The role of atypical protein kinase C iota in cancer invasion

by

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ABSTRACT

Breast cancer is the most frequently diagnosed and the second leading cause of cancer deaths in Canadian women. The most morbid and deadly feature of the disease is the emergence of metastases. Breast cancer progression is a consequence of disruption of normal cell organization, polarity, and cell-cell adhesion, which allow cells to become more motile and invasive. Atypical protein kinase C iota (aPKCἰ) functions as an oncogene and is found to be elevated in lung adenocarcinoma, chronic myelogenous leukaemia, non-small cell lung cancer cell lines, ovarian, pancreatic, colon and breast cancers. In addition, several studies demonstrate an important role for aPKCι in promoting tumour cell invasion and the subsequent formation of metastases in a number of human cancers. Also, aPKCι was suggested to be involved in the extracellular matrix degradation in breast cancer. However, the exact mechanisms by which aPKCι promotes breast cancer cell invasion remain to be elucidated.

The purpose of this thesis is to establish the role of aPKCἰ in breast cancer invasion and metastasis. To achieve this, a 3D collagen *in vitro* assay was chosen to examine the effect of aPKCἰ overexpression on breast cancer cell invasiveness, morphology, the formation of cellular protrusions. I also investigated the potential molecular mechanisms of aPKCι involvement in breast cancer cell invasion.

The work in this thesis demonstrates that the aPKCἰ overexpressing cells are capable of invading further distances into the surrounding collagen matrix and possessed higher speed than the control cell line. Furthermore, these cells changed morphology, as characterized by the formation of multiple protrusions. Additionally, the number of cellular protrusions was found to correlate with the cell invasion velocity. Finally, a study of the the molecular pathway underlying the role of aPKCἰ in breast cancer cell invasion was initiated.

RÉSUMÉ

Le cancer du sein est le cancer le plus fréquemment diagnostiqué et la deuxième cause de décès du cancer chez les femmes canadiennes. La caractéristique la plus morbide et mortelle de la maladie est la formation des métastases. La progression du cancer du sein est une conséquence de la perturbation de l'organisation cellulaire normale, de la polarité et de l'adhérence cellule à cellule, ce qui permet aux cellules d'être plus mobiles et invasives. La protéine kinase C atypique iota (aPKCἰ) agit comme un oncogène et se trouve en taux élevé dans l'adénocarcinome du poumon, la leucémie myéloïde chronique, les lignées cellulaires de cancer bronchique non à petites cellules, et les cancers de l'ovaire, du pancréas, du côlon et du sein. Également, plusieurs études ont démontré que aPKCι joue un rôle important dans la promotion de l’invasion des cellules tumorales et la formation subséquente des métastases dans un certain nombre de cancers humains. En outre, il a été suggéré que aPKCι est impliqué dans la dégradation de la matrice extra-cellulaire dans le contexte de cancer du sein. Cependant, il reste à élucider le mécanisme exact par lequel aPKCι favorise l'invasion des cellules cancéreuses.

L'objectif de cette thèse est d'établir le rôle de aPKCι dans le cancer du sein en termes d'invasion de cellules tumorales et de leur métastase. Pour ce faire, j’ai choisi un essai 3D en collagène *in vitro* pour étudier l'effet de la surexpression aPKCι sur l’invasion des cellules du cancer du sein, leur morphologie, et la formation de protrusions cellulaires. J'ai également étudié les mécanismes moléculaires potentiels de la participation de aPKCι dans l'invasion de ces mêmes cellules.

Le travail dans cette thèse démontre que les cellules surexprimant aPKCι sont capable d'envahir des distances plus grandes dans la matrice de collagène entourante et possèdent une vitesse moyenne plus élevée que celle de la lignée de contrôle. En outre, ces cellules ont modifié leur morphologie, qui est maintenant caractérisée par la formation de plusieurs protrusions. De

plus, le nombre de protrusions cellulaires est en corrélation avec la vitesse de l'invasion cellulaire. Enfin, une étude de la voie moléculaire qui sous-tend le rôle de la aPKCι dans l'invasion des cellules du cancer du sein a été lancée.

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PREFACE AND CONTRIBUTIONS

This is a traditional thesis, which consists of a general introduction, abstract, objectives, literature review, materials and methods, results, discussion, references and figures.

In this thesis, to evaluate the role of aPKCι in cancer cell invasion, I overexpressed a constitutively active form of aPKCι, A 129 E mutant, and followed cell invasion by employing *in vitro* 3D collagen assays. The original protocol for the collagen type I matrix recipe [1] was generously provided by Dr. Elad Katz (University of Edinburgh, Edinburgh, United Kingdom). However, all the protocols for the technical assays to study cellular invasion into the surrounding matrix have been developed and described in this thesis.

ABBREVIATIONS

ADH: atypical ductal hyperplasia

AJ: adherens junctions

aPKCs: atypical PKCs

ATG: aurothioglucose

ATM: aurothiomalate

CTCs: circulating tumour cells

DAG: diacylglycerol

DCIS: ductal carcinoma in situ

DIC: differential interference contrast

DMEM: Dulbecco's Modified Eagle Medium

2D: two-dimensional

3D: three-dimensional

ECM: Extracellular matrix

EGF: epidermal growth factor

EMT: Epithelial to mesenchymal transition

ER: estrogen receptors

ESCC: esophageal squamous cell carcinomas

FEA: flat epithelial atypia

FACS: fluorescence activated cell sorting

F-actin: filamentous actin

FBS: Fetal Bovine Serum

FGF: fibroblast growth factor

GFP: Green fluorescent protein

GTP: Guanosine triphosphatase

GJ: gap junctions

HEK293-LT: Human Embryonic Kidney 293 cells

HEPES: Hydroxyethyl)piperazine-1-ethanesulfonic acid

HER2: human epidermal growth factor receptor 2

IDC: invasive ductal carcinoma

IHC: Immunochistochemistry

ILC: invasive lobular carcinoma

IgG: immunoglobulin

JAM: junctional adhesion molecules

LOX: lysyl oxidase

M: metastases

MMP: matrix metalloproteinases

MOI: multiplicity of infection

N: lymph nodes

NSCLC: non-small cell lung cancer

NMuMG: Normal Murine Mammary Gland

NSCLC: non-small cell lung cancer

NGS: Nottingham grading system

nPKCs: novel PKCs

OCT: Optimal Cutting Temperature

PAR: partitioning-defective

PB1: phox-bem 1

PBS: phosphate buffered saline

PDK1: phosphoinositide-dependent kinase 1

PEG: polyethilene glycol

PI3K: 3-phosphoinositides

PR: progesterone receptors

PKC: Protein kinase C

RFP: red-fluorescent protein

RT: room temperature

SCC: squamous cell carcinoma

T: Tumour size

TDLUs: ductal lobular units

TGF- β: transforming growth factor beta

TJ: tight junctions

TU/ml: transduced units/millilitre

U/ml: unit/millilitre

WHO: World Health Organization

ZO: zonula occludens

µg/ml: microgramme/millilitre

ng/ml: nanogram/millilitre

µm/s: micrometres/second

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Chapter 1 Literature Review and Introduction

1.1 Breast cancer epidemiology worldwide

Cancer is a group of diseases, which can be generally characterized by an abnormal cellular growth, proliferation, and malignant tumour formation. The malignant, or cancer cells, arise from normal tissue cells that have evaded various growth control mechanisms via acquiring a series of genetic mutations. Eventually, these cells may gain an ability to leave the tissue of origin and travel to distant organs, metastases, which often represents the deadliest aspect of the cancers.

Based on the recent data from the World Health Organization (WHO), 14 million of new cancer cases were diagnosed and over 8 million of cancer-related mortalities occurred in the year 2012 worldwide, making this group of diseases the leading cause of death around the world [2]. Specifically, breast cancer was ranked the most frequent type of cancer amongst women worldwide accounting for 1.67 million of newly diagnosed cases in 2012 [2]. Additionally, breast cancer was reported to be the fifth cause of cancer-related deaths in the world and was responsible for over one half of a million of mortalities [2].

1.2 Breast cancer epidemiology in Canada

Cancer is the leading cause of deaths in Canada accounting for the estimated over 187,000 new patients and over 75,000 deaths in the year 2013 [3]. Breast cancer is the most frequently diagnosed and second leading cause of cancer-related mortalities in Canadian women. In fact, almost 24,000 of newly diagnosed cases and 5,000 breast cancer mortalities were projected for the year 2013 [3, 4]. Thus in Quebec, for the year 2013, 1,350 breast cancer mortalities were expected [5]. The incidence of breast cancer has been observed to steadily rise in Canadian women for the past two decades. However, the number of breast cancer-related mortalities has been decreasing, which can be explained by better screening techniques and improved therapeutics [6-8].

1.3 Classification of breast cancer

Breast cancer is a heterogeneous disease explaining the variability in clinical symptoms and the differential patients' responses to therapy. Hence, a number of schemes based on histolopathological appearance, immunohistochemical markers and gene expression profiles have been developed to subclassify breast cancer. The existence of such classification may serve different purposes, such as treatment selection, prognosis and outcome prediction.

1.3.1 Histopathological classification of breast cancer

Histolopathological classification is based on analysing the morphology of the malignant cells within the mammary tumours using light microscopy. Currently, the WHO recognizes 21 histopathological types of invasive breast carcinomas [9]. The two predominant types are the invasive ductal carcinoma (IDC), which accounts for 50%-80% of breast cancers, and the invasive lobular carcinoma (ILC). Amongst other categories are the inflammatory, metaplastic, tubular apocrine and other histopathological types of mammary tumours [10, 11].

The Nottingham grading system (NGS) is used to classify breast cancers based on tumour grade based on the following parameters: (i) formation of tubule, (ii) nuclear pleomorphism and (iii) mitotic rate [12]. Evaluation of each of the three parameters is translated into a numerical value from 1 to 3 and then the cumulative score is translated into the tumour grade. Specifically, grade I represents low malignancy tumours, grade II is intermediate grade and grade III comprises high grade tumours [13].

The TNM system is used to determine the stage of breast cancers and is based on evaluating the primary tumour size (T), the involvement of the regional lymph nodes (N) and the presence of distant metastases (M). According to this system tumours are staged from 0 (*in situ* tumour) to IV (invasive cancer with distant spread) [14, 15].

1.3.2 Immunohistochemical markers in breast cancer

Immunochistochemistry (IHC) is a laboratory technique that aims at identifying the expression of specific proteins in tissues of interest. The three major molecular markers that are used in diagnosis of breast cancers are estrogen receptors (ER), progesterone receptors (PR) and human epidermal growth factor receptor 2 (HER2/neu/ErbB2) [16]. The main growth stimulus for the ER-positive tumour cells is the steroid hormone estradiol. This feature makes the ER-positive breast cancers to be suitable targets for endocrine therapies. PR is a downstream target of ER signalling pathway and hence is considered to be an indicator of an intact estrogen response pathway [17]. Finally, amplification of the *HER2* gene appears in about 13% to 20% of breast tumours [18]. Patients with tumour positive for both ER and PR are considered to have the best prognosis, whereas those with breast cancers positive for HER2 or are negative for all three markers are noted to have significantly shorter disease-free survival [19, 20].

1.3.3 Molecular subtypes of breast cancer

A more recent classification of breast tumours is based on the global gene expression profiles [21]. This method clusters mammary tumours into distinct molecular subtypes including:

luminal A and luminal B, triple-negative, normal-breast-like, and HER2 positive tumours [21, 22]. Luminal A and luminal B are the most common breast cancer subtypes and represent about 40% of mammary tumours. Both of these subtypes are characterized by positive estrogen receptor (ER) signalling, however, luminal B cancers display higher proliferation rates, increased histological grade and a significantly poorer prognosis [11]. Triple-negative is a breast cancer subtype that lacks expression of an estrogen receptor (ER), progesterone receptor (PR), and HER2 receptor, and is subdivided into basal-like and Claudin-low [23]. Basal-like subtype includes tumours that express genes normally attributed to normal basal/myoepithelial cells of the breast. The Claudin-low subtype expresses low levels of genes involved in tight junctions, cell–cell adhesion, and luminal genes [24]. In addition, these cancers are characterized by association with poor prognosis for breast cancer patients [25]. HER2-positive subtypes exemplified by breast tumours with high expression of a number of genes in the ERBB2 amplicon and have a poor prognosis [22]. Additional molecular subtype having poor prognosis has been subsequently identified as molecular apocrine [16, 17].

1.4 Breast cancer origins

Breast tumourigenesis is a complex multistep process that involves multiple aberrant alterations in the normal mammary epithelium and surrounding stroma. To date, two models of breast cancer origination have been proposed to describe tumour heterogeneity: the stochastic model and the cancer stem cell model [26, 27]. The stochastic model suggests that a tumours cell is a product of multiple random mutations that occur in any given cell type within the breast tissue. On the other hand, the cancer stem cell model postulates that a subpopulation of malignant cells have steam cell properties, including self-renewal and giving rise to different malignant cell types in the tumour.

However Visvader suggests that during tumour progression the different cell populations may have different plasticity and that the molecular signatures of the different tumour subtypes might not indicate the properties of the cell of origin [23]. Thus Claudin-low may originate from stem cell, basal-like from progenitor and luminal A/B subtype from differentiated cells [23]. Interestingly, studies have shown that the majority of breast tumours initiate in the terminal ductal lobular units (TDLUs) within the breast [28]. These cells further go through stages of abnormal cellular proliferation, atypia, atypical hyperplasia to a pre-invasive carcinoma *in situ* [29]. Thus breast cancer forms via cell progression from flat epithelial atypia (FEA), atypical ductal hyperplasia (ADH), to malignant ductal carcinoma in situ (DCIS) and to invasive ductal carcinoma (IDC) [30].(Figure 1.1)

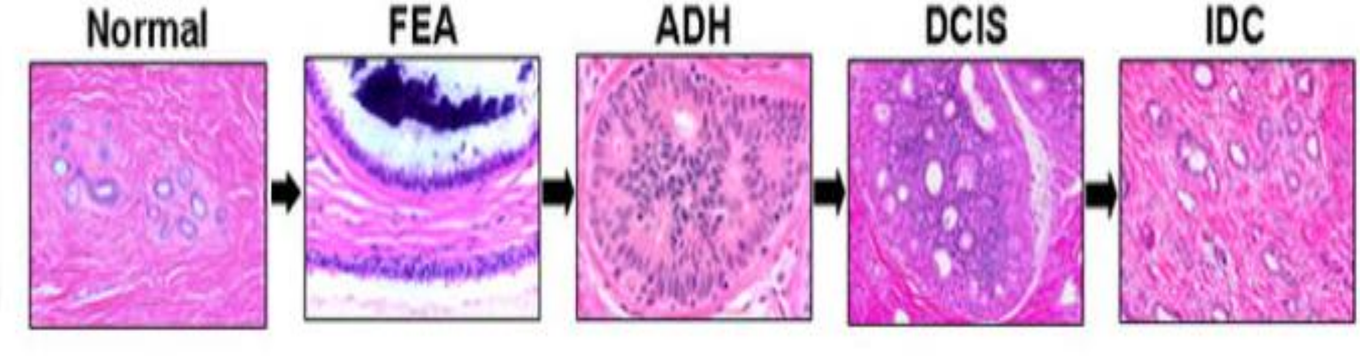


Figure 1. 1 Breast cancer progression model

The classic model of breast cancer progression of the ductal type proposes neoplastic evolution initiates in normal epithelium (normal), progresses to flat epithelial atypia (FEA), advances to atypical ductal hyperplasia (ADH), evolves to ductal carcinoma in situ (DCIS) and culminates as invasive ductal carcinoma (IDC). Immunohistochemical, genomic and transcriptomic data strongly support the evidence of a continuum from FEA to ADH, DCIS and IDC, indicating FEA as the potential non-obligate precursor of ADH. Figure was adapted from [30].

1.5 Invasive breast cancer

One of the most critical events in the process of tumoursigenesis is the transformation of localized carcinoma *in situ* to invasive cancer. In fact, metastases, the spreading of malignant cells to distant organs in the body, account for over 90% of breast cancer-related deaths [31] . Specifically, metastases occur in 25-50% of breast cancer patients and are associated with an average 5-year survival rate of only about 25% [32]. Metastatic cancer spread is a complex multistep process that poses a number of challenges for a tumour cell [33]. First, a cancer cell is required to invade the local microenvironment. Thus some cells loose the contact with the neighbouring cells and others may invade collectively with retained cell-cell contact [34]. The cell then needs to intravasate into local blood or lymph vessels and, importantly, to survive in the circulation. After, the cancer cell must arrest in the circulation and to extravasate into a distant tissue organ. Finally, the malignant cell is challenged to survive and establish in the distinct microenvironment of a foreign organ [35].(Figure 1.2)

Understanding the initial events that enable breast cancer cells to leave the primary tumour site is of a paramount importance, as they represent the foundation of the metastatic progression. These processes involve the deregulations that occur in cell-cell and cell-matrix adhesion within the lesion. Additionally, the malignant cells gain an ability to digest and invade the surrounding matrix that serves as a barrier between cellular compartments. The alterations that occur to cancer cells are a product of both intrinsic and extrinsic modifications. In fact, early metastatic events are a combined result of the aberrant changes occurring within cancer cells and their local microenvironment [34, 36-39]. Hence, it is important to understand these changes in order to develop better therapeutics to combat metastatic breast cancer.

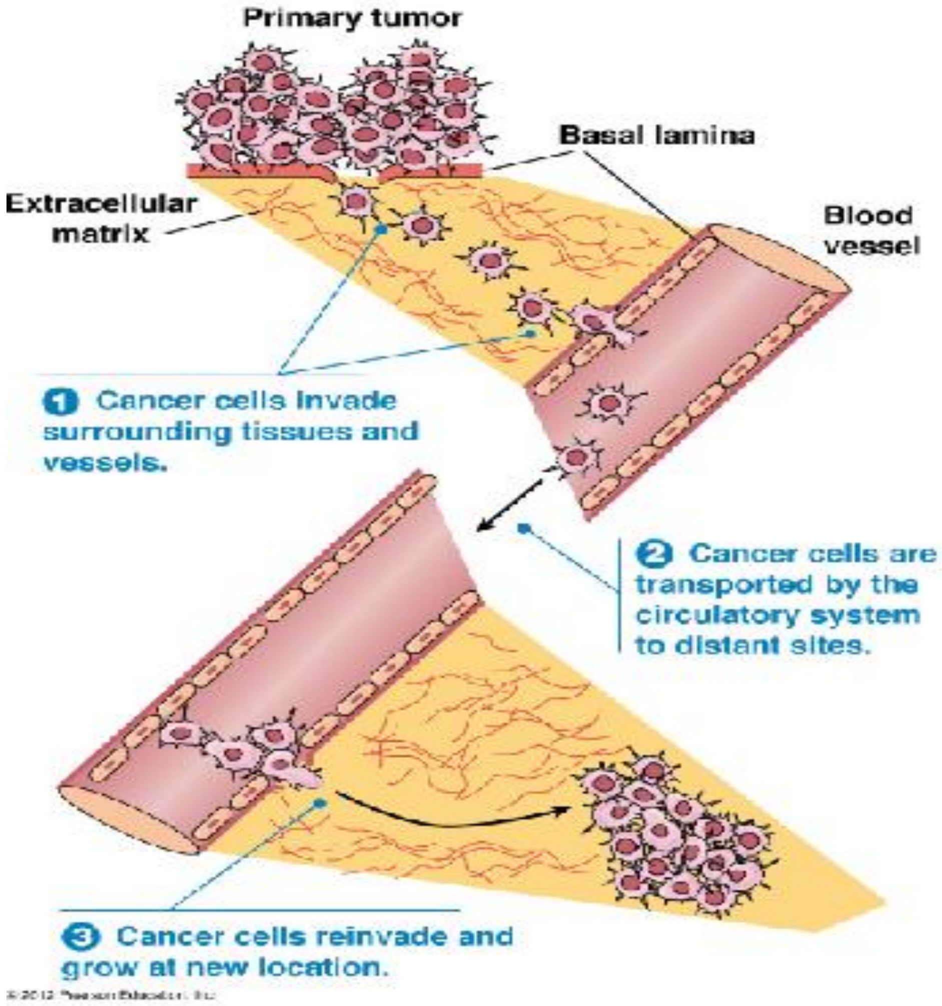


Figure 1. 2 Steps of cancer spread spread

Figure was adapted from Becker's World of the Cell, 8th Edition, by Jeff Hardin, Gregory P. Bertoni & Lewis J. Kleinsmith, , 2012

1.6 Extracellular matrix

Extracellular matrix (ECM) represents a complex and highly organized structure, which surrounds and supports cells in a tissue or an organ and is mainly consisted of fibrous proteins and polysaccharides [40-42]. Collagens represent the major constituents of the ECM and serve as a structural support for the resident cells within the matrix [43]. ECM can be separated into two compartments: interstitial stroma, found between different cell types, and the basement membrane that separates organ compartments [44]. Interstitial matrix is mainly produced by stromal cells and is primarily composed of collagen type I [45], proteoglycans, and various glycoproteins such as tenascin C and fibronectin [43]. On the other hand, the basement membrane is made by epithelial, endothelial, and stromal cells cooperatively. It is a more compact structure in comparison to interstitial matrix and mainly composed of type IV collagen, laminins, fibronectin, and linker proteins such as nidogen and entactin [44]. Despite the initial belief that ECM has purely structural functions, recent data attributes very diverse physical, biochemical, and biomechanical properties to ECM [46] . The interactions between resident cells and ECM are believed to be reciprocal. In fact, ECM is known to be constantly remodelled by various cell types within the microenvironment. On the other hand, these changes in ECM may change properties of other resident cells [47].

Cancer is an aberrant condition and it has been demonstrated that tumour cells are capable of modifying the structure and composition of the surrounding ECM. For example, an increased deposition of type I and type III collagens was reported in human breast cancer *in vivo* [48]. Additionally, the collagen type I fibrils in breast tumours were suggested to become highly linearized, compared to more relaxed and non-oriented fibrils in normal a normal breast tissue [49, 50]. Finally, tumours ECM was found to be up to 10 times stiffer than that of a normal breast, which was attributed to an increase in lysyl oxidase (LOX) activity in these lesions [50, 51].

1.7 Modification of cell adhesion in breast cancer

Changes in both cell–cell adhesions, as well as cell-matrix interactions play essential roles in the progression of malignant cancer. Epithelia have a highly organised architectural structures, which are maintained by a number of regulatory mechanisms that prevent abnormal cell movements. The epithelial cells in mammary ducts are normally found to be tightly packed into tubes by attaching to one another via different junctional complexes: tight junctions (TJ), adherens junctions (AJ), and gap junctions (GJ) [52]. The tight junctions (TJ) are formed by transmembrane proteins such as occludin, claudins and junctional adhesion molecules (JAM), the peripheral anchoring proteins such as zonula occludens (ZO) and TJ-associated regulatory proteins such as α-catenin, cingulin [53]. Thus tight junctions (TJ) control paracellular diffusion of molecules at the apical region of cells and forms belt like structures [52] separating the apical and basolateral membrane domains. The adherens junctions (AJ) mediate cell-cell adhesion and can be subdivided into two groups: classical cadherins such as E-cadherin, N-cadherin and P-cadherin and nonclassical desmosomal cadherins that form desmosomes which connect intermediate filaments from cell to cell. Finally, gap junctions (GJ) often located underneath of TJ [54], forming clusters of channels unable small molecules to pass directly from cell to cell. These channels are formed by connexons made up of six transmembrane connexin subunits [52]. Thus cell-cell adhesion determines the polarity of cells regulated by the interactions with proteins in the ECM [55] and maintain the tissue organization. Reduced intercellular connection leads to the disruption of histological structures which can be defined one of the tumour features that may contrebute to abnormal cell motility and spreading (Figure 1.3).

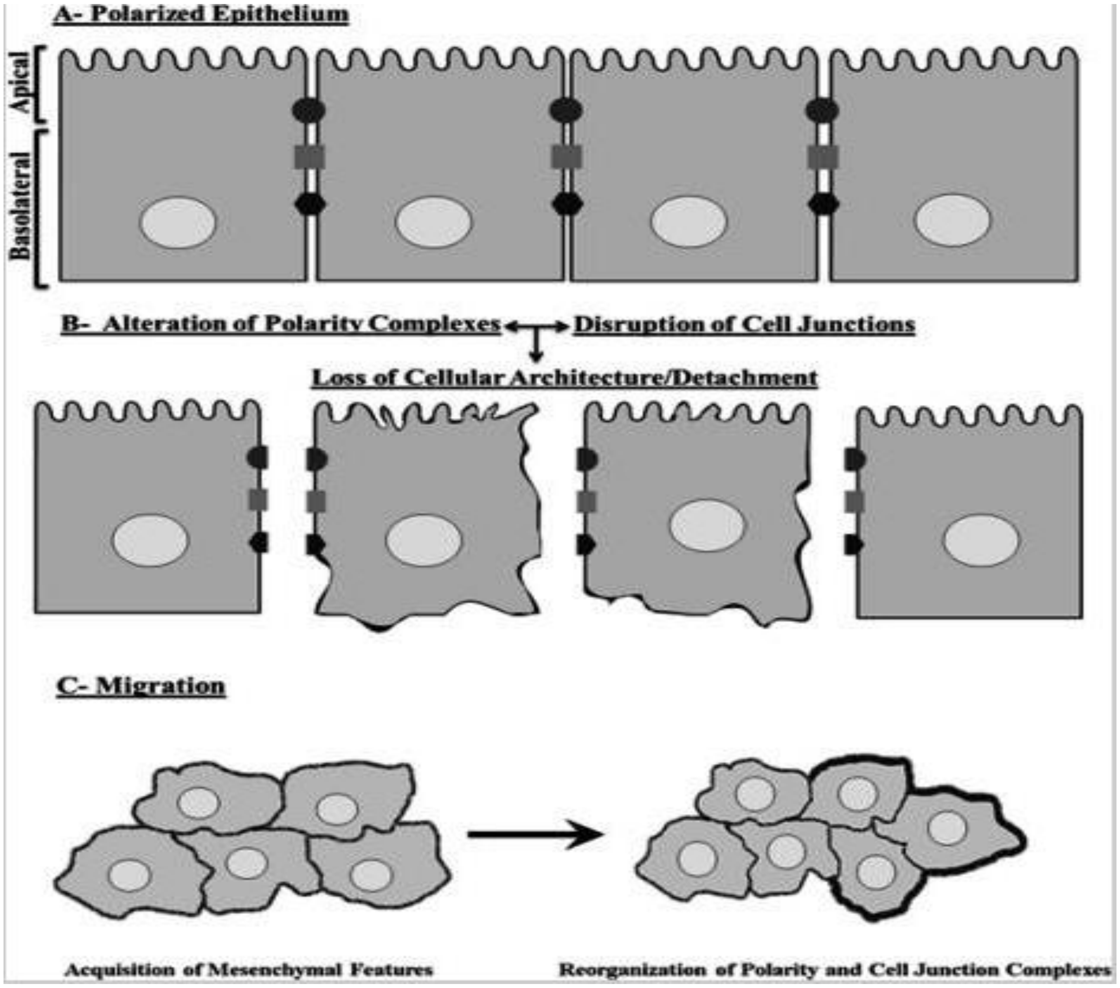


Figure 1. 3 Junctions and polarity

A. Before cancer initiation, cell junctions as in TJ, AJ, and GJ (black circle, grey square and black hexagon respectively) and polarity proteins are properly expressed and localized. **B**. Their disruption leads to the loss of cellular architecture and marks the detachment of epithelial cells from each other within the tissue. **C**. This induces the formation of multilayers of epithelial cells that have acquired mesenchymal features. At this stage, cells need to rearrange their cell junction and polarity proteins to facilitate their collective migration, extravasation and invasion. Figure was adapted from [52]

1.8 *In vitro* models of breast cancer

There are a number of experimental approaches used in studies of breast cancer. One of the most popular approaches to study the progression of the disease is via analyzing breast cancer cell cultures. To model cancer cell invasion *in vitro*, researches frequently utilize two-dimensional (2D) and sometimes three-dimensional (3D) cultures, each with distinct advantages and disadvantages.

1.8.1 2D model versus 3D model of cell invasion

The differences of cell invasion in 2D and 3D formats may be determined by differences between cell to ECM interactions [56] such as high (2D) and low (3D) stiffness, differences in adhesions and ECM components organization, polarity, soluble gradients, and restricted spreading affects cell invasion [56]. And finally, the different modes of cell invasion are observed in 2D and 3D environment.

The preference for studying an invasion processes is given to the 3D format, because the cell invasion in 3D systems more accurately reflects all what is happening *in vivo* and therefore can be considered as more physiological way to study the invasion than 2D formats [57].

1.9 Tumour cell migration

Cancer cells are thought to migrate away from the primary mass via a number of different migration modes depending on two major factors: cell morphology and the local microenvironment [36, 37, 58]. The two main categories of cell migration are individual and collective modes [34]. The individual cell migration mode, as the term suggests, implies a single cell movement following the loss of cell-cell contacts [59]. This mode of migration is further subdivided into amoeboid and mesenchymal types [60]. On the other hand, during the collective migration, cells are moving as a group while maintaining cell-cell adhesions as a clustered or a solid strand [60]. It must be noted, however, that cancer cells are capable of modifying their migration modes depending on the conditions they experience [47].

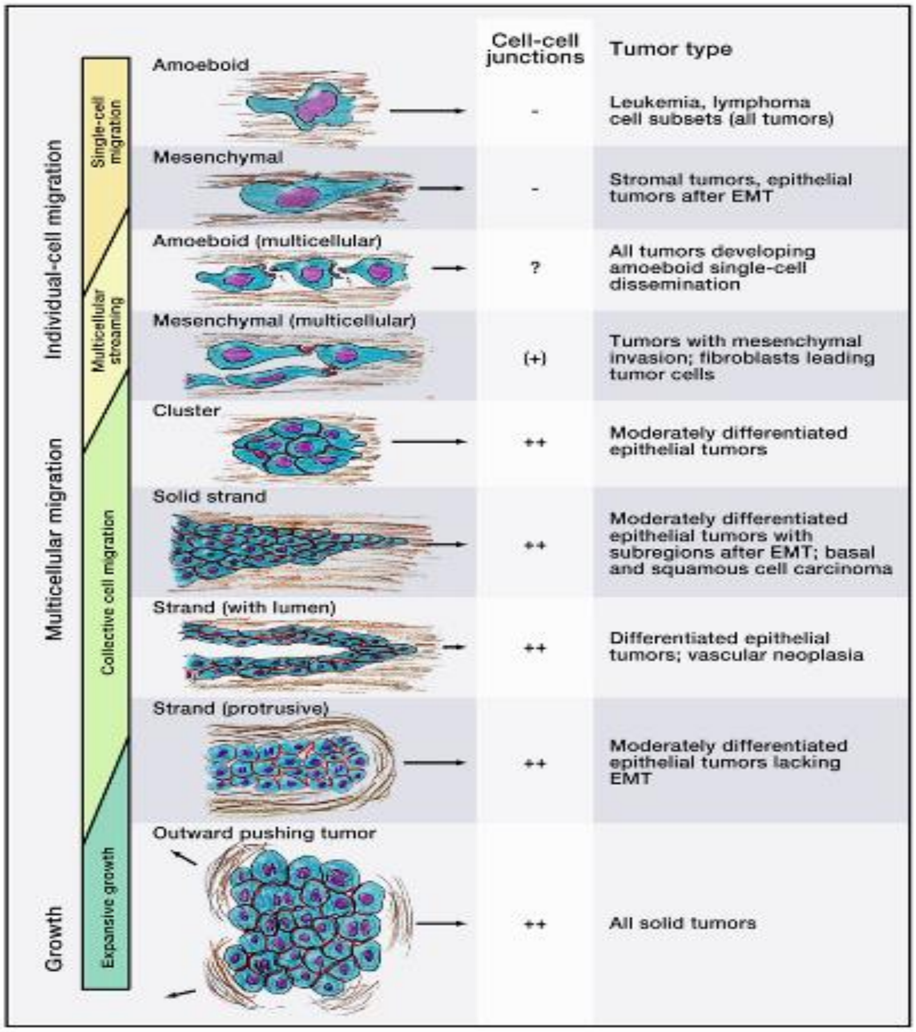


Figure 1. 4 Modes of Cell Movement Implicated in Cancer Invasion and Metastasis

Single-cell and collective cell migration can be further partitioned based on the specific cell-cell junctions, the contractility of cytoskeleton, and the turnover of cell attachments to extracellular matrix (ECM). These modes of migration can be further unstable and change upon alterations of cell-cell interactions, cell-ECM adhesion, or cytoskeletal contractility, resulting in intermediate phenotype. Figure was adapted from [60].

1.10 Proteolytic and non proteolytic cell migration

In order to escape the primary tumour, cancer cells are required to penetrate the basement membrane and enter the underlying interstitial matrix to gain access to the lymph and blood vessels. Thus different classes of enzymes such as cathepsins, serine proteases, and matrix metalloproteinases (MMPs) found activated in the response to changing substrate properties and co-engaged cytokine and growth factor signals [61]. These enzymes degrade ECM via three mechanisms: diffuse proteolysis by invaded cell released proteases, proteolysis generated by membrane-inserted proteases, and intracellular degradation by proteases confined in lysosomes [62-64] characterizing proteolytic cell migration. However, in the absence of proteolytic ECM malfunction or by intrinsic lack of proteases cells migrate in a protease independent manner [65, 66]. Interestingly, in a breast cancer model it has been shown that cells migrate without signs of collagen fiber degradation or reorganization [67].

1.10.1 Amoeboid movement

Amoeboid movement resembles the movement of a soil-living amoeba, *Dictyostelium discoideum*, and is characterized by low cell-environment adhesion force and/or by high actiomyosin contractility [38, 68] with uniformly diffused integrins. Thus, cells change their shape and push through the ECM [69].

1.10.2 Mesenchymal movement

About 40% of carcinomas go through epithelial to mesenchymal transition (EMT) [70]. During EMT, cells lose their epithelial characteristics and gain mesenchymal traits. Mesenchymal mode of invasion is the most well-studied and understood amongst modes of cellular migration. This process induces the migrating cells to have elongated morphology, proteolytic activity and to be able to form focal adhesion points to create contacts with the extracellular matrix [62].

Interestingly, *in vivo* research of circulating tumour cells (CTCs) isolated from cancer patients revealed simultaneous expression of markers for both mesenchymal and epithelial cells, indicating that cells may undergo partial EMT in patients [71].

1.11 Epithelial to mesenchymal transition (EMT)

Epithelial to mesenchymal transition (EMT) is a complex process, via which epithelial cells undergo multiple changes to acquire a mesenchymal phenotype, characterized by a loss of cell polarity and cellular adhesions rendering cells more motile [70]. A large body of work has revealed a number of molecular pathways that are able to induce the process of EMT. These include the molecular pathways downstream of a some potent growth factors (transforming growth factor beta (TGF-β), fibroblast growth factor (FGF), epidermal growth factor (EGF) [72-77], as well as some receptor tyrosine kinases (ErbB2) [78]. Additionally, Wnt/beta-catenin , integrins and Notch [79] pathways have been shown to promote EMT [80]. Although multiple mesenchymal markers have been described, it is believed that EMT is mainly characterized by down-regulation or misslocalization of important epithelial markers such E-cadherin and zonal occludin (ZO-1) followed by reorganization of the actin cytoskeleton [70, 81].

Recently Many and colleagues showed that EMT generates cells with the properties of stem cells thus increasing cancer cell resistance to radiation and chemotherapy [82] and additionally, stimulates breast cancer stem cells self-renewal [83]. Therefore, cells that undergo EMT may not only be able to disseminate, but may also have an increased ability to survive and colonize distant metastatic sites.

1.11.1 Collective mode of invasion

Collective migration implies a group cell movement with retained cell-cell junctions, thus migration occurs as a multicellular unit [37, 84, 85]. The mechanism of collective cell migration resembles the migration of individual cells [86]. However, a simultaneously coordinated polarization of the cells of the leading edge, basement membrane formation surrounding the cell mass, and a coordinated retraction of rear cells occurs during this mode of cell migration [87].

1.11.2 Cell migration processes

Cell migration is a multistep process that contributes to cell invasion and consists of following aspects: (i) protrusion formation at the cell leading edge as a result of actin polymerization, (ii) integrin mediated adhesion to ECM, (iii) proteolysis, (iv) actomyosin mediated cell contraction, (v) cell rear retraction and detachment [88, 89]. This migratory program is used by mesenchymal cells, fibroblasts, endothelial cells, and many tumour cells [90]. However amoeboid movement is characterized by low levels of integrins, weak adhesion and lack of proteolysis [69]. *In vivo,* cells migrate through three types of ECM such as: dense, loose connective tissue and a dense acellular layer forming basement membrane [91]. Thus 3D *in vitro* models mimic this environment and affect cell migratory properties. For example, cells migrating in 3D exhibit matrix adhesions all over their surface, in contrast to 2D where cell adhesions are formed at the ventral surface [92]. In addition, cells cultured on 2D surfaces have a forced apical–basal polarity but in 3D during migration the cell polarity is distributed from front to rear [93]. In addition, cell migratory processes are also affected by physical forces and the ability of cells to invade through ECM is reciprocally influenced by tissue constraint and cell deformation. Wolf and colleagues showed the influence of matrix metalloproteinases (MMPs) exerted on the cell while passing through ECM [89]. Thus MMPs influence on matrix pore diameters as well as integrin and actomyosin dependent force generation influence nucleus deformation of the cell [89].

1.12 Formation of cell protrusion

The first step in the cell migration process is the formation of protrusions, which are extentions of the plasma membrane projected from the front edge of the cell. The process of protrusion formation is driven by polymerization of actin, a globular protein that can polymerize to form filamentous actin (F-actin) or microfilaments. There are several types of membrane protrusion described, however, the most common types are lamellipodia, filopodia and invadopodia [94]. Invadopodias are extended plasma membrane protrusions of invasive cells. These protrusions possess proteolytically active properties and are responsible for the focal degradation of ECM [95]. Invadopodia originates from invaginations of the ventral surface of the plasma membrane [96] and is associated with sites of substrate degradation [97]. These invasive cell protrusions can be identified by their proximity with the Golgi complex [96] and their long lasting activity of up to two hours or more [98].Lamellipodia are thin and flat sheet like extension, whose formation is regulated by the Rac1 GTPase (Guanosine triphosphatase), which stimulates membrane ruffling [99].Additionally, it has been shown that lamellipodia formation can depend on the level of Rac activation [100]. On the other hand, filopodia are thin finger-like membrane extensions, whose formation is regulated by the small GTPase CDC 42 [101, 102]. Both Rac1 and CDC 42 proteins are members of the Rho family of small GTP – binding proteins. CDC 42 is known to interact with multiple proteins. The most studied pathway activated by CDC 42 is the activation of ARP2/3 complex through WASP and N-WASP that promotes actin nucleation [103, 104]. Another pathway which is involved in filopodia formation is CDC 42 signaling via the insulin-receptor substrate p53 (IRSp53) [105]. Additionally, Rac can also activates ARP2/3 through WAVE thus promoting membrane protrusion formation [106].

1.13 Actomyosin complex

Cell contraction is another important step in the cell migration process where actomyosin complex plays a crucial role. The complex is composed of actin filaments associated with myosin II bundles.

The role of myosin is to convert chemical energy into mechanical force, thus driving cell movement processes and the contraction of the actin cortex by generating the force required for retraction of the cell trailing edge [107].

1.14 Signaling and kinases

The communication between cells in an organism is a vital process. This process is carried out by means of the transduction of cell signaling. Signaling is the process of intercellular communication, which comprises the following steps: (i) synthesis and (ii) release of extra/intracellular molecules involved in mediating of the cell-cell or cell-environment responses, (iii) transport of the signal to the target cell by blood or electric impulses, (iv) detection of the signal by specific receptor protein that binds extracellular specific proteins, then (v) cell triggered response, and, finally, (vi) signal termination [108].

There are several groups of highly conserved proteins that play a crucial role in cellular signal transduction such as GTPases, adapter proteins and kinases. GTPase proteins enable hydrolysis of GTP, switching a signal transduction chain between on (when bound to GTP) and off (when bound to GDP) states. Adapter proteins do not have catalytic activity, but instead contain domains that function as docking sites for other proteins. Finally, protein kinases phosphorylate substrates, which activates or inactivates them and thus participate in signal transduction cascades. There are two types of protein kinases, tyrosine and serine/threonine specific groups. Their catalytic activity can be regulated by phosphorylation, direct binding to other proteins, and by changes in the levels of various second messengers [108].

However the role of phosphatases is also important in signaling pathways to balance the role of kinases. Protein phosphatases are classified as protein serine-threonine or protein tyrosine phosphatases. They dephosphorylate proteins by removal a phosphate group and thus controlling the signaling pathways [109].

1.15 Protein kinase C (PKC)

Protein kinase C (PKC) isoenzyme belongs to the serine/threonine family of kinases and was first discovered by Nishizuka and colleges while studying extracts from rat brains [110]. The PKC family had been subsequently associated as key signaling molecules involved in many cellular processes including cell proliferation, differentiation, polarity and survival [111]. Currently the PKC family consists of at least 12 distinct lipid-regulated protein kinases. Based on their structure and the activation mechanisms the PKC family is divided into three groups: (i) conventional, (ii) novel, and (iii) atypical [112]. The conventional group of PKCs is represented by PKCα, PKCβI, PKCβII, and PKCγ, which require diacylglycerol (DAG) and calcium (Ca^{2+}) for activation. The novel PKCs (nPKCs) include PKCδ, PKCε, PKCθ and PKCη, which are also activated by DAG and phospholipids, but are Ca^{2+} independent. Finally, the third group, atypical PKCs (aPKCs), encompasses aPKCι/λ and aPKCζ, which do not require either DAG or calcium for activation.

1.15.1 Regulation of aPKCs

As discussed above, aPKC include two protein isoforms, aPKCζ and PKCι/λ, where aPKCλ is not a distinct isoform but is the mouse ortholog of human aPKCι [113].

Both isoforms consist of N-terminal regulatory regions and a C-terminal kinase domain. At its N terminus aPKCs has phox-bem 1 (PB1) domain followed by an atypical C1 domain that is unable to bind DAG [114]. However, aPKCs have a pseudosubstrate sequence on their regulatory domains, which maintains kinases in their inactive state in the absence of

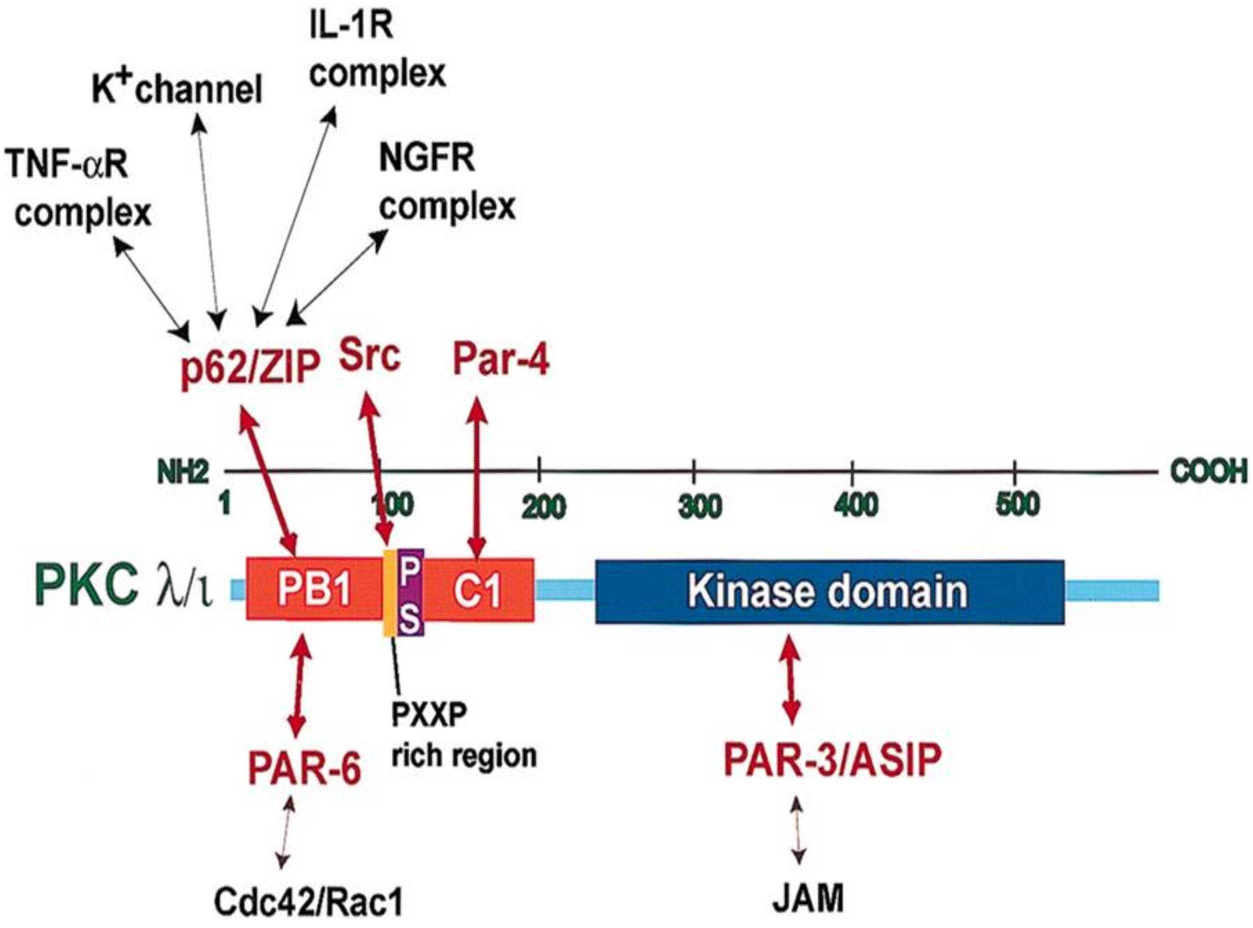


Figure 1. 5 The structural domains of PKCλ/ι and their interactions with aPKC-specific binding proteins (red).

Recent studies have shown that PKCλ/ι has various protein-protein interfaces including a cysteine-rich sequence (C1) in its N-terminal regulatory domain. The most N-terminal PB1 domain of aPKC interacts with OPR/PC/AID motif–containing proteins, such as PAR-6 and p62/ZIP. The PB1 domain of aPKCs is also suggested to embed the OPR/PC/AID motif within its core structure. PS: pseudosubstrate. Figure was adapted from [114].

activation signals [115, 116]. In addition, the activity of aPKCs can be regulated by 3-phosphoinositides (PI3K) through phosphoinositide-dependent kinase 1 (PDK1) phosphorylation [117] and protein–protein interactions with upstream effectors, such as Ras [118].

It has been shown that aPKCs are involved in the establishment of normal cell proliferation, differentiation, cell polarity and cell survival [119]. Cell polarity is a fundamental biologic process, which determines asymmetric distribution of cellular components resulting in formation of at least two cell surfaces such as apical and basal. aPKCs have been found to be a part of the PAR complex, a known regulator of cell polarity [120]. In fact, it has been shown to associate with the members of the PAR complex, Par 3 and Par 6, to localize to tight junctions and play an important role in the tight junctions formation [121-123].

The human gene encoding aPKCι protein is PRKCI, which is located on chromosome 3q26 and is a frequent target for mutations in a number of human cancers [60]. The PRKCZ gene encodes aPKCζ protein and is located on chromosome 1p36. Interestingly, it has been shown that depending upon the cellular context aPKCζ acts as either a tumour suppressor or tumour promoter [124, 125], however aPKCι protein functions as an oncogene [126, 127].

1.16 PKC role in different diseases

The members of protein kinas C (PKC) family have been implicated in many diseases such as central nervous system dysfunctions, Alzheimer’s disease, neuronal degeneration, cardiovascular disorders atherosclerosisis and cancer [128]. In addition, some of the PKC members are found to be overexpressed in most solid tumours and play an important role in cancer progression such as cell migration, invasion and metastasis [129] Moreover, a crucial role of PKC in cancer progression was further emphasized when these proteins have been implicated in a number of oncogenic signalling pathways such as Ras, Myc and Fos [130].

1.16.1 aPKCι as a prognostic marker

To date a number of studies implicate an oncogenic role for aPKCι. Precisely, overexpression of aPKCι is found in non-small cell lung cancer cell lines [127] and ovarian cancers [131, 132]. In addition, the 3q26 chromosome amplification was found in squamous cell carcinoma (SCC) of the oesophagus [133], cervix [134], head and neck [135] and lung [136] cancers. Moreover, the aPKCι protein expression was found to be elevated in lung adenocarcinoma, chronic myelogenous leukaemia [137], pancreatic [138] and colon cancers [126]. Finally, aPKCι protein is overexpressed in 80% of the breast carcinomas [139], where it is activated by PI3K signalling [140]. Therefore, PKCι expression profiling can be a valuable prognostic marker in diagnostics of various cancers.

Importantly, a correlation between PKCι protein overexpression and poor prognosis has also been reported. Specifically, high aPKCι expression was found to correlate with poor patient outcome in NSCLC [127], ovarian [131, 132], pancreatic [141], bile duct [142], prostate [143] and breast [139] cancers.

1.16.2 aPKCι as a therapeutic target for treatment of cancer

aPKCι was shown to be involved in a number of oncogenic signaling pathways. Thus, the inhibition of aPKCι activity presents an attractive therapeutic venue in treatment of cancer patients. It has been shown that aPKCs have protein-protein interaction PB1 domain that is critical for aPKCs activation [144]. Thus PB1 domain blockage will potentially lead to the inhibition of the signalling pathway downstream of aPKCs. Intriguingly, Stallings-Mann and colleagues demonstrated that small molecules such as aurothioglucose (ATG) and aurothiomalate (ATM) that can disrupt the PB1-PB1 domain interaction [145] were able to supress tumourogenesis in *in vivo* and *in vitro* models of non–small-cell lung cancer (NSCLC) [146].

1.17 Rational and approach

The main cause of breast cancer-related deaths is metastases to distant organs, which arise from a disruption of normal cell organization, polarity, and cell-cell adhesion making the malignant cells more motile and invasive.

Members of the mammalian protein kinase C (PKC) superfamily play key regulatory roles in a multitude of cellular processes such as regulation of normal cell proliferation, differentiation, polarity and survival. In fact, the members of the atypical protein kinase C (aPKCs) subfamily have been implicated in the development of a number of human diseases including cancer. aPKCs are involved in both tumourogenesis and metastasis. A member of aPKC subfamily, aPKCι, has been identified as a human oncogene and has been used as prognostic marker with a potential of a novel therapeutic target. However, the precise mechanism of an aPKCι involvement in breast cancer progression has not been shown.

The first step in the spread of metastatic cancers is the movement of cancer cells into the tissue that surrounds the tumour. To do so, cancer cells must acquire motile and invasive phenotypes. aPKC has been previously shown to promote tumour cell invasion in a number of cancers. Consistently, aPKCι activity was found to be required for extracellular matrix degradation and invasion of Src-transformed fibroblasts [147]. In addition, aPKC overexpression was shown to correlate with increased invasion and metastasis in esophageal squamous cell carcinomas (ESCC) [148] and increased metastasis formation in breast cancer [139]. Furthermore, McCaffrey and colleagues showed that the disruption of Par3 polarity complex causes an aPKC-dependent activation of the Jak/Stat3 signaling pathway that results in increased matrix metalloproteinase-9 expression, degradation of the extracellular matrix, and tumour invasion and metastasis *in vivo* [149]. The previous studies demonstrate an important role for aPKCι in promoting tumour cell invasion and the subsequent formation of metastases in a number of human cancers. Additionally, aPKCι was suggested to be involved in the extracellular matrix degradation in breast cancer [149, 150] . However, whether aPKCι is sufficient to promote breast cancer cell invasion remains to be elucidated and thus were chosen as a focus of research of this thesis.

Thus I hypothesised that amplification of aPKCι promotes breast cancer invasion. To achieve my objective and in order to determine how aPKCι promotes breast cancer invasion, I chose to use 3D collagen *in vitro* assays. I have used murine mammary gland cancer cell lines that have been engineered to overexpress a constitutively active form of aPKCι as a model system. I employed live imaging microscopy to observe the effects of aPKCι overexpression on the dynamics of breast cancer cell invasion into the surrounding matrix in real time.

Additionally, I examined the effects of aPKCι overexpression on mammary gland cancer cell morphology and the formation of cellular protrusions. Finally, I used small molecule inhibitors to investigate the potential molecular mechanisms of aPKCι involvement in breast cancer cell invasion.

Chapter 2 Experimental design

2.1 Cell culture

The Normal Murine Mammary Gland epithelial cell line (NMuMG) was previously engineered to overexpress a constitutively activated form of the mouse receptor tyrosine-protein kinase ErbB2 [151] and was generously provided by Dr. Peter Siegel (Rosalind and Morris Goodman Cancer Research Centre, Montreal, QC, Canada). This cell line was cultured in DMEM media supplemented with 10% FBS, insulin (5 μg/ml), penicillin/streptomycin (100 U/ml).

The Neu NDL (Neu deleted) cell line was derived from Neu transgenic mice [152] and was kindly provided by Dr. William Muller (Rosalind and Morris Goodman Cancer Research Centre, Montreal, QC, Canada). This cell line was cultured in DMEM/F12 media, supplemented with 10% FBS, insulin (5μg/ml), hydrocortisone (1μg/ml), EGF (10ng/ml), penicillin/streptomycin (100U/ml).

The Human Embryonic Kidney 293 cell line (HEK293-LT) was obtained from (ATCC, Manassas, Virginia). These cells were cultured in DMEM media supplemented with 10% FBS, penicillin/streptomycin (100U/ml).

2.2 DNA constructs

A constitutively active form of the Protein kinase Cι (aPKCι), where the alanine residue at the position 129 was substituted by a glutamate residue (A129E), was cloned into the pWPI plasmid to be expressed under the EF1α promoter (EF1α-A129E-IRES-GFP). The construct was previously made in Dr. McCaffrey's laboratory.

The pWPI plasmid was obtained from Dr. Trono, through Addgene. pWPI (Addgene#: 12254)

The pWPI-RFP-Histone plasmid was previously made in Dr. McCaffrey's laboratory.

pMD2.G (Addgene#: 12259) encodes for the envelope protein VSV-G and psPAX2 (Addgene#: 12260) is the packaging plasmids were provided by Dr.Trono (http://www.tronolab.unige.ch).

2.3 Virus production and infection

HEK293-LT cells were plated at a density of 6.5x10^6 cells per 15 cm culture dish. Cells were transiently transfected using PolyJet (Frogga Bio SL 100688) with 20.8 µg of either pWPI-aPKCι-A129E-GFP or pWPI-GFP lentiviral constructs together with 10.5 µg of pMD2.G and 6.3 µg psPAX2. After 4 hours of transfection, cells were washed and fresh media added. The packaging cells were cultured for 48h to produce lentivirus, which was subsequently concentrated by polyethylene glycol (PEG) precipitation. For this 40% PEG was added into the viral supernatant in a volume 1 to 3 by gentle pipetting. This mixture was incubated at 4^{0}C for 12h, followed by 30 minutes centrifugation at 4^{0}C. Concentrated virus was titered in HEK293-LT cells using serial dilutions and microscopic analysis of GFP positive cells. Dilutions with less than 10% of cells expressing GFP were selected for analysis using the following formula $TU/ml = \frac{average\ \%\ GFP\ positive\ cells\ \times 20000\ cells\ plated \times 1000\mu l}{quantity\ of\ the\ dilution\ in\ \mu l}$. NMuMG-ErbB2 and NeuNDL cells were then infected with lentivirus at a multiplicity of infection (MOI) of 10. Pure populations of infected cells were obtained by fluorescence activated cell sorting (FACS). FACS procedure was generously provided by Ken McDonald, Life Sciences Complex Flow Cytometry Core Facility, McGill University's Department of Physiology (Montreal, QC, Canada).

Green fluorescent protein (GFP) positive cells were sorted by Fluorescence-activated cell sorting (FACS) technique with the similar fluorescent intensity for both control and mutant cells.

The pWPI-RFP-histone plasmid was transfected into the HEK293 cells similar to the method described above. The resulting virus was used to infect NMuMG-ErbB2-GFP and NMuMG-ERbB2-aPKCι-A129E cell lines analogues to the protocol outlined above.

2.4 Western blotting analyses

NMuMG-ErbB2-GFP, NMuMG-ErbB2-aPKCι-A129E and NeuNDL-GFP, NeuNDL–aPKCι-A129E cells were lysed using radio-immunopreciptation assay (RIPA) buffer (1M Tris-HCI 2.5ml, Bioshop TRS0012, 10% NP-40 BioShop CAS# 9016-45-9, 10% Na-deoxycholat Bio Basic CAS# 302-95-4, 10% SDS BioShop SOD 002.205, 4M NaCl BioShop SOD 002.205). Total protein concentration was determined by Bradford Protein Assay (BioRad, Cat. # 5000006) and 15 µg of protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Immobilon FL_IPFL_ 00010). The membranes were then blocked with (milk and incubated with the following antibodies: PKCι (BD Transductions Laboratory, Cat #610175 1:5000) , PKCζ (Cell Signaling Technologies, Cat. #9368S 1:5000), PKCζ (Santa Cruz Biotech, Cat #CS-216 1:5000) and α-Tubulin (Sigma, Cat #T9026 1:10000). The blots were then incubated with horseradish-peroxidase-conjugated secondary antibodies: Mouse IgG whole molecule (Jackson ImmunoResearch, Cat. #015-000-03 1:5000), Rabbit IgG whole molecule (Jackson

ImmunoResearch, Cat #011-000-03 1:5000). Finally, proteins were visualized with an enhanced chemiluminescence detection system (BioRad and exposed to autoradiographic film (Santa Cruz Biotech, SC 201697).

2.5 3D collagen type I invasion assay

The original recipes for collagen type I matrix assay [1] was generously provided by Dr. Elad Katz (University of Edinburgh, Edinburgh, United Kingdom). Specifically**,** the following mixture was prepared in the DMEM/F12 medium: purified collagen peptide (Nitta Gelatin Inc, Osaka, Japan, Cat. #: 17100017) (1.3mg/ml), 0.1% acetic acid and 0.22M sodium hydroxide. Next, $1x10^5$ cells were resuspended in the resulting matrix and incubated at 37^oC for 1h to prepare a cell plug. Then, this cell plug was inserted into the larger volume of the empty matrix mixture and incubated further at 37^oC for 1h. Finally, after the gels were solidified, the complex was topped up overlayed with culture medium.

2.6 Immunofluorescence

Cells in the Collagen I gel matrices were fixed with 4% paraformaldehyde (PFA) for 1.5h, then Optimal Cutting Temperature (OCT) (VWR, CA 95057-838) embedded and frozen at -80^oC. Samples were then cryo-sectioned at 50-60μm thickness. Sections were washed with phosphate buffered saline (PBS), and stained with Phalloidin-Alexa555 (Invitrogen, Cat #A34055 1:750) and Hoechst 333258 (Biotium, Cat # 40009 1:10000) for 1h RT.

2.7 Inhibitors

Inhibitors were used to examine molecular pathway via which aPKCι influences protrusion formation. RAC1 inhibitor (100µM) -NSC23766 (EMD/Millipore, Cat. #553502), myosin II inhibitor –Blebbistatin [153] (50µM) (Cedarlane, Cat # BML-EI 315-0005) were added to the 3D collagen gel cultures and the ability of cells to form protrusion were analysed as described above. Both inhibitors were applied 24h following invasion and left for 24 h.

2.8 Live-Imaging and Cell Tracking

The ability of cells to invade the surrounding matrix was followed by the live-imagingmicroscopy. The procedure was performed using the LSM700 confocal laser scanning microscope Zeiss with the objective lenses (20X dry, NA 0.8, Zoom 2X) and the lasers 555 at 0.2-1% power for 16h under the following conditions: (i) plates with the cells were held at 37^oC during the entire procedure and (ii) 10 mM of 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffer was added into the plate 20 min before the microscopy. The differential interference contrast (DIC) and fluorescent images were taken as a series of frames, one image every 15 minutes, for the total of 64 frames, allowing capturing the dynamics of the invasion process. The motile cells were subsequently tracked and analyzed using the Image J software program. Specifically, MTrackJ command was placed under the Plugins [154] section to allow for manual cell tracking. The option “Tracking” allows choosing a “snap feature” and a” snap range”. The “Bright centroid” and “23×23” pixels were chosen respectively.

To track the cells, both aPKCi overexpressing and the control NMuMG ErbB2 cell lines were infected with the lentivirus to expressing red-fluorescent protein (RFP)-Histone (see section 2.3 virus production and infection). Nuclear staining allows for an easier cell tracking compared to cytoplasmic staining due to continues changes in the cell shape during movement within the matrix. In addition, only motile cells travelled 10μm were included, since this distance is approximately one cell diameter, and is sufficient to exclude changes in position due to cell shape changes, or minor inaccuracies of the automated microscope stage.

2.9 Measurement of cell movement persistence

The persistence of cell movement was calculated as the ratio between distance traveled by a cell and its displacement (Figure 4.5 *A*):

$$Persistence = \frac{Distance}{Displacement}$$

The distance was calculated using ImageJ software and the plugin properties explained above and represents the total distance of a given cell's trajectory. The displacement variable was determined to be the shortest distance, which connects the start and the end points of a cell's trajectory and was calculated using the Pythagorean equation (Figure 4.5 *A*):

$$Displacement = \sqrt{d^{\,2} + c^2}$$

where d and c can be found as:

$$d = y2 - y1$$

$$c = x2 - x1$$

where point A has coordinates (x_1, y_2) and point B has coordinates (x_2, y_1).

2.10 Angle displacement

Angle displacement was used to analyze cell directionality (Figure 2.1). Importantly, the angles included in the analysis were the ones obtained from the trajectories of only the cells that had traveled at least 10µm.

Specifically, I first drew vectors along cell trajectories for every 10µm of cell displacement (Figure 3.1). I then calculated the cosine of the angle α formed between the two adjacent vectors A and B using the following formula:

$$\cos\alpha = \frac{A * B}{|A|\,|B|}$$

$$A * B = A_x * B_x + A_y * B_y$$

$$|A| = \sqrt{{A_x}^2 + {A_y}^2}$$

$$|B| = \sqrt{{B_x}^2 + {B_y}^2}$$

$$A_x = x_2 - x_1$$

$$A_y = y_2 - y_1$$

$$B_x = x_2 - x_1$$

$$B_y = y_2 - y_1$$

where $(x_1, y_1); (x_2, y_2)$ are the vector coordinates.

Finally, the displacement angles were deducted from the cosines using trigonometric tables (Microsoft Excel 2010).

2.11 Statistical analysis

Statistical significance values for determination of the area of invasion, the percentage of invading cells, cell velocity and persistence were obtained by performing a two-sample variance, two-tail, student's t-test with confidence interval of 95% where *p*-values of $p < 0.05$ were considered statistically significant. GrafPad Prism and Microsoft Excel 2010 used for the statistic test performance.

The *Chi*-square test was performed to analyze the directionality of cell movement, the existence of a distinct phenotype and was used to analyze protrusion formation on blebbistatin treated cells. It was calculated using 2 x 2 Contingency Table and Microsoft Excel 2010.

Pearson sample correlation coefficients between the number of protrusions a cell produced and its velocity were computed using Microsoft Excel 2010.

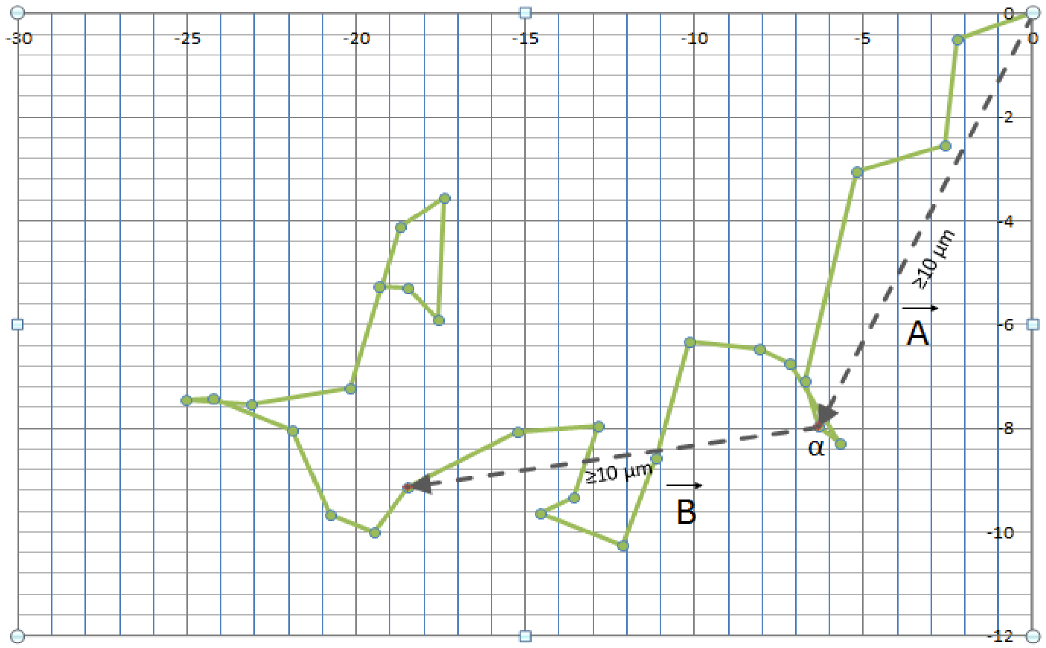


Figure 2. 1 Calculation of the displacement angle.

A representative cell trajectory is shown with the green line. The dotted lines show the A and B vectors of the cell's directionality. The angle α is formed between the vector A and the vector B

Chapter 3 Results and analysis

3.1 The effect of aPKCἰ expression on ErbB2 breast cancer cell invasion

To test my hypothesis, I first examined if expression of activated aPKCι affects invasion of mammary cancer cells *in vitro*. NMuMG-ErbB2-GFP and NMuMG-ErbB2-aPKCι-A129E-GFP cells were embedded into collagen type I and their invasion into the surrounding cell-free matrix was studied by light microscopy (Figure 3.1).

I found that both cell lines are capable of invading the collagen type I matrix. However, individual aPKCι−A129E-expressing cells were capable to invade farther (Figure 3.1), suggesting that aPKCι may facilitate the cellular invasion of cells through collagen type I matrix.

3.1.1 Determination of the area of invasion and the percentage of invading cells in collagen type I matrix with activated aPKCἰ.

At 72 hours post-cell embedding in collagen type I matrix, I found that both the control NMuMG-ErbB2-GFP cell line, as well as the aPKCἰ-expressing cells were able to invade the surrounding matrix. Therefore, I quantitatively investigated and compared areas of invasion in both lines (Figure 3.2). To do so, I performed three independent 3D invasion assays in triplicate and utilized ImageJ software program to determine the area of cell invasion without the area of the plug.

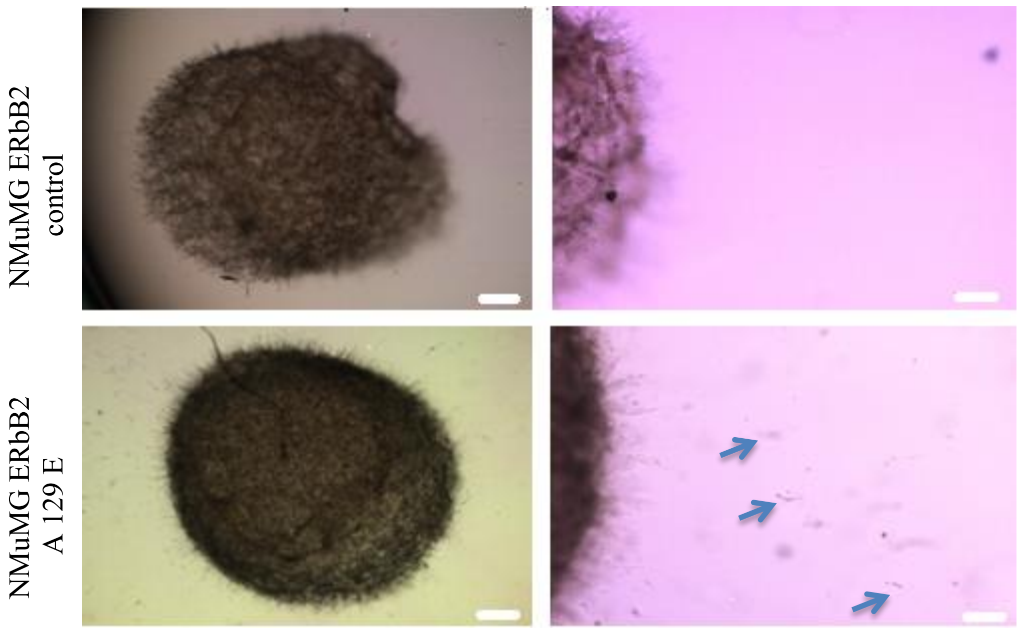


Figure 3. 1 Cell invasion in collagen type I assay.

Images of NMuMG-ErbB2-GFP (control) and NMuMG-ErbB2-aPKC*i*-A129E-GFP cells were taken 72h post cell embedding into collagen type I matrix. Left panels show representative cell colonies formed in collagen type I matrix. Higher magnification of the colony edges (right panels) demonstrate that both cell lines are capable of invading the collagen type I matrix. The arrows in the right lower image point out cells invading further out. The scale bars represent 500 µm (left panels) and 200 µm (right panels).

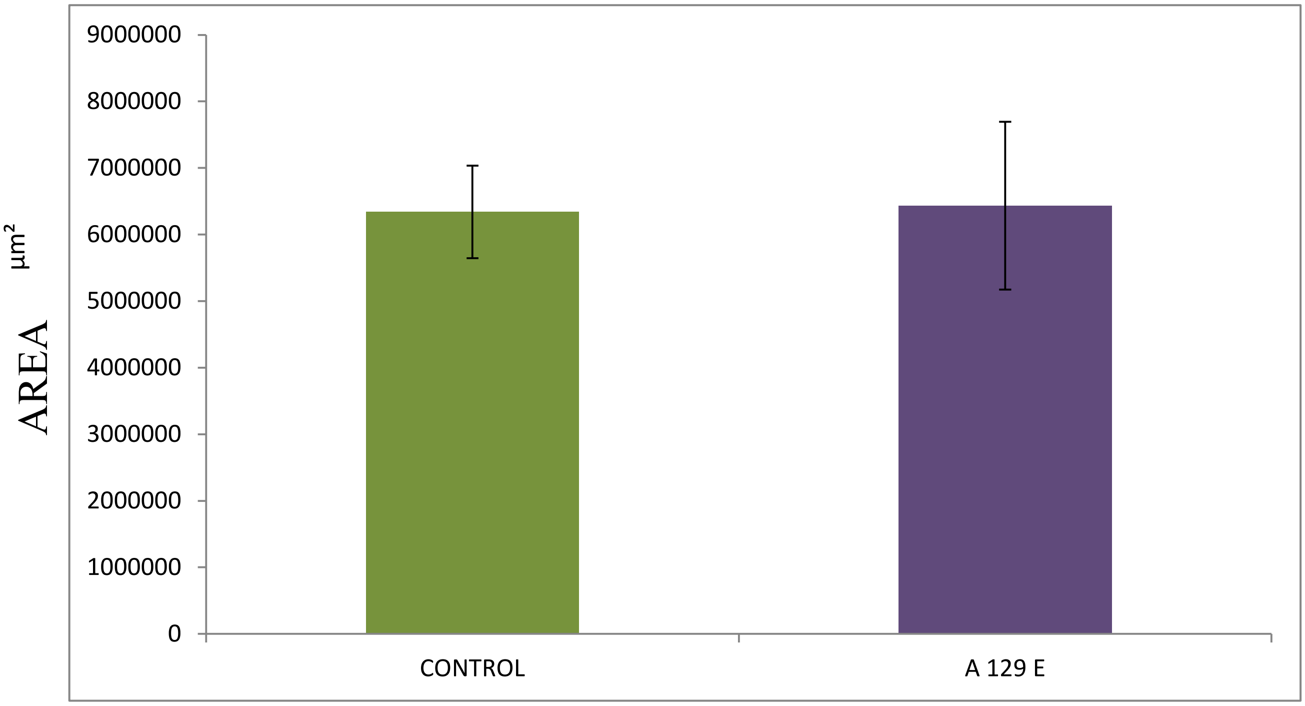


Figure 3. 2 Areas of invasion.

Areas of invasion were measured 72 hours post cell embedding into the collagen type I matrix. The aPKC*i* A129E showed a 1.014 fold enhancement in cell invasion over the control cells with no statistical significance (p = 0.857216). The error bar shows standard deviation.

The average area of invasion of the control cell line was determined to be 6433069.674μm^2, which is 1.014 fold smaller than that of the mutant line 6339839.109μm^2. However, this difference was not statistically significant ($p = 0.857216$).

To gain further insights into a potential role for activated aPKCι in promoting invasion, I used live fluorescence microscopy to examine cells that invade the collagen I matrix away from the main colony (Figure 3.3). The analysis was performed on the fluorescent images allowing capturing the dynamics of the invasion process. Both the control and the mutant cell lines were infected with the lentivirus expressing the RFP-Histone since nuclear staining allows for an easier cell tracking compared to cytoplasmic staining due to continues changes in the cell shape during movement within the matrix. Interestingly, I found that not all cells are invasive; 36.6 % of the control cells and 34.8 % of the mutant cells were able to leave the cell colony and invade the surrounding collagen I matrix. However, there was no statistically significant difference between the two cell lines. Therefore, expression of activated aPKCι does not alter the proportion of cells that are invasive.

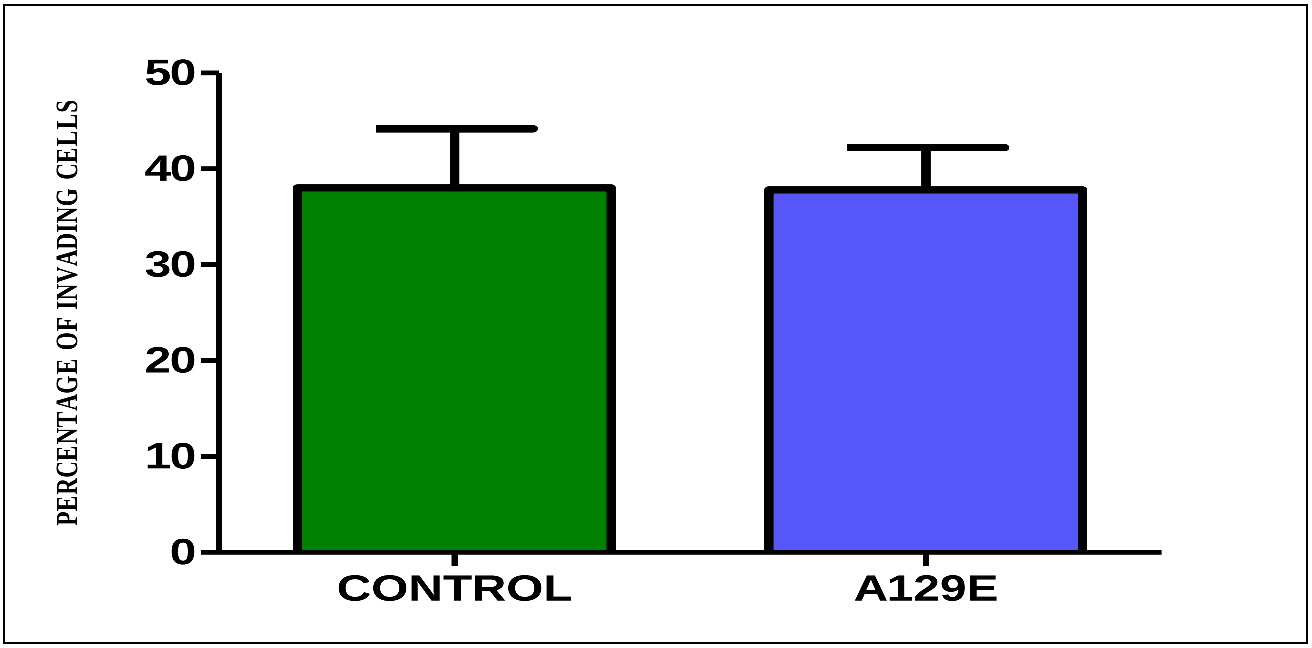


Figure 3. 3 The percentage of invading cells was measured using fluorescent microscopy. The control line showed 36.6% of invading cells and the aPKCi A129E showed 34.8% of invading cells. The error bars represents mean + SEM. The data is not statistically significant (p = 0.9801).

3.2 Analysis of cell speed and invasive persistence.

To understand why some mutant cells were found to invade farther out from the bulk colony when compared to the control cells, I next analyzed the velocity at which the aPKC-expressing cells move within the collagen I matrix in comparison to the control cells (Figure 3.4). Secondly, I analyzed the persistence of cellular movement in both aPKCi overexpressing and the control NMuMG ErbB2 cells (Figure 3.5 *A* and *B*).

To perform these analyses I employed the live imaging microscopy, as it permits to collect the data about the dynamics of cell movement over a period of time. I carried out three independent experiments, each performed in triplicate. Additionally, images were taken from three independent areas of each triplicate. Motile cells were tracked using Image J software (Figure 3.6).

Importantly, I employed a set of criteria applied uniformly to cells for invasion analyses: (1) To standardize tracking, only cells with nuclei positive for RFP-Histone were selected. If two or more RFP-Histone-positive cells came in contact and tracking became ambiguous, the cells were excluded; (2) Since cells stop moving during division, non-dividing cells were excluded; (3) Cells that went out of focus or out of the imaging area in fewer than 10 frames; and (4) Only cells that were motile were included. For this, only cells moving at least 10μm were included, since this distance is approximately one cell diameter, and is sufficient to exclude changes in position due to cell shape changes, or minor inaccuracies of the automated microscope stage. A total of 50 mutant cells and 77 control NMuMG-ErbB2-GFP cells met these stringent selection criteria.

3.2.1 Cell velocity

Although aPKCἰ does not influence the proportion of invasive cells, it does appear to influence their ability to invade farther. One possible mechanism is that aPKCἰ causes cells to invade faster. To test this, I compared the velocity of invading NMuMG-ErbB2-GFP and NMuMG-ErbB2-aPKCι-A129E-GFP cells within the collagen I matrix (Figure 3.4). Whereas control NMuMG-ErbB2-GFP cells invaded with a velocity of 0.001 μm/s, the mutant cells showed a 2-fold increase in invasion with average velocity 0.0023 μm/s. The velocities of both groups are statistically different with $p < 0.0001$. Therefore, aPKCἰ promotes increased invasion velocity, which is consistent with these cells migrating farther than the control (Figure 3.1).

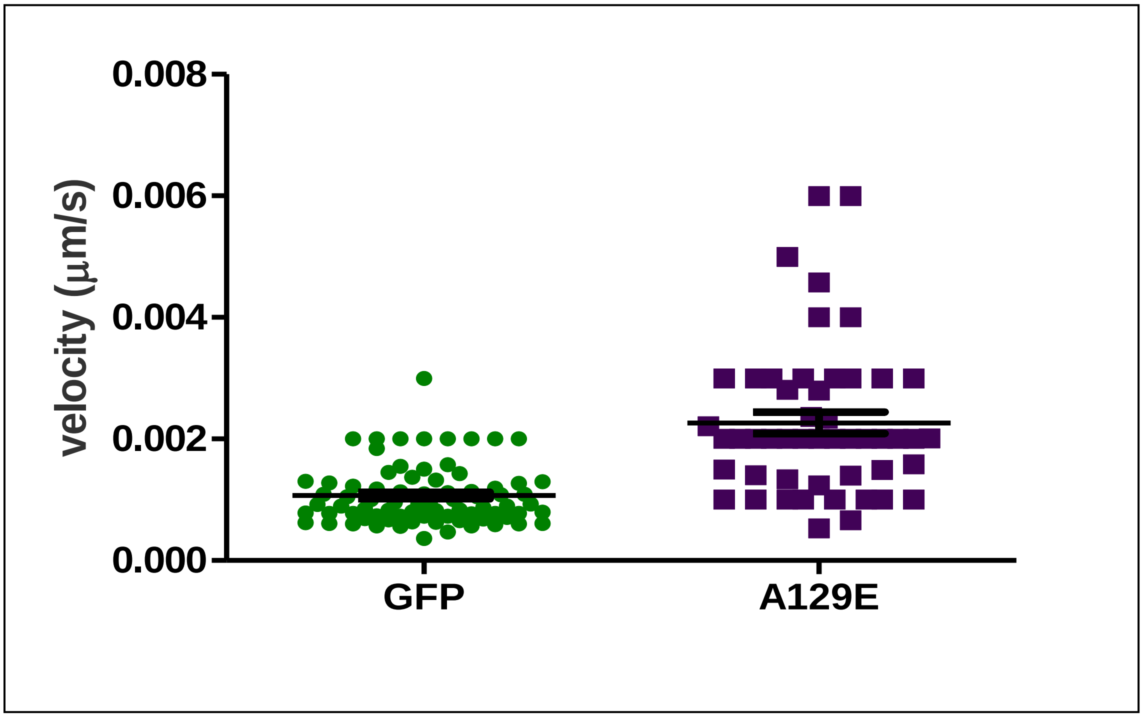


Figure 3. 4 NMuMG ERbB2 cells velocity was measured using Image J software program. Each point on the chart represents an average speed of an individual cell. Green points are average speed of control cells; purple points are average speed of aPKC*i* A129E mutant cells. The mutant cells displayed 2 times higher velocity than control.The error bars represent mean +/- SEM. $p < 0.0001$.

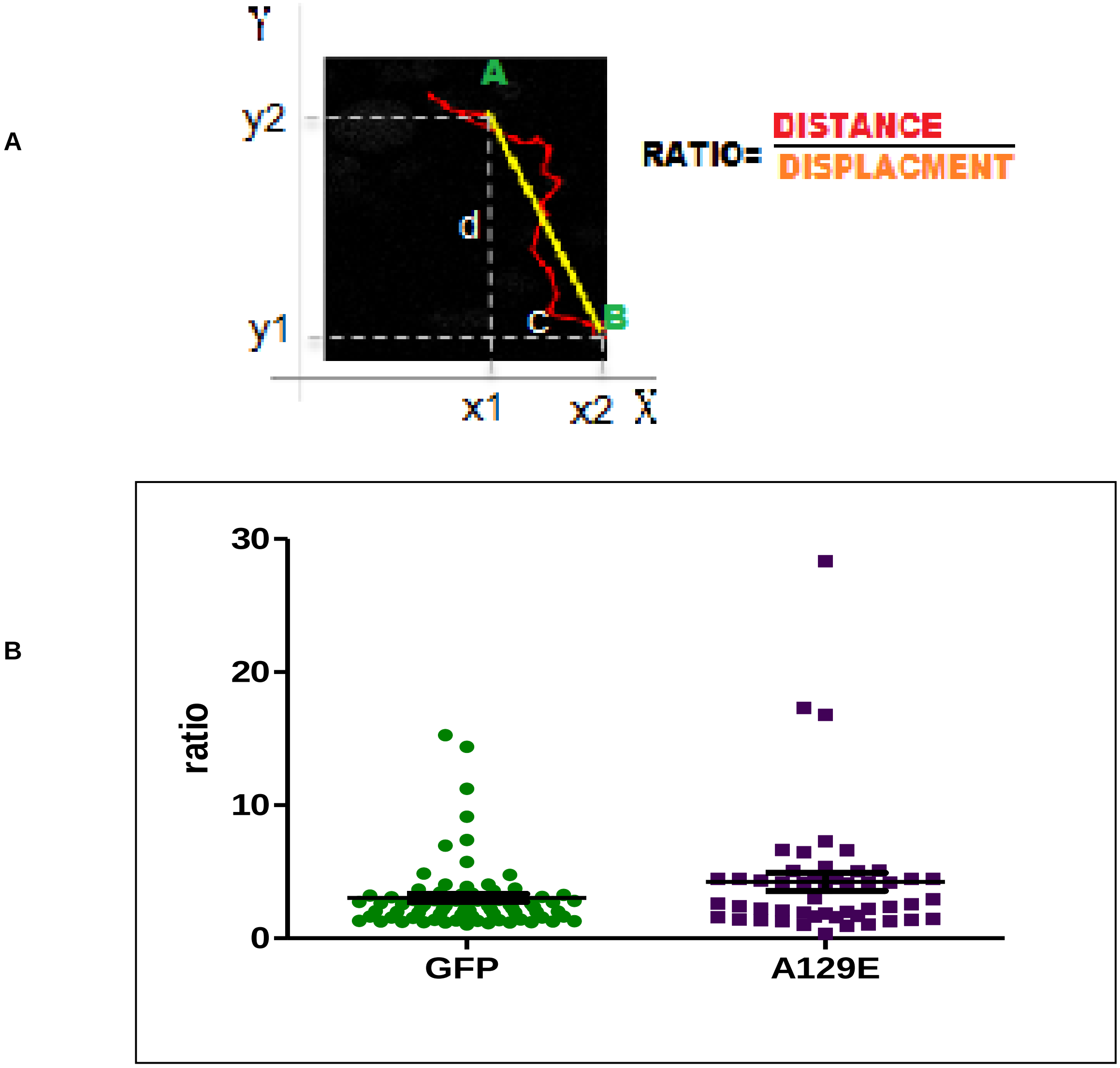
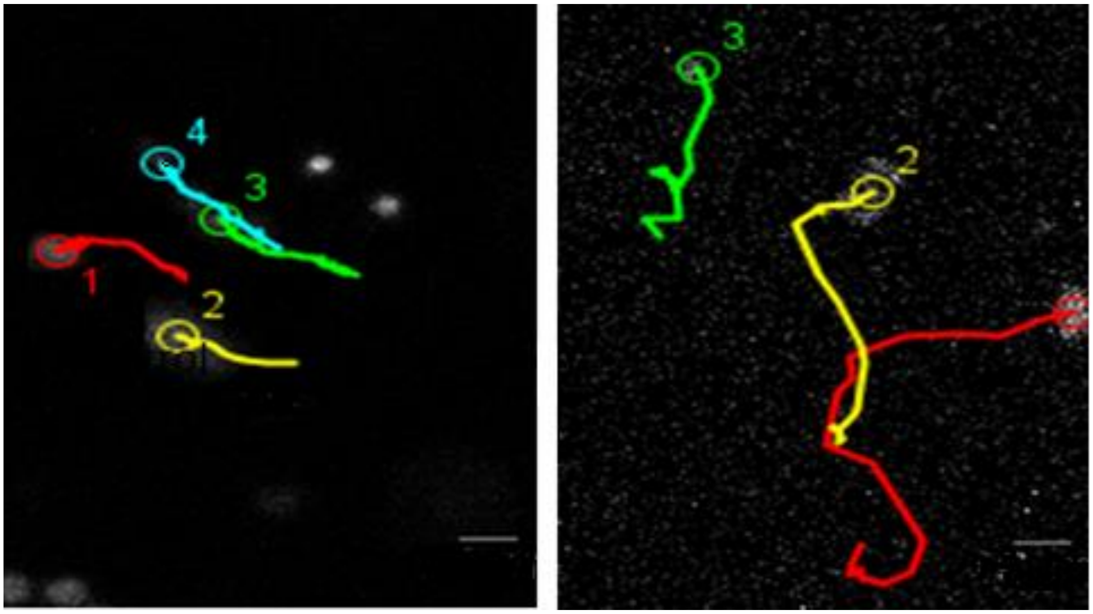


Figure 3. 5 *A* Cell trajectories calculation.

Cell trajectory was calculated as ratio of the distance travelled by a cell (red line) to the cell's displacement (yellow line). Points A and B represent start and end of the cell track respectively. ***B*. Analysis of cell directionality** is obtained as a distance to the displacement ratio. Each point on the chart represents cell ratio; green points are the ratios of control line and purples are aPKC*i* A129E mutant. Difference in ratio between groups is not statistically significant. p = 0.067.



control

A129 E

Figure 3. 6 Cellular trajectories.

Cellular trajectories appeared as a result of cell track using Image J software tool. Colored lines are NMuMG ERbB2 cells trajectories. Left panel shows cell trajectories in the control line; Right panel shows cell trajectories of the aPKCi A129E line. The scale bars represent 15µm.

3.2.2 Persistence of cell invasion

The ability of aPKCi-A129E-expressing cells to invade farther could also be due to differences in invasive persistence. To investigate this possibility I employed two complementary approaches. I first evaluated cell movement persistence by calculating the distance-to-displacement ratio (Figure 3.5 *A* and *B*). Second, I determined the directionality of the cell movement by analyzing changes in direction during invasion (Figure 3.7).

To evaluate cell movement within the collagen type I matrix, I calculated the ratio between the actual distance a given cell travels and the displacement the cell experiences in space (Figure 3.5 *A*). For example, a smaller ratio will be obtained if a cell travels in a more targeted trajectory, persistent moving. This is because this cell will need to move for the minimal actual distance to obtain the largest displacement. On the other hand, a larger ratio will indicate that a cell is moving in a non-targeted manner, non-persistent moving. Such a cell will move a large actual distance with a minimal displacement. Thus, this method of assessing cellular movement distinguishes between cells with a persistent or targeted trajectory, from those that move in a non-persistent, random way.

My analysis reveals that the control MNuMG-ErbB2-GFP cells had an average distance to displacement ratio of 3.0 and the mutant cells had a ratio of 4.2 (Figure 3.5 *B*). Although there was a modest difference between the two cell types, it was not significantly different statistically $p = 0.057$.

As a complementary approach, I analysed dynamics of directional cell invasion through collagen type I gels. To do so, I calculated the direction changes of invading control and aPKCι-A129E-expressing cells. Direction changes were designated into 4 groups: Group I, 0^0 to 44^0; Group II, 45^0 to 89^0; Group III, 90^0 to 134^0, and Group IV, 135^0-180^0 angles. After a direction change, the new forward direction was set to $0^{0.}$ In this way, cells, whose change of angle fell in the range of Group I, from 0^0 to 44^0, were considered to continue moving forward. Furthermore, the cells with the change of angles from Group II, 45^0 to 89^0 and 90^0 to 134^0, Groups II and III, were considered to turn left or right. Cells with the angle change of 135^0 to 180^0, Group IV, were considered to have a directional movement that was reversed (Figure 3.7).

I observed that the majority of the displacement angles fell into Group I (0^0 to 44^0) for both the control NMuMG ErbB2 cells (63%) and the mutant (54%) cells, indicating that cells predominantly migrate forward. However, only 7% control changes of angles and 11% of mutant belonged to the Group IV (135^0 to 180^0). Finally, 28% control and 34% of mutant displacement angles were found in Group II (45^0 to 89^0) and Group III (90^0 to 134^0). However, there was no significant difference in the changes of angles between the control and mutant cells in either of the four groups $p = 0.59$. Therefore, these observations indicate aPKCί-A129E expression did not alter the turning dynamics of cells. Collectively, this demonstrates that aPKCι does not have a major effect on the persistence of cell invasion under these conditions.

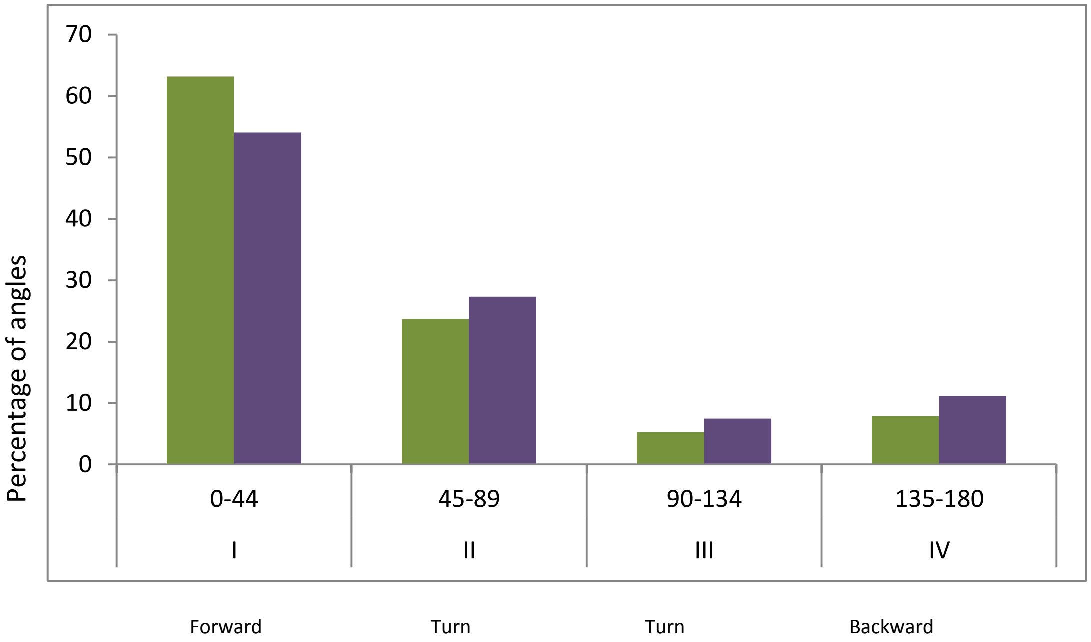


Figure 3. 7 Direction changes of invading cells.

Percentages of angles designated on 4 groups. Green represents control NMuMG-ErbB2-GFP cells; purple represents the aPKC*i* A129E mutant cells. Data shows no differences in cell directionality. p Value = 0.59

3.3 Cell morphology analysis

Actin filaments make a part of a cell cytoskeleton, playing a central role in cell morphology and participate in cell migration processes [155]. Actin polymerisation drives protrusion formation and, by making a part of actomyosin complex, participates in cell contraction process. Hence I next aimed at investigating the effects of aPKCι overexpression on the cellular morphology of NMuMG ErbB2 cells.

3.3.1 Actin cytoskeleton in aPKCι overexpressing NMuMG ErbB2 cells

I first intended to examine the architecture of the actin cytoskeleton with the mutant cells and compared it to the control NMuMG-ErbB2-GFP cells. To achieve this I froze the Collagen type I matrices that contained the invading aPKCι overexpressing or the control NMuMG ErbB2 cells, sectioned and stained the slides with phalloidin (Figure 3.8). Interestingly, I noticed that the mutant cells formed longer and thinner cell protrusions compared to those formed in the control cell line (Figure 3.8). This observation led me postulate that the aPKCι overexpression in the NMuMG ErbB2 cells influenced the actin cytoskeleton reorganization in these cells.

After observing the effect of the aPKCι overexpression on the actin cytoskeleton in the NMuMG ErbB2 cells, I investigated whether similar results would be obtained in and independent cell system. For this experiment I chose another breast cancer cell line, NeuNDL. Similar to previously described approach, the NeuNDL cells were infected with the lentiviruse expressing GFP alone, control, or with a constitutively active form of aPKCι in addition to the GFP, aPKCι-A129E. These cell lines were then embedded into the collagen type I matrices. In

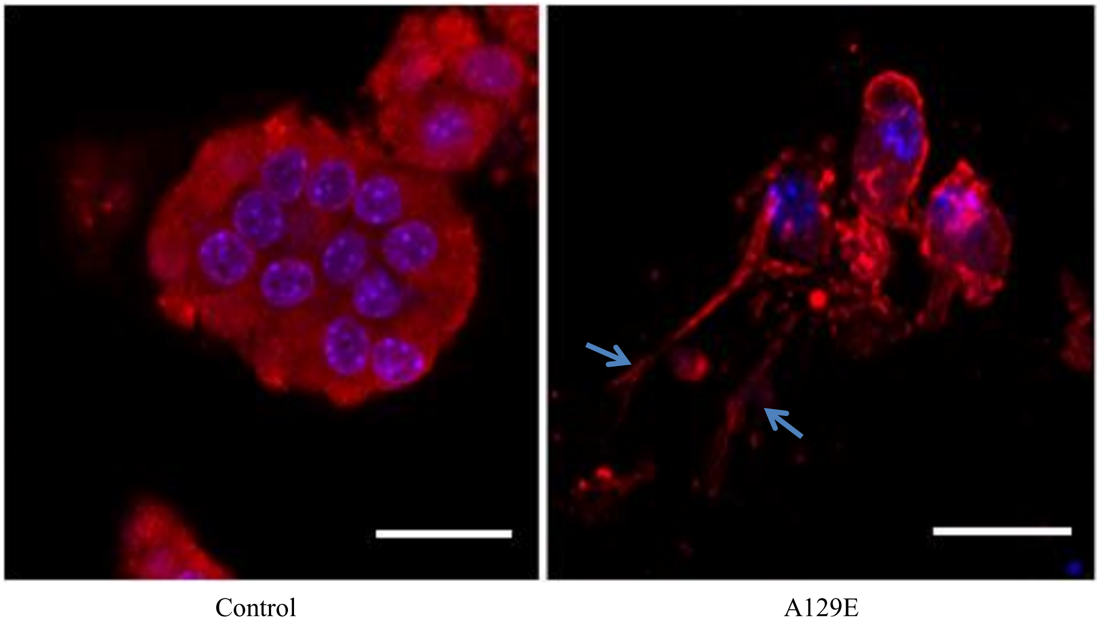
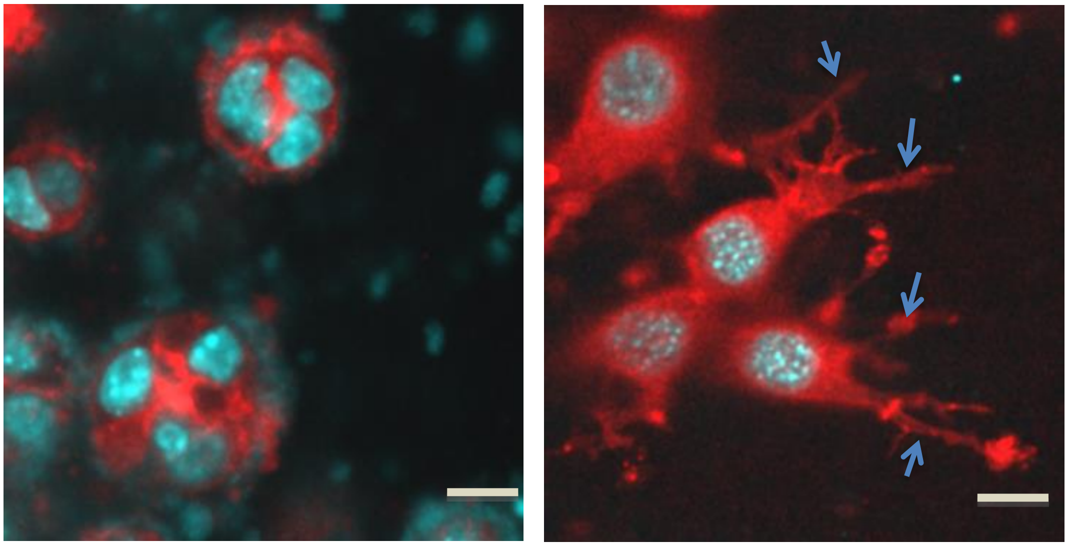


Figure 3. 8 Actin stained cells.

Immunostaining of NMuMG ERbB2 cells 72 h post cell embedding into collagen type I matrix. Left panel shows control cells. Right panel shows image of aPKC*i* A129E cells with reorganization of actin filaments and arrow pointed cell protrusions formation. The scale bar represents 10μm. Hoechst 33342 - blue; Phalloidin – red.



CONTROL A129E

Figure 3. 9 Actin stained Neu NDL cells embedded

Neu NDL cells embedded into collagen type I 72 h following invasion. Left panel is Neu NDL control cell line. Right panel is aPKC*i* A129E cells, with reorganization of actin filaments and arrows pointed cell protrusions formation. Hoechst 33342-blue; Phalloidin-red.

72 hours post-embedding the matrices containing the aPKCι overexpressed or the control NeuNDL cells were frozen and stained with phalloidin (Figure 3.9).

I found that the overexpression of aPKCι in the NeuNDL cells resulted in the formation of longer and thinner protrusions similar to those observed in the NMuMG ErbB2 cell system. Taken together, this indicates that aPKCι protein plays an important role in the actin cytoskeleton architecture in at least two independent breast cancer cell lines.

3.3.2 Effect of aPKCι on cell morphology

Previously it has been shown that aPKC regulate several aspects of neuronal migration [156]. Thus aPKC controls cell polarity in migrating astrocytes [157] and Par6-Par3-aPKC complex regulates orientation of the protrusions [158] , these results suggest the involvement of these protrusions in cell migration.

My previous results indicate that the overexpression of aPKCι in breast cancer cell lines leads to longer and thinner cellular protrusions. I thus aimed at quantifying this result by counting the NMuMG ErbB2 cells with and without protrusions (Figure 3.10 *A* and *B*). Furthermore, I divided the cells lacking protrusions into mesenchymal and rounded subcategories since, it has been shown that differently shaped cells have different migration mode [34, 159](Figure 3.10 *A*). I also distinguished the cells possessing one protrusion from those with two or more protrusions (Figure 3.10 *A*).

My analysis shows that 20% of aPKCι overexpressing cells displayed one protrusion compared to only 3% of the control NMuMG ErbB2 cells. Furthermore, 45% of the aPKCι overexpressing cells displayed multiple protrusions, whereas none of the control NMuMG ErbB2 cells had this feature (Figure 3.10 *B*). This data is statistically significant, p = 6.27x10(superscript)-41. Collectively these results show that the overexpression of aPKCi protein alters the cytoskeleton architecture of cells by stimulating the formation of cellular protrusions. This, indicates that overexpression of aPKCi changes the invasive cell phenotype.

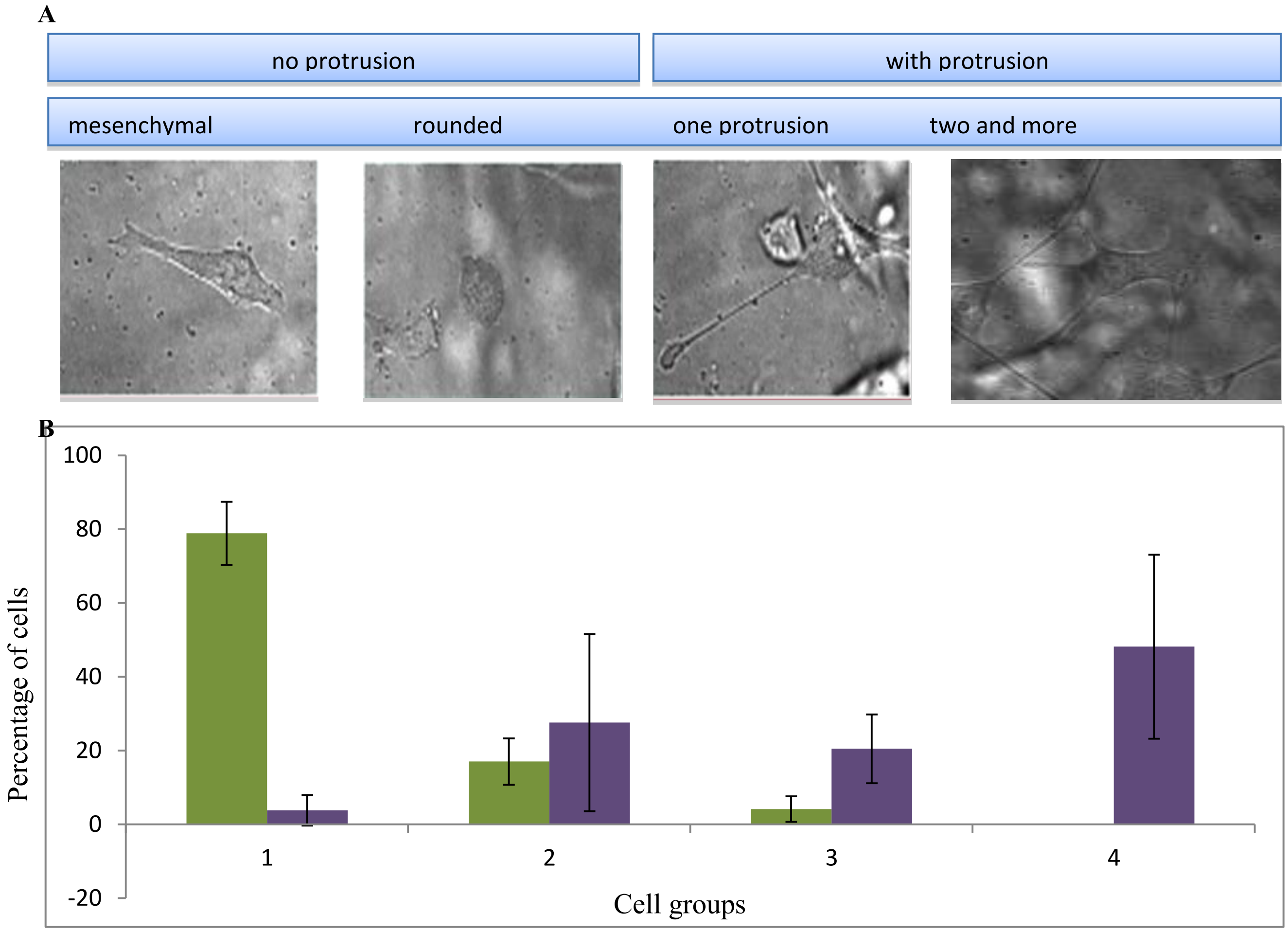


Figure 3. 10 *A*. Based on the morphology cells are designated on 4 groups.

Cells of group 1 and group 2 does not have protrusions, group 3 represents cells with one protrusion and cells of group 4 forms multiple protrusions. *B.* Percentages of cells per group. Green rectangles represent percentages of the control cells; purple rectangles represent percentages of the aPKC*i* A129E mutant cells. Mutant line forms distinct phenotype. The error bars show standard deviation.

p = 6.27E-41.

3.3.3 Cells with different morphologies have different velocities

To assess whether the morphologic changes in cells induced by aPKCι might explain the increased velocity of aPKCι-A129E cells, I measured the velocities of individual cells with different morphologies as they invade collagen type I matrix. Thus overlaying the differential interference contrast (DIC) and fluorescent images allowed visualization of invading cells morphology and their trajectories with displayed velocity. Mesenchymal cells making only one protrusion during the entire imaging time were considered as cells with one protrusion. However during invasion some of the cells change their morphology from mesenchymal to forming one or several protrusions. Then these cells were assigned to the group with one or group with multiple protrusions respectively.

Interestingly, I discovered that the mesenchymal, rounded, cell with a single protrusion and cells with multiple protrusions moved at different velocities (Figure 3.11). Previously it has also been shown that morphologically different cells invade in different mode [34, 60, 159] and amoeboid cells move 10–30-fold faster than mesenchymal [36].

The mesenchymal cells exhibited the slowest speed in both the control NMuMG ErbB2 (0.001+/-0.00005 μm/s) and the aPKCι overexpressing (0.0016+/- 0.0001 μm/s) cell lines. The aPKCι overexpressing cells with one protrusion moved at the velocity of 0.002 μm/s, whereas these cells with multiple protrusions had the speed of 0.004 μm/s. Importantly, I found a statistically significant correlation between the number of protrusions a cell has and its velocity. R = 0.959

Of note is that the control NMuMG ERbB2 cells with one protrusion are very rare and constituted only 3% of the population. Additionally, none of these cells remained RFP positive making them impossible to track. The rounded cells lacked the ability to move in the collagen type I matrix. Therefore, these groups of cells were not included in the velocity analysis.

Interestingly, that during the invasion some mutant cells change their shape from mesenchymal to having one and/or multiple protrusions; therefore, I repeated the correlation analysis by looking on cell speed by splitting cell movement on the intervals depending on cell shape (Figure 3.12). Thus every point on this chart represents the time interval during which cell retain only one of the shapes. The mesenchymal cells exhibited speed 0.001761 ± 0.0001μm/s, cells with one protrusion moved at the velocity of 0.001872 ± 0.0002μm/s, whereas the cells with multiple protrusions had the speed of 0.004588 ± 0.0004μm/s. As the previous data (Figure 3.11), this result represents the correlation existence between the different cell morphologies and cell velocity.

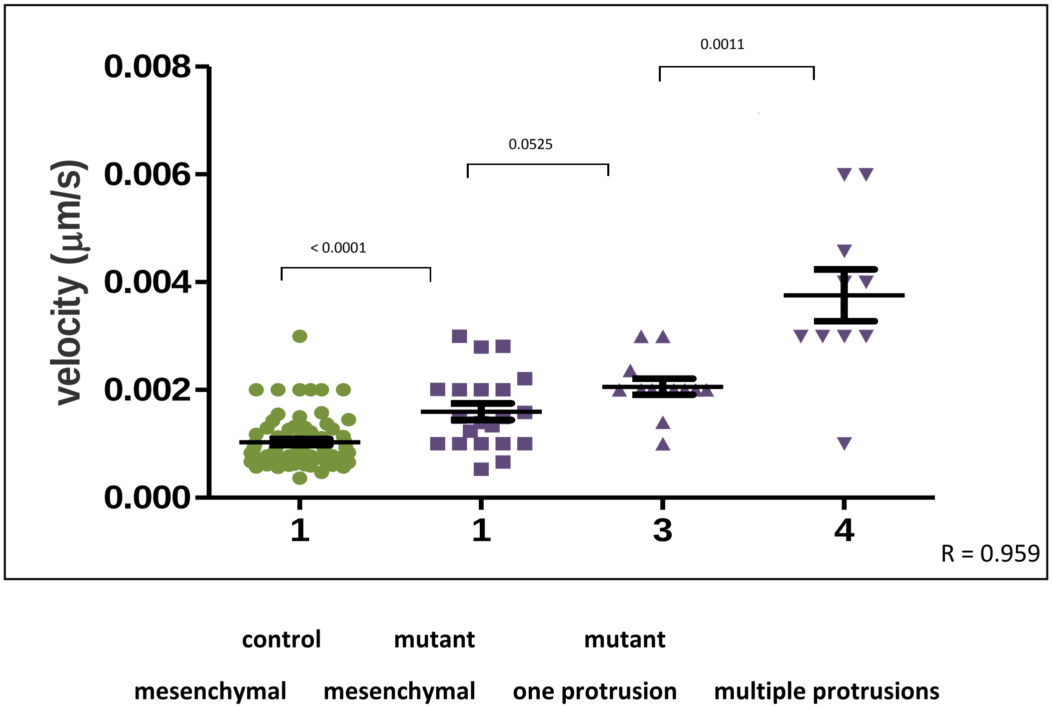


Figure 3. 11 Cell phenotype and cell velocity correlation.

Cell groups have different velocity and the velocity correlates with the cell morphology. Green points (group 1) are mesenchymal cells formed by the control group cell line. Purple color marks mutant cells. Purple squares (group 1) are mesenchymal cells of the mutant line; purple triangles (group 3) show the cells formed one protrusion and purple triangles (group 4) represent cells with multiple protrusions. Numbers above show p values; the error bars represent mean +/- SEM. Correlation coefficient R = 0.959

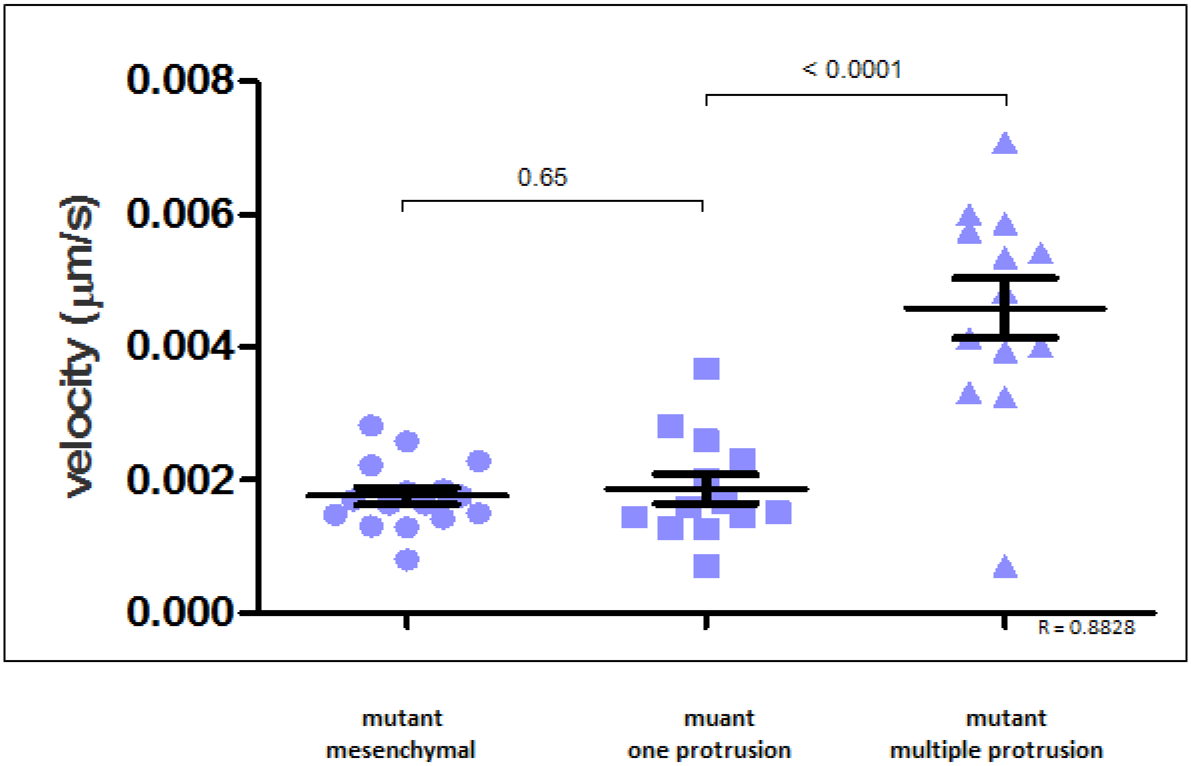


Figure 3. 12 Cell shape and velocity correlation

Each point represents one cell of aPKC*i* A129E cell line with constant morphology during a period of time and its speed can be found on Y – axis. Rounded group are mesenchymal cells, the squared dots represent cells with one protrusion and the triangles are cells with multiple protrusions. Numbers above show p values; the error bars represent mean +/- SEM. Cell shape correlates with the cell velocity. R = 0.8828

3.4 Molecular pathways

Previously, I showed that overexpression of aPKCἰ influences protrusion formation but the molecular pathway still need to be elucidated.

Since Rac1 protein is a known regulator of actin dynamics [160-162] and inducer of podia formation [100, 163], I hypothesize that aPKCἰ may influence cell protrusion formation and invasion via Rac1.

To test if Rac1 is required for protrusion formation I treated the cells embedded into collagen type I with Rac1 inhibitor (Figure 3.13). Thus the cells plated into collagen type I matrix were left for 24 hours to allow the cells to invade. After that, the Rac1 (NSC23766) inhibitor was added on top of the invading cells and left for an additional 24 hours. Analysis of the treatment was done by confocal microscopy.

The results indicated no difference in protrusion formation between control and mutant cell lines, suggesting that aPKCἰ does not influence protrusion formation via Rac1. However, based on the literature, NSC 23766 blocks Rac1 activation by inhibiting select Rho-GEFs, particularly TIAM1 and TRIO, but is inactive against others. Thus, the role of Rac1activated by other GEFs cannot be excluded. It has been shown that aPKCι–Par6–ECT2 complex drives non-small cell lung cancer (NSCLC) cell transformation [164].

Additionally, it has been shown that partitioning-defective (PAR) proteins modulate the actomyosin complex and microtubule cytoskeletons [165]. Thus another protein via which aPKCἰ can influence cell protrusion formation and invasion is myosin. Myosin protein is a major contractile protein that binds actin filament, forming cytoskeleton complex. In such a way a specific myosin II inhibitor, Blebbistatin, was added to the cells emebeded into the collagen type I 24 hours post initiation of invasion with following cells phalloidin staining. (Figure 3.14).

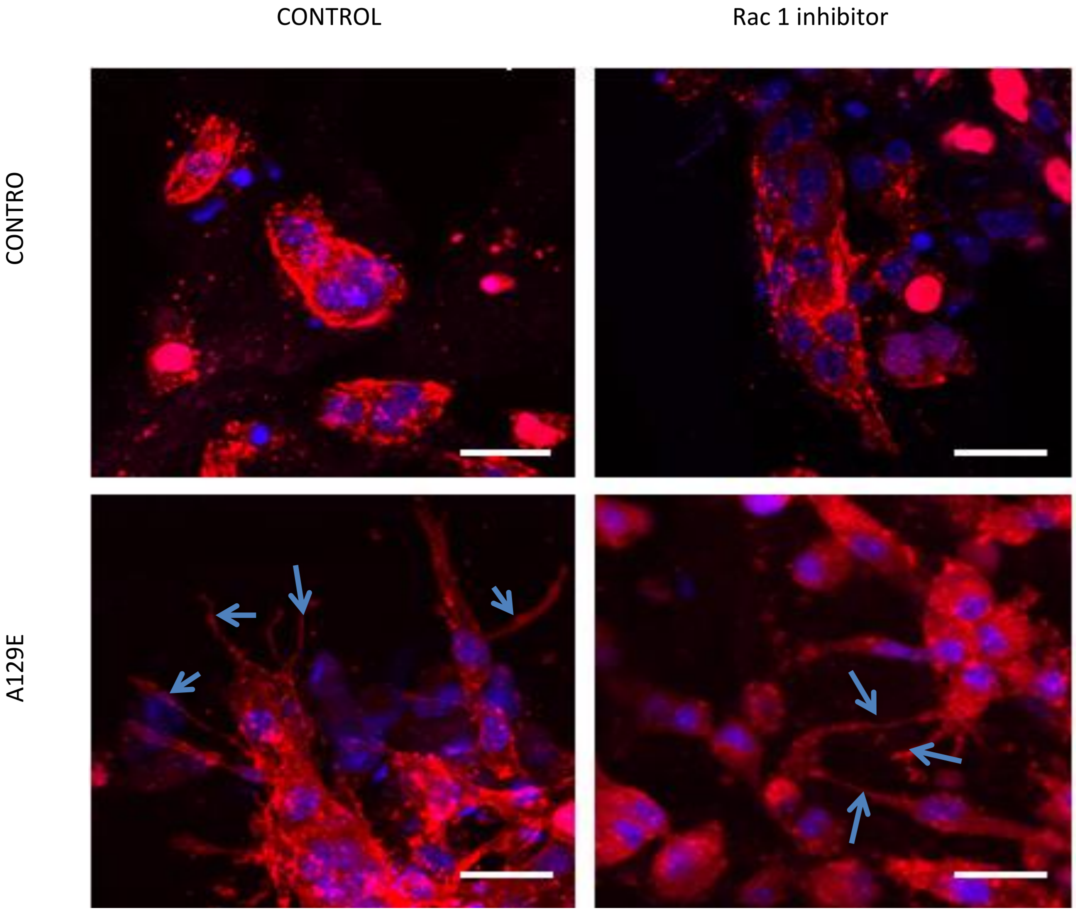


Figure 3. 13 Immunostaining of NMuMG ErbB2 cells embedded into collagen type I matrix and treated with Rac 1 inhibitor (NCS 23766).

Left panel shows untreated control (upper image) and untreated aPKCi A129E mutant cells (bottom image). Right panel (upper image) shows Rac1 inhibitor treated control cells after 24h of treatment and Rac1 inhibitor treated aPKCi A129E cells (bottom image). Arrows point out the protrusions. The scale bar represents 20μm. Hoechst 33342 - blue; Phalloidin – red.

Sections with blebbistatin did not greatly influence protrusion formation in the mutant line, only some slight deformation and micro-spike formation was observed. However, following blebbistatin treatment of the control cell line, cells formed protrusions. Interestingly, this phenotype was previously inherent only to the mutant line. This result demonstrates that Blebbistatin treatment affected cell protrusion formation with statistically significant difference. P = 0.002514. (Figure 3.14).

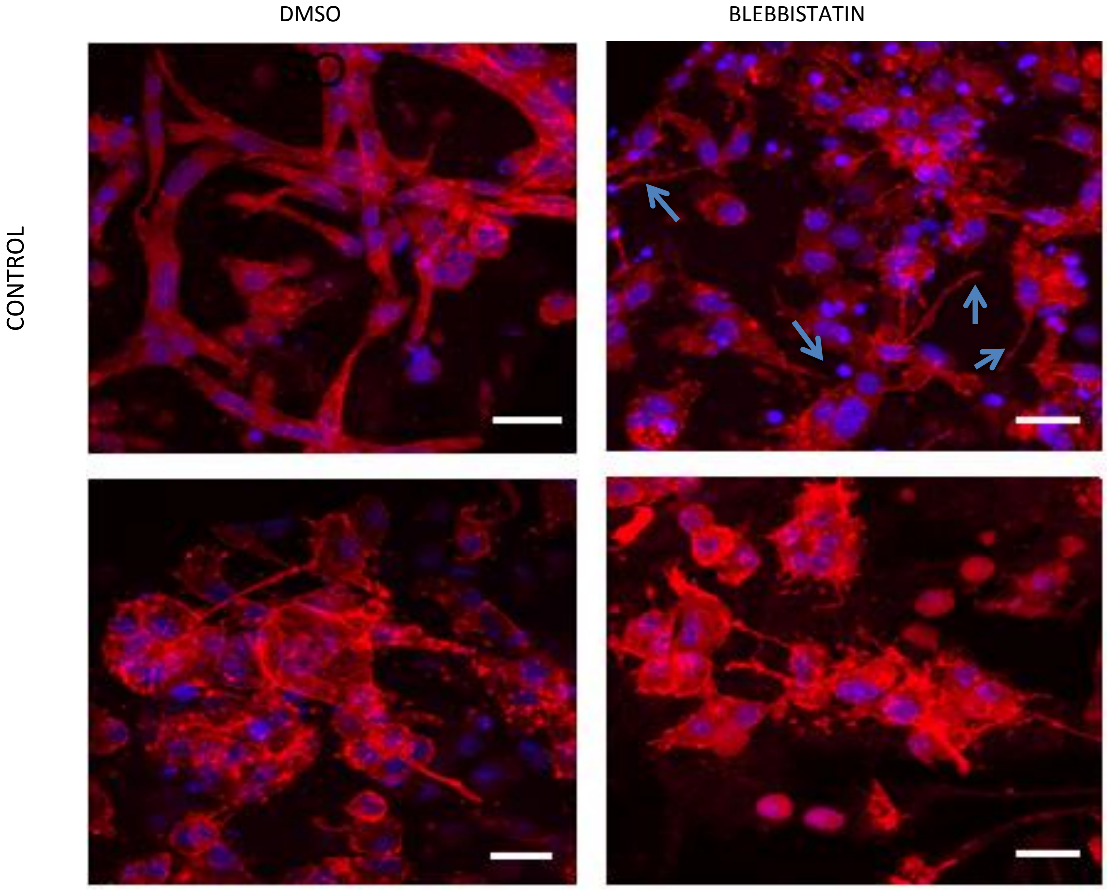


Figure 3. 14 Immunostaining of NMuMG ErbB2 cells embedded into collagen type I matrix and treated with blebbistatin

Left panel shows untreated control cells (upper image) and untreated aPKCi A129E mutant cells (bottom image), however DMSO vehicle used. Right panel shows images after 24 hours of treatment, blebbistatin treated control cells (upper image) and blebbistatin treated aPKCi A129E (bottom image). Following blebbistatin treatment of the control cell line, cells formed protrusions. Arrows show the protrusions appeared in control blebbistatin treated cells. *Chi*-test p = 0.002514. The scale bar represents 20μm. Hoechest - blue; Phalloidin – red.

Chapter 4 Discussion and Conclusion

An important aspect in the malignant breast cancer progression is the ability of tumour cells to invade trough the surrounding extracellular matrix (ECM) and stromal cells in the local tumour microenvironment. The mechanism of cancer invasion is comprised of the changes in both the intrinsic cell properties and the cell microenvironment that happen as a result of the disease progression [60]. As described above aPKCἰ is overexpressed in many cancers including breast cancer [122-131] as well as correlates with poor prognosis and increases cancer cell invasion [142-143].

Previous studies have shown that aPKCἰ is not only overexpressed but is also found activated in some cancers. Particularly, Paget's group found that aPKCἰ protein is activated by PI3K phosphorylation in breast cancer cells [140]. Balendran et al showed that activation of aPKC λ/ι requires phosphorylation of the threonine residue 403 by 3-phosphoinositide-dependent kinase 1 (PDK1) within the activation loop [166]. In addition Rodriguez and colleagues have shown that aPKC activation is required for migration of Src-transformed cells, where aPKC is activated as a result of its tyrosine phosphorylation [147]. Moreover *in vivo* experiments by Wooten et al., revealed that aPKCι activation requires the tyrosine phosphorylation by Src on residue 325 [167]. Thus, aPKCι have a variety of phosphorylation sites and require different proteins for its activation. In our study, we employed an aPKCι mutant in which the alanine 129 was substituted by a glutamic acid residue. This mutation allows a conformational change of the protein and as a result a release of the kinase domain making a number of amino acid residues available for phosphorylation. The limitation of this mutant is the inability to control the exact residue that will be phosphorylated. Additionally, it is difficult to predict which kinase is responsible for the phosphorylation of the aPKCι mutant. To overcome these limitations, a number of aPKC mutants with amino acid substitutions mimicking constitutive phosphorylation can be engineered.

To evaluate the role of aPKCι in cancer cell invasion I observed the ability of cancer cells to invade in the 3D collagen assays. I first showed that in both the control NMuMG-ErbB2-GFP and the NMuMG-ErbB2-aPKCι-A129E-GFP cell lines only about 35 % of cells were capable of invading the surrounding collagen matrix. Interestingly, I observed that even though there was no difference in the number of invading cells between the control NMuMG-ErbB2-GFP and the NMuMG-ErbB2-aPKCι-A129E-GFP cells, the aPKCι overexpressing cells were capable of invading further distances into the surrounding matrix. This result suggests that aPKCι enhances the invasive properties of the cells, which already possess an invasive phenotype. Consistent with our results, an i*n vivo* study by Sahai and colleges showed, that only a small proportion but not all of the tumour cells can gain motility (<0.1% of tumour cells *in vivo* per hour) [39]. Thus it was shown that the changes in cancer cell behaviour and the possibility of the cells to gain the motile property can be explained as a combination of cell intrinsic modifications and external changes in ECM, which provoke the cell motility response but only in the cells that are able to respond [39]. In addition, an aPKC involvement in ECM regulation has been shown by McCaffrey and colleagues. They found that PAR3 depletion is associated with increased MMP9 matrix degradation, which also correlates with active aPKC and cancer cell invasion [149]. Moreover, Litherland assessed the role of aPKC in ECM remodeling by the induction of collagenases, matrix metalloproteinase (MMP)-1 and MMP-13 by IL-1 [168].

We found that aPKCι overexpression increases the speed at which cells move within the 3D collagen matrix. Also, we discovered that the aPKCι overexpression changed cellular morphology that has previously been reported by Rodriguez in Src-transformed cells [147]. Finally we observed a correlation between the cell speed and the number of cellular protrusion.

We found that aPKCι overexpression increased the invasive speed of the cells *in vitro* by 2-fold. In fact, the control NMuMG-ErbB2-GFP cells invaded the collagen matrix with an average velocity of 0.06 μm/m, whereas NMuMG-ErbB2-aPKCι-A129E-GFP counterpart did so with an average velocity of 0.14 μm/m.

Wolf and Friedl have previously shown that cancer cells undergoing mesenchymal migration have velocity of 0.1 to 2 μm/m [159]. However my data shows that the cells undergoing mesenchymal migration in the mutant line have velocity of 0.096+/-0.009 μm/m and the cells from the control line have speed 0.06+/-0.003 μm/m. In addition our data revealed statistically significant difference in speed between mesenchymal cells of both cell line groups (Figure 4.11). This data supports that aPKCι overexpression might influence breast cancer cell motility.

It has been shown that the cell protrusions define the mode of cell migration and that during development, as well as in certain physiological situations, cells may alter their mode of invasion [169]. Consistently, Wolf *et al.* showed that many cancer cell lines are able to change their mode of migration, switching from the lamellipodia-based mesenchymal mode to the amoeboid mode at different velocities [66]. Therefore, in our study we aimed to investigate if overexpression of aPKCι in breast cancer cells will change cell morphology and speed. In fact, I observed that the aPKCι overexpressing cells exist in following shapes: mesenchymal, rounded, and cells that have a single or multiple protrusions. Additionally, I found out that the cells of different morphologies move at different velocities within a collagen matrix. Interestingly, I noticed a direct correlation between the number of cellular protrusions and the speed of cells. In fact, whereas the mesenchymally shaped cells moved at a relatively slow speed of 0.096 μm/m, the cells with multiple protrusions demonstrated a significantly higher average speed of 0.24 μm/m. Further statistical analyses of these data revealed a strong correlation between the cell shape and its speed. Thus I show that cells change the velocities depending on their morphology that is consistent with the results received by Wolf and colleagues.

To elucidate a potential molecular mechanisms of the aPKCι influence on cell speed and protrusion formation we used small molecule inhibitors. Previously, a number of publications have shown Rac1 involvement in the organization of the actin cytoskeleton [160-162], and cell protrusion formation [100, 163].

Thus, we have applied a Rac1 inhibitor (NSC 23766) on the control NMuMG-ErbB2-GFP and the NMuMG-ErbB2-aPKCi-A129E-GFP cells embedded into the collagen type I matrix. However, after the application of NSC 23766 on the cells I noticed no differences in cell protrusion formation between the control and the mutant cell lines, suggesting that aPKCi does not influence protrusion formation via Rac molecular pathway. However, based on previous studies, NSC 23766 blocks Rac1 pathway activation by inhibiting select Rho-GEFs, particularly TIAM1 and TRIO [170], but is inactive against others. Therefore, Rac1 activated by TIAM and TRIO is unlikely involved in aPKCi-mediated protrusion formation and invasion of breast cancer cells. However, the role of Rac1 on cellular protrusion formation cannot be completely excluded. Therefore, Rac1 specific shRNA or a broader Rac1 inhibitor would further define a role of Rac1 in cell motility and protrusion formation in breast cancer cells in future investigation. For example, a novel Rac1 inhibitor (EHT 1864) was introduced for the research purposes and its further usage in our research may help to shed a light on the molecular mechanisms involved in cell invasion [171, 172].

Additionally, another protein via which aPKCi can influence cell protrusion formation and invasion is myosin. In fact, myosin is a major contractile protein that binds actin filament, forming a cytoskeleton complex. Therefore, we utilized a specific myosin II inhibitor, Blebbistatin, on the cells embedded into the collagen. Following this experiment, we have only been able to observe some minor changes on the protrusion formation, characterized by a deformation and micro-spike formation, in the mutant cell line and long protrusions were formed in the control cell line.

Previous work has shown that a blebbistatin treatment promotes the formation of the cellular projections in neurons, neurites, via regulating the activity of myosin II [173]. Similarly, our data shows that the control NMuMG-ErbB2-GFP cell line formed protrusions in response to the blebbistatin treatment. As both the blebbistatin treatment and the aPKCι overexpression in the NMuMG-ErbB2-GFP cells led to a similar phenotype, the increase in the protrusion formation, I hypothesized that aPKCι potentially inhibits the myosin function. This hypothesis is supported by data obtained by Ishiuchi and collegues, who found that the Par3-aPKC-ROCK pathway controls epithelial apical morphology [174]. They showed that aPKC phosphorylates ROCK. However, ROCK is known to phosphorylate myosin by inactivating myosin phosphatase, leading to promotion of myosin/actin interaction and as a result stress fiber development and contraction [175]. In summary, I hypothesize that aPKC may inhibit myosin via ROCK and therefore influence cell invasion and protrusion formation. However, further research needs to be done to reveal a complete pathway of protrusion formation by aPKCι.

4.1 Conclusions and perspectives

There are many reasons to believe that the future research will improve our understanding of cancer cell motility and will identify new molecular players that regulate the spread of cancer by affecting cell motility. The work presented in this thesis has established that overexpression of aPKCι influences cell motility by increasing the cell speed by 2-fold, which strongly correlates with a change in cellular phenotype exhibiting extensive cell protrusions. Currently the drug representing an aPKC inhibitor, aurothiomalate, is in the phase I clinical trial for advanced non-small-cell lung, ovarian and pancreatic cancers [176]. However our data show that aPKC influences the speed of the invading cancer cells, it does not provide full information on how aPKC influences the cancer cell motility and invasion. Therefore more studies are required to elucidate the exact molecular mechanism through which aPKCι regulates tumour cell protrusion formation and invasion.

Finally, the understanding of this mechanism will help in the development of new therapeutics that will target cancer cell motility by restricting cancer cell spread and may lead to better treatment for cancer in the clinic.

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