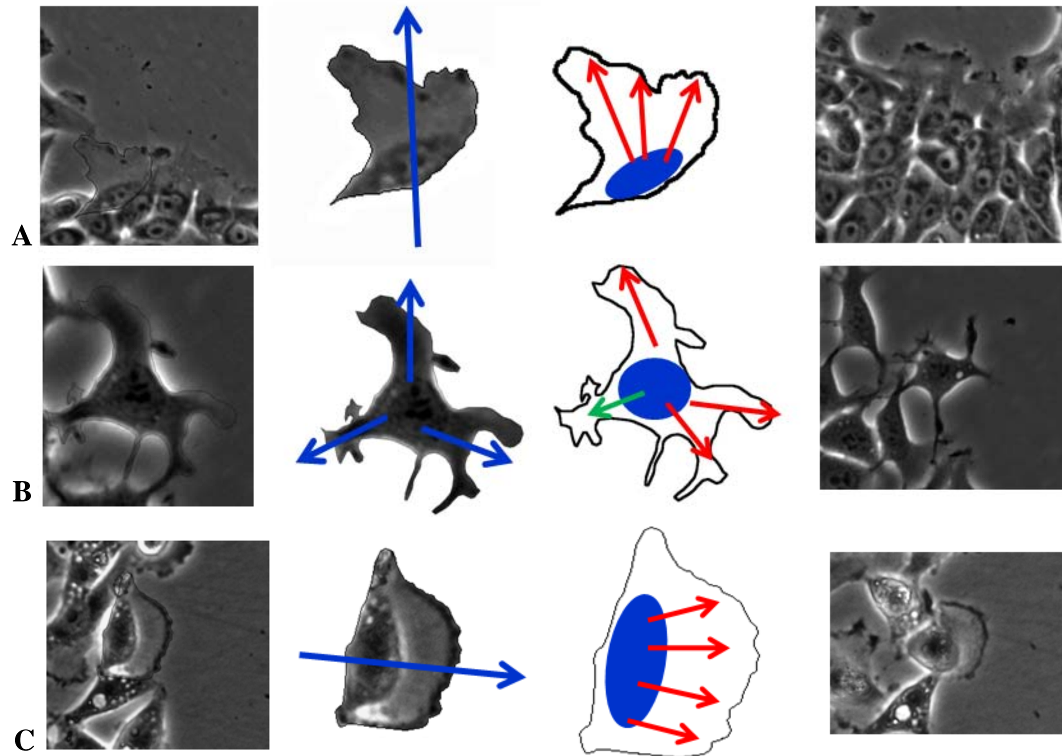


Figure 6.6 Schematics showing lamellipodial polarization in effecting directional movement of cells. (A) MCF-10A (B) MCF-7(C) MDA-MB-231. The images in the first column show the retrieved cells in the collective context. The images in the second and third columns show the directional vector and the role of lamellipod polarization respectively. The images in final column represent the corresponding final frames of the 2.5 hour video clips from which these cells were extracted. (See text for more explanation)

6.5.4 Implications of Combined Analysis on Actual Diseases and the Biological Environment

The results from our analyses of collective cell migration and individual cell motility in benign and malignant breast epithelial derivatives are consistent with the expected behavior of the cells in the disease of their origin. As mentioned in the literature review the MCF-10A cells are derived from the fibrocystic breast disease characterized by formation of multiple cystic structures. Such cystic structures can be envisaged as ductal structures with closed ends while normal breast ducts are usually formed by epithelial cells and have an open end. The benign cells, derivative of the ductal epithelial cells, however, can form cystic structures as they still possess epithelial sheet like migratory behavior with intact intercellular adhesion. The closed cyst formation could be a result of combination of an enhanced proliferative capacity in these cells and a loss of spatiotemporal polarization that is characteristic of benign tumors.

The MCF-7 and MDA-MB-231 cells were isolated from metastatic pleural effusions, but represent solid tumors. Their collective cell migration behavior can be typical of metastasizing solid tumors. The MDA-MB-231 had haphazard track patterns which are consistent with highly invasive metastatic solid tumor. Here it has to be mentioned that low cell migration observed for MCF-7 cells on 2D surfaces might not be the same in 3D environments. It is in this context that the individual cell motility analysis comes to the rescue. As mentioned in the literature review, in a 3D environment, it is clear that a two point attachment of cells on fibers is due to the restriction of aggregation of focal adhesions of these points, resulting in only two directions of possible movement. Thus, it is possible that if the focal adhesion points of MCF-7 are

limited to two regions or even in case of multiple points, aligned in a uniaxial direction, the contractility of these cells could result in enhanced migration.

Furthermore, the MCF-7 cells are known to be non-invasive despite them being obtained from metastatic pleural effusions. Therefore it is possible that these variants have somehow lost their invasive property or that migration alone with other factors such as angiogenesis facilitating spillage of cells into circulation is sufficient for metastasis. In this regard, it would be interesting to test the hypothesis that MCF-7 migration might drastically improve in a 1D/3D environment with further experiments.

6.6 Conclusions

In this chapter, we effectively demonstrated how individual cell motility studies support and help in the interpretation of collective migration analysis of breast cancer cells. The collective migration track patterns of breast cancer cells were shown to reflect the motility mechanisms in the individual cells. In this context it was shown that the cells of the benign and the highly invasive cancer cell lines had advantageous lamellipodial protrusive properties, as revealed by the kymographic analysis of lamellipodial protrusions, which could be correlated with the cell migration track data. These analyses have also helped to correlate the cell migratory behaviour to the actual disease conditions.

In effect, it was shown that the lamellipodial protrusion parameters could be used to explain the development of smooth tracks with fewer deviations for both the benign and highly invasive cell lines. However, due to differences in intercellular adhesion properties and the loss of contact inhibition, the highly invasive cell lines showed cell

tracks with a characteristic mesenchymal pattern. Also, the lateral displacement of cell bodies in MCF-7 cells was shown to be the reason for their highly tortuous cell migration tracks. The underlying defective motility mechanism implicated these displacements were identified to be simultaneous and multiply located lamellipod formations. In this context, we established the importance of lamellipod polarisation in the development of persistent and directional migration. This led to the hypothesis that disruption of lamellipod polarisation can disrupt the collective cell migration in cells.

In the next and final chapter, we will test this hypothesis on the epithelial cell lines by performing the 2D non-wounding migration assay and a complete analysis including collective cell migration by cell tracking and individual cell motility analysis.

Chapter 7
Effect of Disruptions of Internal Motility Mechanisms in Epithelial Cell Lines

7.1 Introduction

So far, we have reported our results on collective cell migration and individual cell analysis. 2D-non wounding cell migration assays were used in all experiments for analysing the collective cell migration. As a result of single cell analysis in breast cancer cells, we arrived to the conclusion that lamellipodial polarisation affects directionality of cell migration. Further, results from Chapter 4 showed that cancer cells capable of migrating with higher speed were found to have highly organized stress fibres i.e., stress fibre formation in static cells correlates with the dynamic migrational capability in 2D.

In this chapter, we pursue the hypothesis that lamellipodial polarisation affects directional cell migration. To attain this objective, we tried to induce disrupted collective migratory behaviour of cancer cells, in normal epithelial cell lines, with the use of a specific inhibitor of the downstream effector of Rho i.e., Rho Kinase (ROCK). We used the scrambled shRNA treated HCE-T as the epithelial cell lines because of ease of availability. Since tests revealed that introduction of the scrambled sequences did not affect the morphology and cell migration, these cells can still be considered representatives of normal epithelial cells. In the following sections the scrambled shRNA cells will be referred to as HCE-T cells. The choice of the inhibitor used was difficult, but the rationale was to induce disruption of lamellipodial polarization without interrupting the lamellipodial formations. Rho proteins facilitate the development of benign tumours by inducing the loss of cell polarisation in benign

tumours (Parri and Chiarugi 2010). Previous research by Brock and Ingber, 2005 had shown that a balance in the activities of Rac and Rho was necessary for directional extension of lamellipodia (Brock and Ingber 2005). In particular, activation of Rac or inactivation of Rho limited lamellipodial protrusions on edges of micropatterned ECM islands, but combining these activities in the cells by transfectional intervention involving recombinant proteins, rescued the formation of lamellipods. However, Rac is involved in lamellipodial formations (Ridley et al. 2003) with decreased levels of its activity favouring directional persistence. Furthermore, our analysis has shown that lamellipodial polarization is affected in MCF-7 and several proteomic studies (Nagaraja et al. 2006; Lai et al. 2010) have shown that several Rho proteins and ROCK isoforms are upregulated in MCF-7 cells. Therefore, to avoid disruption of lamellipodial formation and to induce defects in intracellular contractility, we have resorted to the ROCK inhibitor.

The method of assay was similar to the previous experiments, except that in these experiments the time lapse monitoring was performed for 8 hours duration and all the cells in the field of view were tracked. The tracked data was subjected to collective migration analysis as performed in Chapter 4. Individual cell motility analysis was performed on 2.5 hour video sequences with 1 minute frame intervals.

7.2 Collective Migration Analysis of HCE-T Cells

The collective migration behaviour of HCE-T cells showed characteristics that are expected of epithelial cell sheets and were similar to those observed in Chapter 6. The track patterns had directional quality and the cells migrated in clusters as observed by color coded clustering of cell tracks. 2D plots of cell tracks (Figure 7.2 A) confirmed this fact as well as demonstrated that lateral dispersion was low, that is consistent with

coherent collective cell migration. This showed that these epithelial cell lines had intact intercellular adhesion resulting in organized migration as sheets. The leader cell behaviour was also prominent. The HCE-T cells treated with the ROCK inhibitor, Y26732 however showed a drastic change in collective behaviour (Figure 7.1 B). The directional quality of the track patterns was completely lost (Figures 7.1-7.2), but clustering of the cells was also affected as observed on video sequences, although most of the cells still appeared to maintain relative positions on still images. It is possible that the loss of clustering was not prominent due to the short time period of monitoring used in these experiments. However, the 2D plots revealed a high degree of lateral dispersion in the ROCK inhibited cells suggestive of individualistic cell migratory mechanism (Figure 7.2B). These results indicated that the intercellular adhesion could be affected, but the haphazard nature of cell track patterns confirmed the disruptive effect on the underlying motility mechanisms.

7.2.1 Cell Migration Parameters

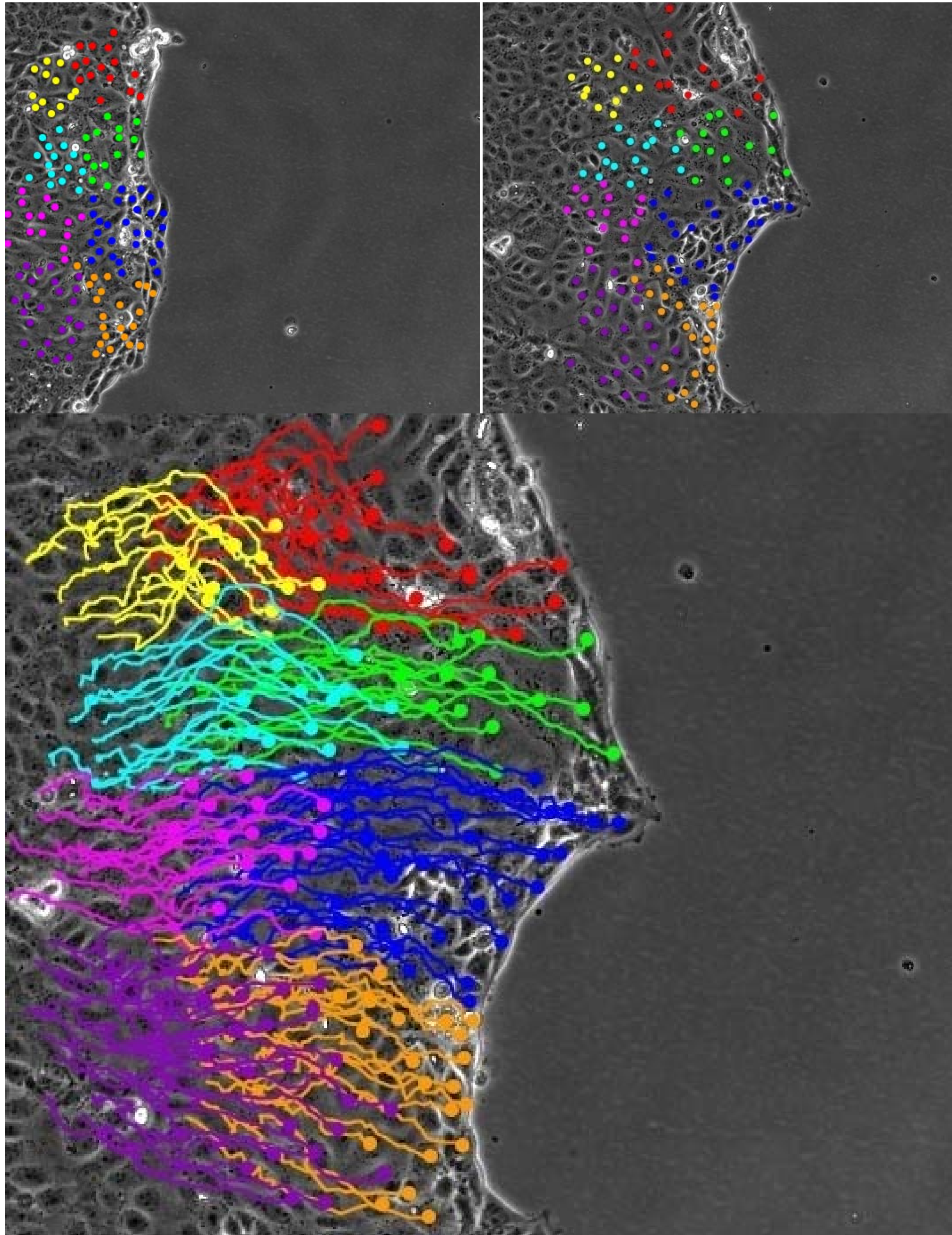
Quantitative analysis of cell track data showed significant decrease in cell migration distance (AD) as well as cell migration speed (Figure 7.3 A and D) in the Y27632 treated cells. However the Euclidean distance (Figure 7.3 B) remained unchanged. This was unexpected, since track patterns were drastically altered and so we performed statistical analysis of the maximum ED achieved by the cells during the 8 hour duration. This ED max (Figure 7.3 C) significantly reduced in the ROCK inhibitor treated cells. However, according to the collective migration track patterns, a significant difference in the mean ED was expected in the results. This discrepancy in ED can be explained as follows. The disruption of intracellular contractility, which caused the cells to lose directional migration, resulted in highly tortuous paths. But

duration of monitoring was not enough to introduce sufficient difference in the monolayer growth between the tested conditions. Therefore, mean ED's which are less sensitive since long time periods are required to increase their values, failed to reflect the difference. ED is the mathematical displacement between the initial and final position of the cells. ED mean is the average of the EDs of all the different positions of the cells, from its starting position, while the EDmax is the ED of the farthest position reached by the cell during the entire time lapse recording. Since the duration monitored was short, the maximum EDs were significantly different between the tested conditions, than the mean EDs, because control cells retained directional quality and both the types of ED's were possibly closer in values to the AD's. Therefore, averaging the EDs produce no difference while recording the highest value achieved (EDmax) reproduced the existing difference. This was confirmed by the significant changes in the CR ratios measured.

7.2.2 Confinement Ratios and Migrational Angles of Deviation

Due to the short time period of monitoring used for these experiments, mean EDs were not sensitive to the difference in the migratory behaviour. But CRs are more sensitive indicators since they are ratios of the distances, and showed statistically significant differences between the control and the ROCK inhibitor treated conditions. A reduction in CR of the ROCK inhibited HCE-T cells (Figure 7.4) indicates the loss of straightness of cell track paths and hence directional migration, which is consistent with the findings of track patterns. The migratory angles of deviations (Figure 7.5) were in the higher range for the ROCK inhibitor treated cells, with a significant increase in mean migratory angle of deviation. This suggests that ROCK inhibitor

mediated disruption of intracellular contractility leads to loss of directionality in epithelial cells.



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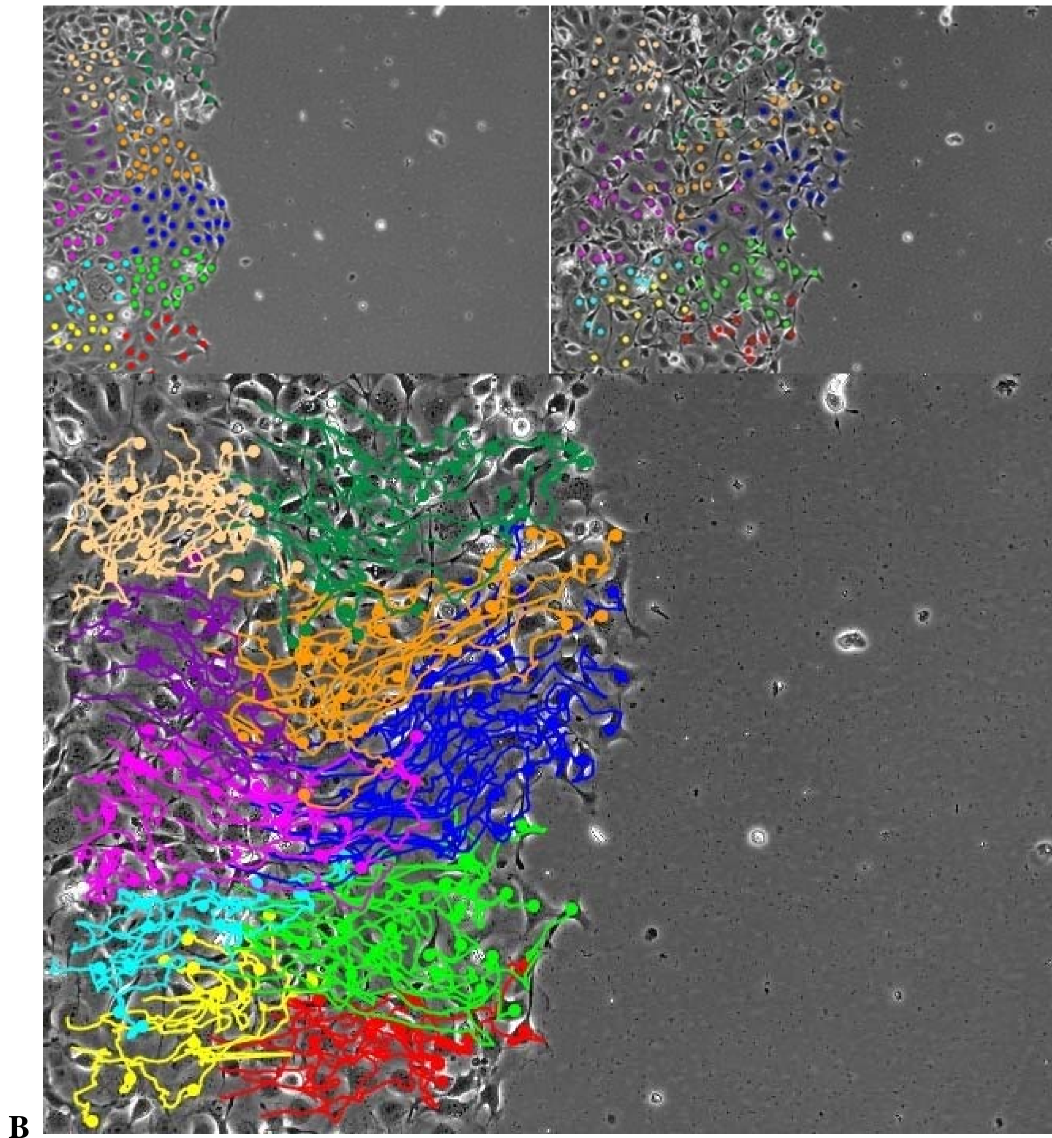

Figure 7.1 Collective Cell Migration Analysis of control (A) and ROCK inhibitor Y27632 (B) treated scrambled shRNA HCE-T cells. Top: initial and final frames with overlapped cell track endpoints. Bottom: Track overlay. Note the lateral dispersion in the drug treated cells evident by the loss of clustering.

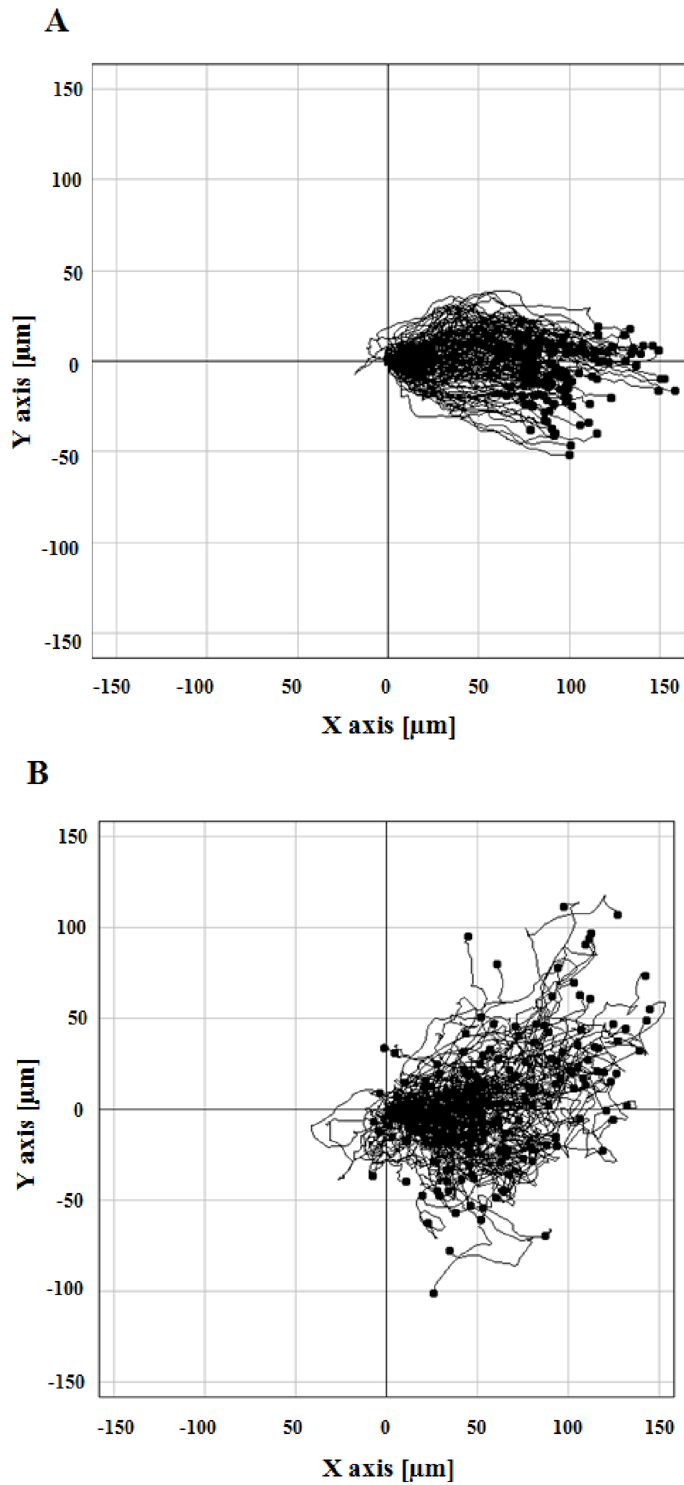

Figure 7.2 2D plots of collective cell migration in control (A) and Y27632 treated (B) scrambled shRNA HCE-T cells. The starting points of the cell tracks are shifted to the origin.

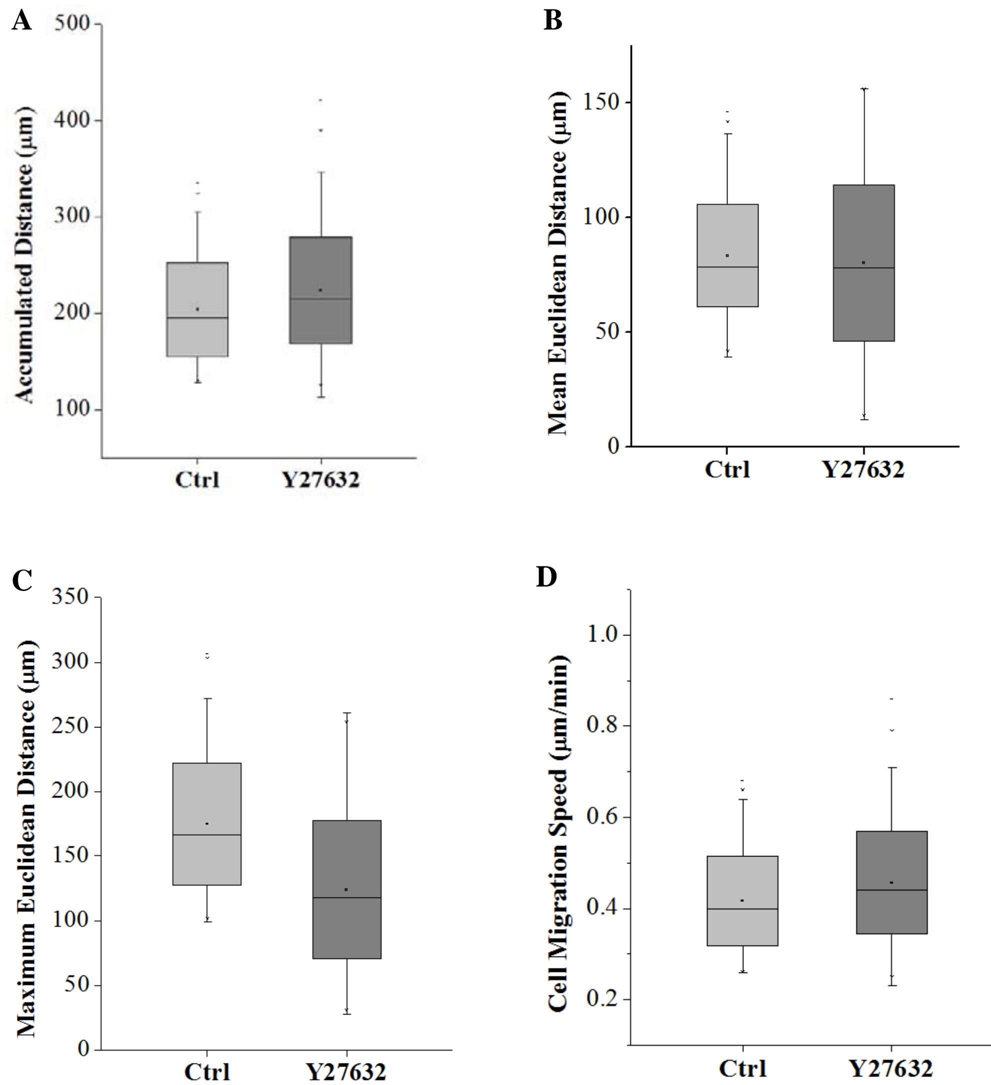

Figure 7.3 Cell Migration Parameters. Box edges represent standard deviation; the small square within the box represents the mean and the divider the 50% value. Outliers are represented by x.

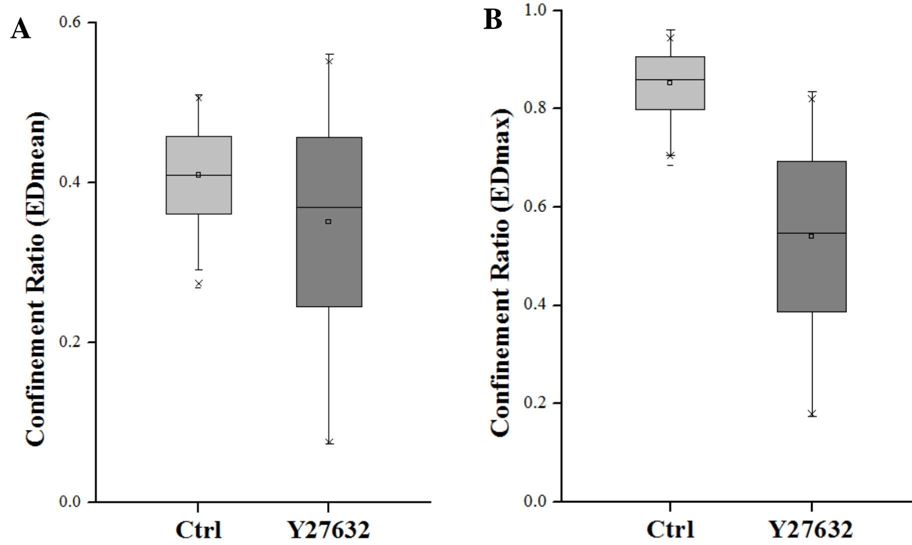

Figure 7.4 Confinement Ratios. (A) CR-EDmean and (B) CR-EDmax calculated from the respective ED measures. EDmax reveals the difference more than EDmeans. (See text for explanation). Box edges represent standard deviation; the small square within the box represents the mean and the divider the 50% value. Outliers are represented by x.

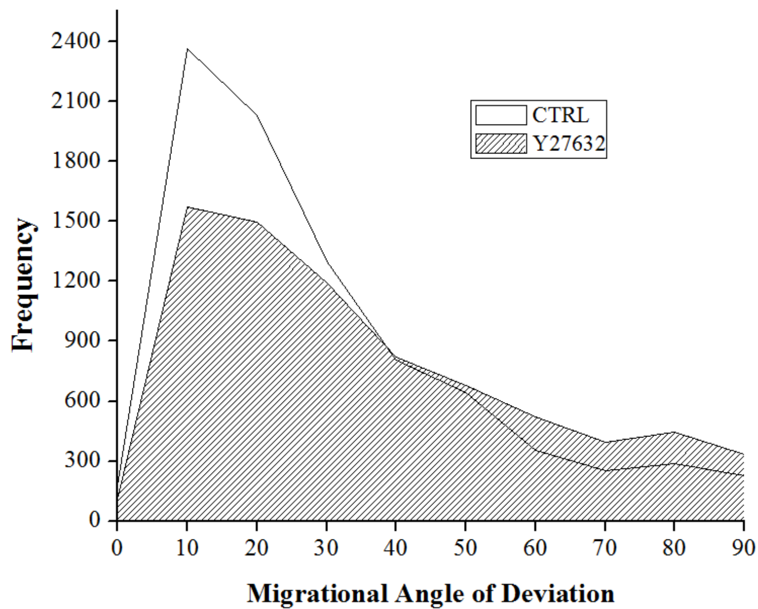

Figure 7.5 Migrational angle of deviation in control and Y27632 treated HCE-T cells. A statistically significant (p=0.05) redistribution of cells with higher angles of deviation was noticed in the ROCK inhibited cells.

7.3 Individual Motility Analysis of HCEC Cells

Kymographic analysis was performed as described in the previous chapter. These analyses proved to be particularly useful in these experiments because the short duration of monitoring for collective migration affected the sensitivity of the cell migratory parameters. FIGURE 54 shows representative kymographs of the control and Y27632 treated HCE-T cells. The control HCE-T cells (Figure 7.6 A) show a steady progression of lamellipodial protrusions with prominent and long lasting retraction phases appearing at the edge of the lamellipods. The movement of the lamellar region of the cell is however constantly in the forward direction even during the retraction phases, thereby showing that RPs do not affect cell movement but are indicators of intercellular adhesion. The Y27632 treated cells have protrusion dynamics similar to those observed in, but not as prominent as, the MCF-7 cells, with thin regions in the cell body representing the lateral movement of cells from the selected pixel wide region of interest. These thin regions occur only once in a kymograph essentially differentiating their altered motility from that of the MCF-7 cells. The essential difference was a sustained change in the directions of the cells instead of the tortuous and alternating pattern of the MCF-7 cells. The retraction phases in the lamellipodial protrusions are also infrequent and irregular compared to the control cells.

We have subsequently performed the measurements of lamellipodial protrusion parameters described in Chapters 3 and 6. The results of this analysis are as follows.

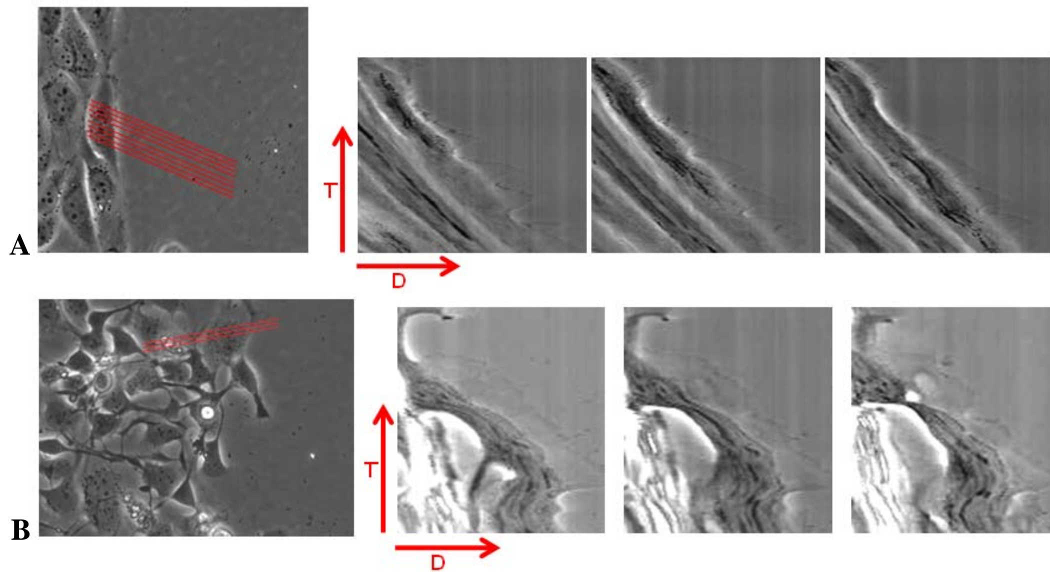

Figure 7.6 Kymography of Lamellipod Protrusion (A) HCE-T Control cells and (B) Y27632 treated HCE-Tcells. Note the regular and frequent retractions in lamellipod formations in the control cells in contrast to the ROCK inhibited cells. The latter also demonstrate total movement of cells in the lateral direction reflective of disrupted directional and collective migration.

7.3.1 Lamellipod Protrusion Parameters

The differences in all the lamellipod protrusion parameters between the control and the Y27632 treated cells were statistically significant (p=0.05) (Figure 7.7). The LPS's were higher for the Y27632 treated cells while the rest of the parameters including RPL, LES and LPT were significantly lower than the control cells. All these results correlate with the cell migration speeds reported by quantitative cell track analysis. As shown in the previous chapter, the LPS is a good indicator of directional cell motility. An increase in LPS here in the context of reduced LPT indicates spurting movements, evidently due to altered intracellular motility. An apparent puzzle was the increase in LPS and decrease in RPL alongside the severe loss of directionality in the Y27632 cells. However, a significant reduction in RPL reveals absence of cell interactions in the form of intercellular adhesion, further facilitating the speed of the cells during the shorter time periods (spurting movements) and thus

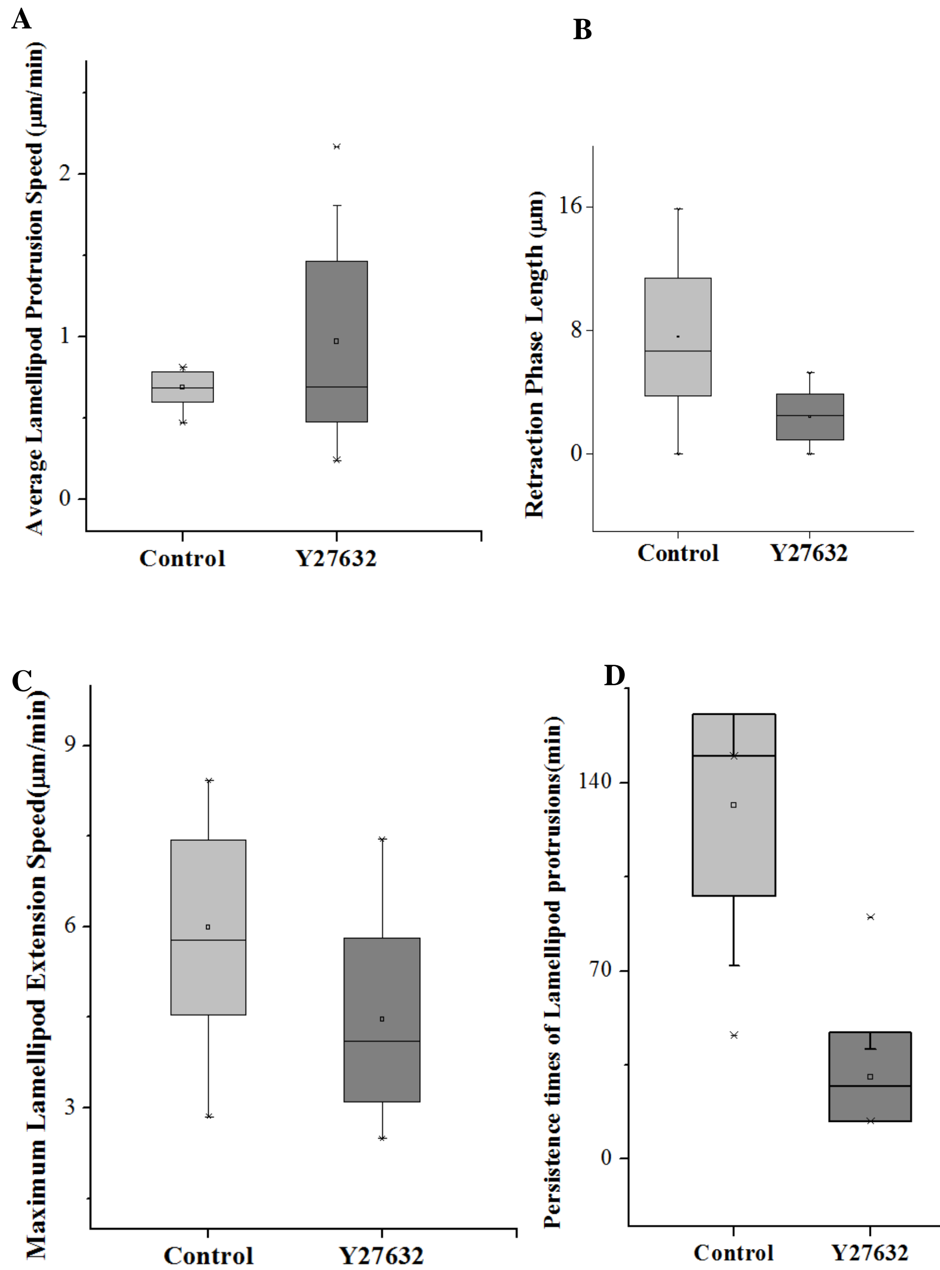

Figure 7.7 Lamellipod Protrusion Parameters in control and Y27632 treated HCE-T cells. Box edges represent standard deviation; the small square within the box represents the mean and the divider the 50% value. Outliers are represented by x.

explaining the concomitant results of high LPS and low directionality as revealed by CR and migrational angle of deviation. The lamellipod polarization was affected in the Y27632 cells as evidenced by the frequent change in directions, but simultaneous multiple locations of lamellipod formation was not observed as seen in the MCF-7 cells. These results show that Y27632 inhibited cells show mixed behavior of the breast cancer cell variants. This is because lamellipodial polarization is just one factor along with others affected in this study such as intracellular contractility. It is impossible to isolate the effect of one component alone, but nevertheless, these results do show that lamellipodial polarizations are necessary for directional migration of cells. Furthermore, once again lamellipod protrusion parameters through kymographic analyses were shown to be useful in interpreting and supplementing the results obtained from collective migration analysis of cell tracks.

7.3.2 Ruffle Thickness

Ruffle thickness (Figure 7.8) was significantly reduced in the Rock inhibitor treated HCE-T cells when compared with the control condition. This was consistent with the lamellipod protrusion parameters which showed increase in the lamellipodial formation and speed in general. As mentioned before, ruffle thickness is an indicator of decreased lamellipod formation and adhesion leading to lesser migratory rates. Thus, a decrease in RT was a facilitatory factor in the higher cell migration speed of the ROCK inhibited cells.

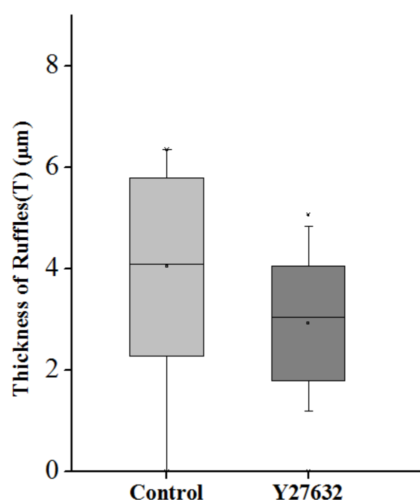

Figure 7.8 Ruffle Thickness. Box edges represent standard deviation; the small square within the box represents the mean and the divider the 50% value. Outliers are represented by x.

7.4 Discussion

7.4.1 ROCK Inhibitor Disrupts Collective Migrational Pattern in HCE-T cells

ROCK inhibitors have been shown to increase cell migration and affect adhesion in trabecular meshwork cells (Koga et al. 2006) and in colon cancers (Adachi et al. 2011). However, their effect on collective migration behaviour has not been elucidated. Our study not only reports the effect of ROCK inhibitor on collective cell migration, but also the details of the underlying mechanisms that are responsible. The collective migration analysis revealed increased migration speeds and total migration distances traversed by the HCE-T cells treated with the ROCK inhibitor, Y27632. The increase in these parameters was supplemented by the results of individual cell motility parameters. In particular, a significant increase of LPS and a decrease in RT was noted in the ROCK inhibitor treated HCE-T cells which was consistent with the increased cell migration speeds. These results suggest increased motility in the ROCK inhibited cells. Furthermore, the decrease in RPL revealed that intercellular adhesion

was affected and therefore cell movement was individualistic rather than coordinated. This was consistent with the track patterns which showed loss of directionality and a haphazard migratory behaviour indicative of disturbed collective migration in contrast to the normal HCE-T cells. These findings are also consistent with studies reported in research.

Thus, the above results effectively show that collective migration of HCE-T cells are disrupted by inhibition of the Rho kinase. The mechanism of action of ROCK inhibition for this effect involves increased cell contractility as well as affected intercellular adhesion.

7.4.2 ROCK Inhibitor Affects Lamellipodial Polarisation in HCE-T

The effect of Rho-kinase inhibitor extends not only on cell contractility, but also affects lamellipodial morphology as revealed by our analysis. Here we report the specific findings that strongly suggest this effect. Although the cell migration speed was increased as reflected by collective migration analysis as well as individual cell motility parameters, the confinement ratios and migratory angles of deviation recorded a decrease in directionality of the ROCK inhibitor treated cells. This indicates increased motility in the ROCK inhibited HCE-T cells with loss of directionality. These results were corroborated by the concomitant increase in LPS and a decrease in LPT.

As described in Chapter 6, LPT signifies directionality of individual cells and a decreased LPT in migrating cells can only mean frequent changes in directions of individual cells. Also 2D plots of cell migration and kymographic analysis showed an increase in lateral cell displacements. These results together point to the disruption of

the normal lamellipodial polarization, observed in the control HCE-T cells, as the underlying mechanism for the altered cell motility. Therefore, our results have proved that ROCK inhibitor disrupts the lamellipod polarization and alters the motility of the cells thereby affecting individual cell migration and thus collective migration.

7.5 Conclusions

In this chapter, we have tested the hypothesis that lamellipodial polarizations can affect the directionality of individual cells and hence the collective migratory behaviour in such cells. We have shown that collective migration of cells is affected when epithelial cells are treated with the ROCK inhibitor, Y27632. The resulting migratory track patterns were comparable to a mixture of behaviour of the cancer cell migration involving frequent multidirectional lamellipodial formation as well as increased motility of the cells. Careful analysis has revealed that this caused by the action of ROCK inhibitor on the intracellular contractility as well as lamellipodial formation. Our findings in this study suggest that ROCK is involved in regulation of the lamellipodial polarisation of epithelial cells, and inhibition of ROCK activity abolishes the directional polarisation leading to the haphazard and mesenchymal type of collective migration pattern.

Thus, by this experiment we have shown that the lamellipodial polarization is indeed required for the directional migration of epithelial cells. Also, all these results confirm the usefulness of the non-wounding migrational assay in combination with the technique of kinematic and single cell migrational analyses. In conclusion, non-wounding 2D migrational assay method combined with individual cell track and motility parameter analysis is an efficient method to study collective migration in cells. Further, this method also helps in delineating the components of individual cell

motility that might be targeted for more efficient treatment of different cancers and other diseases.

Chapter 8
Conclusions and Future Work

Study of collective migration is an important adjunct to research in cell biology and biomechanics. Migrational assays are frequently used to assess the progression and metastatic potential of cancers as well as the effects of anticancer agents. Improving the details of studying collective migration can therefore be beneficial in achieving accuracy of the reported results as well as enhance our knowledge of the collective behaviour imparted by the self propelling machinery that is characteristic of living cells.

In this thesis, we focused on the study of collective migration of breast cancer cells. During the course of the work, we improved the current 2D migrational assay methods by modifying the ring assay technique. We also used time lapse phase contrast microscopy to observe the kinematics of the collective cell migration and developed single cell migration parameters to aid in the analysis of collective cell migration patterns. The following sections summarise the efforts of the study along with notes on some interesting findings and future work.

8.1 Conclusion

Effectively, a modified migration assay technique involving the use of glass cylinder barriers has been used in this work to achieve an intact monolayer of cells in the study of collective cell migration. To study the accuracy of the method in reporting migration in typical research scenarios, it was performed for normal epithelial cells as well as cancer cells conditioned with knock-down of different proteins. The results from the method corroborated with those obtained by other established and commonly used methods. Particularly, the proteins DP103 and TG-2 were shown to be necessary

for cell migration in breast cancer cells and normal corneal epithelial cells, respectively. In this way it was shown that this technique is reliable in the study of cell migration.

To further improve the informative capabilities of migrational assays, analytical method involving parameters for single cell motility mechanisms was developed. This method was used to correlate with the results obtained for collective migration by individual cell tracking, and successfully explain the development of collective cell migratory patterns that were observed. In combination with the results from the tracking method of collective migration analysis, single cell analysis increased details and accuracy of the final reports.

Kinematic study of collective cell migration in breast epithelial derivatives revealed the nature of individual interacting units and the effect of intercellular adhesion in regulating the development of patterns. The migratory patterns in MCF-7 cells were zig-zag fashion and most of energy was lost in unnecessary cell body movements. The individual cell track patterns of MCF-10A and MDA-MB-231 cells were smooth and had the quality of directional persistence. The clustering of cell tracks in MCF-10A cells revealed the role of strong intercellular adhesion in the development of the organized collective migratory patterns. In contrast, the collective migratory pattern of MDA-MB-231 cells had spaghetti-like appearance due to affected intercellular adhesion and contact inhibition, reflecting the swarming movement that is reminiscent of mesenchymal migration.

Single cell migration analysis has shown that individual characteristics like lamellipodial polarisation dynamics and intercellular adhesion can explain the collective pattern of cancer cells. The defective lamellipodial polarisation in MCF-7

cells was shown to reduce the efficiency of cell migration by affecting its directional persistence. The uniform lamellipodial polarisation of MDA-MB-231 cells and MCF-10A cells allowed for efficient cell migration, although the decreased intercellular adhesion in the former proved to be beneficial in increasing their individual speed as well as overall migration distance covered.

The results of this study have provided more insights into cancer cell collective cell migration in several respects. For instance, considerable concerns had been expressed in literature regarding mesenchymal status of cancer cells. Here, it is shown that the highly invasive cancer cells indeed exhibit individualistic migratory behavior due to altered intercellular adhesion and contact inhibition of locomotion. Typically, location of adjacent cells had no effect on the migration of highly invasive cancer cells. Although, the same effect was observed in the MCF-7 cell lines, the underlying cause was their defective motility on 2D surfaces. Furthermore, 2D migration can reveal defects/altered motility that might not be appreciated in a 3D environment. For example, MCF-7 has defective but increased contractility (since its overall migratory distance, but not the displacement, was similar to that of the benign cells), which may be beneficial in the 3D situation.

Also, disruption of components of single cell migration can affect collective migration of cells as evidenced in normal epithelial cell lines. In normal corneal epithelial cells it was observed that the ROCK inhibitor Y27632 disrupted the lamellipodial polarisation in addition to contractility of the cells and thus affected collective cell migration patterns which resembled a mixture of the collective migratory behaviour of the two cancer cell lines. Moreover, it was demonstrated for the first time using

kymographic analysis and individual cell motility parameters, that ROCK inhibition affects lamellipodial polarization.

8.2 Disadvantages of the methodology

In this subsection, we summarize some of the advantages as well as hurdles and drawbacks encountered in this work. The method followed in this study yields more qualitative as well as quantitative information than the usually performed scratch assays. An accurate assessment of behavior of cells is possible at both gross and individual levels of cell migration. Also, measuring the actual migration of individual cells is more informative about the migratory behavior of the cells than monolayer edge distance. Furthermore, the migrational angle of deviation is much more reliable than the usual index of confinement ratio, while studying the efficiency of migration.

However, a major hurdle in terms of methodology is the manual tracking method which is tedious and impractical for large data. Automated tracking can compensate this problem, but needs concessions such as luminescent protein tagging of nuclei. This will still be a challenging task for cell lines like MDA-MB-231 which exhibits mesenchymal mode of migration with frequent overlapping of track paths. Semi-automated (interactive) tracking (Zicha et al. 1998) will augment the method but again limit the throughput.

Another important concern is related to the individual cell analysis. It is mostly limited to 2D while most of physiological migration occurs in a 3D environment. 2D migrational study will still be useful since the components of the motility machinery remains the same whether in 2D or 3D. Furthermore, some of the parameters may be inaccurate for cells with defective motility mechanisms. For example, higher

retraction phases in MCF-7 do not reflect the strong intercellular adhesion, as in other epithelial cells, but rather a defect in polarized focal adhesion and lamellipodial formation and the resulting abnormal contractility.

Mitotic agents/suppressors, which will have shed more information on the effect of cell proliferation on the migratory patterns, were not used in this work. Of concern was that mitotic inhibitors alter physiological condition (native states) of the cells which would not be desired for the motive of this study. However, more importantly, it has been argued that the growing cell monolayers exist in a global state of tensile stress and thus their expansion is not influenced by cell proliferation (Trepat et al. 2009). Also, since the doubling times were similar for these three cell lines and experiments were conducted post 24 hrs of seeding for an additional 24 hrs, any effect would be similar across the cancer cell lines studied. Finally, since individual cells were tracked, this factor (cell population pressure) alone cannot affect the conclusions of this work. Therefore, although this is not a critical disadvantage, inclusion of this part could be considered to have provided adjunct information and can be open for future work.

8.3 Interesting Findings and Speculations

Several observations were made with the monitored videos that deserve mention and perhaps further detailed inquisition. These were not followed because it was not in the primary goals of the study, and might be separate projects on their own, but nevertheless are very interesting. The first of these is the density of the cell seeding. When sparse cell patches resulted during some failed sample preparation, we went ahead and performed the monitoring in the hope of learning something. What we have noticed is that certain patches of cells of about 50-200 cells will not show migration at

the edge, even though the cells show broad morphologies. This leads us to speculate that there might be a critical size of the cell monolayer when the cumulative intracellular motility of all the peripheral cells taken together breaks the binding force of intercellular adhesion and other factors, resulting in outward movement of the cells. This is a tricky hypothesis to prove, but if dealt with, can show the importance of the critical factors that are responsible for individual and collective cell migration.

Another observation refers to the increased motility but yet decreased migration of the MCF-7 cells. We would speculate that it is likely that if MCF-7 cells are confined to 1D movement their migration could be significantly benefited by the dimensionally restricted intracellular contractility. It is easy to visualize that instead of formation of multiple focal adhesion sites, if focal adhesions are restricted to form at only two ends of the MCF-7cells, the directionality and efficiency will improve especially in a chemotactic environment. It might even explain why MCF-7 cells, although considered non-invasive, were still obtained from metastatic pleural effusions.

Finally, we have also noticed that cells observed after 72 hours of seeding are comparably slower than those seeded after 48 hours. It is natural that post 72 hours the diameter of the cell monolayer is larger than that observed post 24hours of seeding. But considering the inner diameter of the cylinder being 6 mm, the difference between the cells at the periphery adds to 4um on each side of the cells, which is not much for well spread cells measuring 30-40um across. This increased speed in migration could be the result of several factors, but increased cell contractility during the initial several hours after attachment could be important.

8.3 Future Work

Several questions have surfaced during the execution of the work in this thesis. It would definitely be useful if these are answered by further research. Also, many of these are fundamental questions that have been difficult to prove due to limitations inherent to the current facilities. A brief list of some of these directions will be mentioned here.

1. Effect of cell population on collective migrational behaviour: Does the effect of migration only in the outer cells exist? Or does this affect only cells with strong intercellular adhesion. Since different number of cells had to be seeded in our experiments due to size restrictions of the various cell lines, it was not possible to estimate the effect of population pressure. Further, as mentioned in the above paragraphs, cell migration was restricted in the confluent centers.
2. Effect of disruption of intercellular adhesion in isolation, on collective cell migration. As described in our analysis, intercellular adhesion and lamellipod polarization form crucial factors that dictate collective cell migration. We have shown in this work that lamellipod polarization is indeed a key factor. It would be interesting to observe the collective migratory patterns in cell lines with disrupted intercellular adhesion.
3. Effect of 3D environment on MCF-7 cell migration. The results in this work has proved that the defects in lamellipod polarization in MCF-7 contributed to their abnormal and inefficient movement. However, it has also been suggested that these defects are possible where multiple focal adhesion patches are possible for these cells. In directionally restricted environments such as 3D, the guidance provided by the fibres might actually enhance the migratory

potential of these cells. Thus, patterned microsurfaces to study migration of MCF-7 cells and other slower cells, might reveal the effect of topology and the relieving of lamellipodial polarisation dynamics in the 3D environment, particularly in the collective migration of cancer cells.

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Appendix A. Differential Expression of Proteins in Breast Epithelial Cell Derivatives

Table 1. List of Identified Differentially Expressed Proteins, affecting the cytoskeleton, intercellular interaction and cell motility, across MCF-10A, MCF-7 and MDA-MB-231 Breast Cells Obtained after 2D-DIGE Coupled with MALDI-TOF Mass Spectrometry Analysis. Table modified and reproduced from (Lai et al. 2010). S=secreted, T=Total Cellular Proteins

Accession Code	Protein Name	Type of Protein	Subcellular Location (a)	Functional Class (a)	MCF-7/ MCF-10A (b)	MDA231/ MCF-10A (b)	MDA231/ MCF-7 (b)
P60709	Actin	**S**	Cytoplasm	Cytoskeleton	**2.57**	1.17	-2.14
P60709	Actin	**S**	Cytoplasm	Cytoskeleton	**2.21**	-1.04	-2.25
Q9C0G6	Dynein heavy chain 6	**S**	Cytoplasm	Cytoskeleton	**5.07**	2.87	-1.71
O75083	WD repeat-containing protein 1/Actin-interacting protein 1	**S**	Cytoplasm	Cytoskeleton	-1.26	1.76	**2.3**
P35240	**Merlin/Neurofibromin-2**	**S**	Plasma membrane	Cell motility/ signal transduction	**4.77**	1.54	-3.03
P26038	**Moesin**	**S**	Plasma membrane	Cell motility/ cytoskeleton	-3	1.95	**6.04**
P23528	Cofilin-1	**T**	Cytoplasm	Cell motility/ Ca regulation	**1.33**	-3.8	-5.06
P20472	Parvalbumin alpha	**T**	Nucleus	Cell motility/ Ca regulation	**16.77**	10.64	-1.52
O00151	PDZ and LIM domain protein 1/LIM domain protein CLP-36/Elfin/CLP36	**T**	Cytoplasm	Cell motility/ Ca regulation	**-3.96**	**-1.46**	**2.8**
P67936	Tropomyosin alpha-4 chain/Tropomyosin-4	**T**	Cytoplasm	Cell motility/ Ca regulation	-1.72	1.38	**2.46**
P47756	**F-actin-capping protein subunit beta**	**T**	Cytoskeleton	Cell motility	**1.53**	-1.38	-2.1
P47756	**F-actin-capping protein subunit beta**	**T**	Cytoskeleton	Cell motility	**1.2**	-1.81	-2.18
P60660	**Myosin light polypeptide 6**	**T**	Cytoplasm	Cell motility	**5.49**	-1.79	-9.48
O14950	**Myosin regulatory light chain MRLC2**	**T**	Cytoplasm	Cell motility	**1.84**	1.8	**1.16**

P19105	**Myosin regulatory light chain MRLC3**	**T**	Cytoplasm	Cell motility	**-3.9**	**-1.93**	**2.09**
P30084	**Myosin-IXa**	**T**	Cytoplasm	Cell motility	**-1.03**	**-2.08**	-1.95
P07737	**Profilin-1**	**T**	Cytoplasm	Cell motility	-1.35	1.2	**1.62**
P07737	**Profilin-1**	**T**	Cytoplasm	Cell motility	-1.29	1.16	**1.5**
P42331	**Rho GTPase-activating protein 25**	**T**	Cell Membrane	Cell motility	**1.54**	1.11	-1.17
Q13017	**Rho GTPase-activating protein 5/p190-B/ARHGAP5/RhoGAP 5**	**T**	Cell Membrane	Cell motility	**1.93**	1.31	-1.47
O75116	**Rho-associated protein kinase 2/ROCK2**	**T**	Cytoplasm	Cell motility	**5.16**	-1.56	-8.07
O75116	**Rho-associated protein kinase 2/ROCK2**	**T**	Cytoplasm	Cell motility	**8.04**	-2.56	-20.57
P26038	**Moesin**	**T**	Cell Membrane	Cytoskeleton	-2.33	3.51	**8.46**
Q15691	Microtubule-associated protein RP/EB family member 1 (End-binding protein 1)	**T**	Cytoplasm	Cytoskeleton	-1.36	1.44	1.97
P13797	Plastin-3/T plastin	**T**	Cytoplasm	Cytoskeleton	**-3.88**	**-1.67**	2.32
P19012	Cytokeratin 15	**T**	Cytoplasm	Cytoskeleton	**-6.26**	**-11.21**	-1.73
P05787	Cytokeratin 8	**T**	Cytoplasm	Cytoskeleton	17.75	1.67	-10.66
Q04695	Cytokeratin 1	**T**	Cytoplasm	Cytoskeleton	1.54	1.34	-1.15
P08670	Vimentin	**T**	Cytoplasm	Cytoskeleton	-3.55	4.14	15.21
P08670	Vimentin	**T**	Cytoplasm	Cytoskeleton	-3.77	2.65	10.32
P09382	Galectin-1	**T**	Cytoplasm	Cell-cell interaction	5.99	-1.18	-6.83
Q14993	Collagen alpha-1(XIX) chain/Collagen alpha-1	**T**	Secreted	Cell-cell interaction	3.23	3.84	1.23

Table 2. Differentially Expressed Proteins Identified by Representational Difference Analysis (RDA). Table compiled from the reference (Nagaraja et al. 2006)

	Upregulated	**Downregulated**
MCF-7/MCF-10A	keratin 19	caldesmon
	serine protease	annexin A1
	amyloid β precursor protein	epithelial membrane protein 1
	neuropeptide Y	S100A2
	receptor Y1	keratin 15
MDA-MB-231/MCF-10A	vimentin	keratin 15
	epithelial membrane protein 3	cystatin A
	cadherin 11	cadherin 1
	GPCR 116	CD24
	collagen type XIII α1	calcium-activated chloride channel
	Bcl2-associated athanogene 2	S100P
		GPCR 87
MDA-MB-231/MCF-7	GPCR11	GREB1
	cadherin 11	S100A8
	annexin A1	amyloid β precursor protein
	vimentin	claudin 3
	lactate dehydrogenase B	cadherin 1

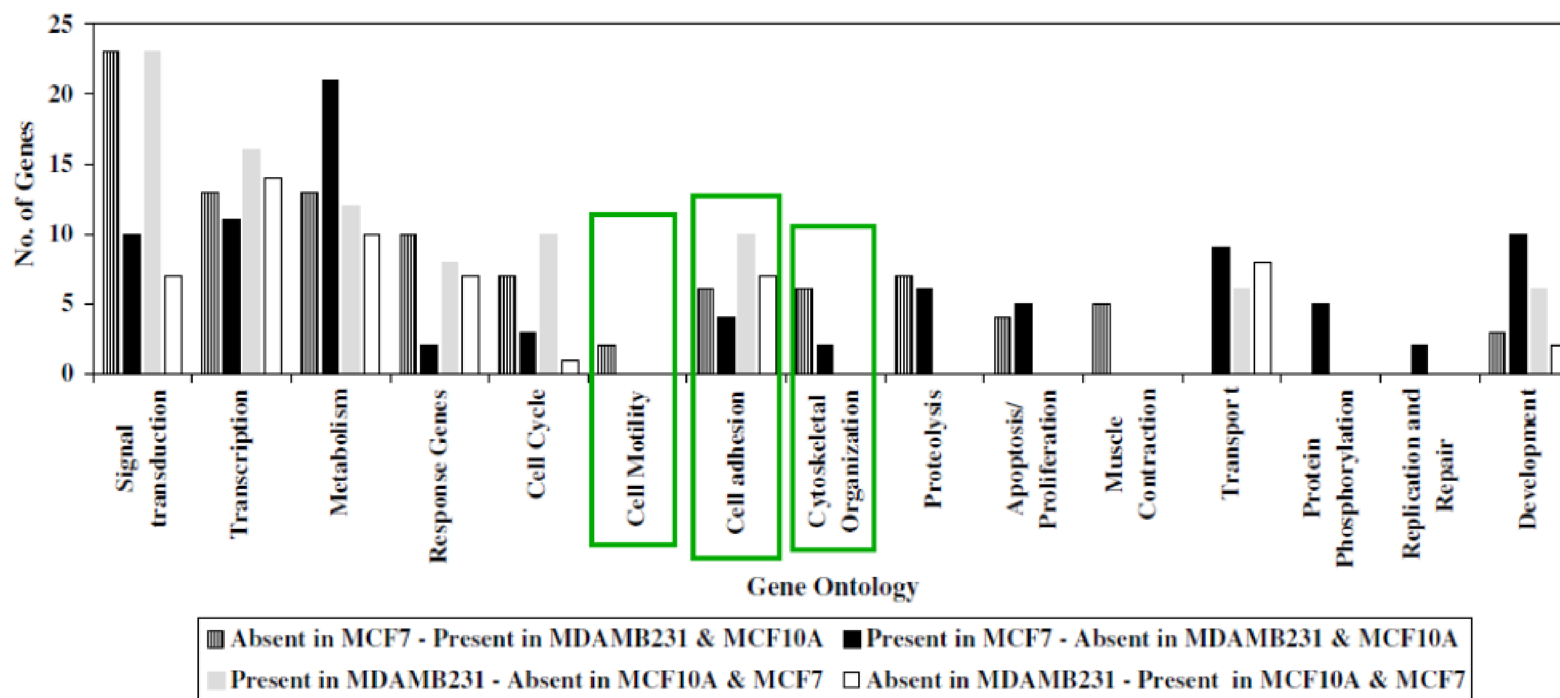

Figure 8.1 Funtional classes of transcripts that differentiate cell line pairs. The transcripts identified as present or absent were classified based on their functional importance. Green rectangles highlight the proteins related to motility, adhesion and cytoskeletal organization. (Figure and legend modified and reproduced from (Nagaraja et al. 2006))