The role of PKCα localisation in prostate cancer

Honours Proposal

Amanda Oliver

Supervisor: Michelle Hill

Co-supervisor: Rob Parton

Statement of Authorship

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I, Amanda Oliver confirm that the work presented in this research proposal has been performed and interpreted solely by myself except where explicitly identified to the contrary*. I confirm that this work is submitted in partial fulfilment for the degree of BSc Hons in Biomedical Science and has not been submitted elsewhere in any other form for the fulfilment of any other degree or qualification.

Word Count (excluding reference list, fig. Legends, tables and appendices): 4,320

Dated: 01/04/2014

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* A student may often find that some work may be performed on their behalf. For example, histology preparations may be created by a member of the technical staff or surgery may be performed by another member of a laboratory. Such assistance must be accurately attributed and acknowledged.

I, Michelle Hill confirm that I have seen a copy of the work presented in this research proposal as the supervisor of Amanda Oliver.

Date: 01/04/2014

Signature: 

Contents

List of Abbreviations

CAV1, CAV2, CAV3	Caveolin -1, -2, -3
CKAP4	Cytoskeleton associated protein 4
cPKC, aPKC, nPKC	Conventional PKC, atypical PKC, novel PKC
DAG	Diacylglycerol
DAPI	4’,6-diamindino-2-phenylindole
DNA	Deoxyribonucleic acid
DRF	Detergent-resistant fraction
DRM	Detergent-resistant membrane
ECM	Extracellular matrix
ERM	Ezrin, radixin and moesin
GFP	Green fluorescent protein
MMP-9	Matrix metalloproteinase 9
MURC	Muscle-related coiled coil protein – referred to as cavin-4
PBS	Phosphate buffered saline
PINA	Protein Interaction Network Analysis
PKC, PKCα, PKCδ, PKCθ	Protein Kinase C – alpha, delta, theta
PKCDBP	Protein Kinase C Binding Protein – referred to as cavin-3
PPI	Protein-protein interactions
PS	Phosphatidylserine
PTRF	Polymerase transcript release factor – referred to as cavin-1
RACK	Receptors for activated C-kinase
SDPR	Serum deprivation response protein – referred to as cavin-2
TNE	Tris, NaCl, Edetic acid (EDTA)

The role of PKCα localisation in prostate cancer

Overview

Prostate cancer is responsible for 6% of cancer related deaths in males worldwide (Jemal et al., 2011). As a localised tumour progresses into an advanced, metastatic tumour the chance of survival decreases (Kirby et al., 2011). Recent studies have shown that the structural proteins in caveolae, caveolins and cavins, are commonly altered in prostate cancer (Gould et al., 2010; Hill et al., 2008; Tahir et al., 2009). Caveolin-1 (CAV1) is associated with aggressive, late stage prostate cancer and is thought to promote tumour progression (Li et al., 2001). However, co-expression of cavin-1 has been shown to attenuate the tumour promoting effects of CAV1, which is partly due to a reduction in migration (Aung et al., 2011; Moon et al., 2013). A known regulator of cell migration, protein kinase C α (PKCα) is recruited to caveolae in prostate cancer cells (Hill et al., 2012). Cavin-1, cavin-2 and cavin-3 have all been identified as PKC binding proteins (Hill et al., 2012; Izumi et al., 1997; Mineo et al., 1998). In addition, there is evidence that cavin-1 alone is sufficient to recruit PKCα to lipid rafts (Hill et al., 2012). Therefore this project aims to further investigate the recruitment of PKCα to caveolae and its impact on cell migration in prostate cancer.

Background

Prostate cancer

Prostate cancer is the second most diagnosed cancer and the sixth leading cause of cancer deaths in the male population (Jemal et al., 2011). Advanced prostate tumours are difficult to treat and are therefore associated with low survival rates (Kirby et al., 2011). Characteristics of advanced prostate tumours include androgen independence and the

ability to metastasize (Rini and Small, 2002; Sturge et al., 2011). Metastatic cells typically develop alterations in their morphology, have reduced contact to their surroundings and as a result show increased migration (Hanahan and Weinberg, 2011). The plasma membrane is a key player in maintaining contact-dependent growth and regulating migration. Specific membrane microdomains called caveolae have been implicated in the progression of prostate cancer.

Caveolae: a membrane microdomain implicated in prostate cancer

Caveolae are 60-80nm diameter, flask-shaped invaginations on the plasma membrane (Parton and del Pozo, 2013; Parton and Simons, 2007). Membrane composition is crucial for caveolae formation, cholesterol depletion has been found to perturb caveolae structure (Hailstones et al., 1998). Caveolae are a type of lipid raft, membrane microdomains rich in cholesterol, sphingolipids and lipid anchored proteins. Lipid rafts exhibit the biochemical property of resistance to solubilisation by non-ionic detergents (Lingwood and Simons, 2007). Therefore, they are commonly isolated as detergent-resistant membranes (DRMs) however this method does not separate planar lipid rafts from caveolae. The abundance of caveolae varies between cell types, for example, fibroblasts, adipocytes and endothelial cells are rich in plasma membrane caveolae (Parton and del Pozo, 2013). A major cellular function of caveolae is in endocytosis, which is partly regulated by PKC (Prevostel et al., 2000). Other functions include calcium signaling, lipid regulation and mechanosensing (Le Lay et al., 2006; Rizzo et al., 2003; Wang et al., 2005). Additionally, various signalling molecules, including PKC, are enriched in lipid rafts showing a key role in signal transduction (Simons and Toomre, 2000).

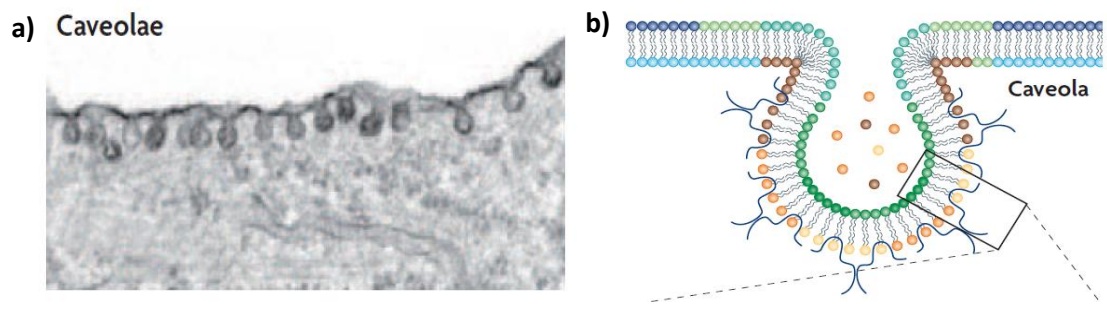

Figure 1: Caveolae structure and features. Caveolae are 60-80nm invaginations in the plasma membrane that are a subtype of cholesterol rich membrane microdomains called lipid rafts. **a)** Electron micrograph of caveolae in adipocytes. **b)** Caveolae structure, the blue hairpin represents caveolin-1 a membrane embedded protein involved in the formation and structure of caveolae. (Adapted from Parton and Simons, 2007)

Caveolins

Caveolins, a family of integral membrane proteins, were the first identified structural components of caveolae (Parton and Simons, 2007). Three mammalian caveolin proteins have been identified, caveolin-1 (CAV1), caveolin-2 (CAV2) and caveolin-3 (CAV3) (Rothberg et al., 1992; Scherer et al., 1996; Way and Parton, 1996). Expression of CAV1 and CAV2 generally occurs in cell types other than skeletal muscle, in which CAV3 is the main caveolin family member expressed (Way and Parton, 1996). Caveolae in non-muscle cells contain approximately 150 caveolin molecules which oligomerise and bind to cholesterol (Murata et al., 1995; Pelkmans and Zerial, 2005). Genetic ablation of CAV1 and CAV3, but not CAV2, has been shown to cause loss of caveolae in their respective cell types (Drab et al., 2001; Galbiati et al., 2001; Razani et al., 2002). Furthermore, ectopic expression of CAV1 results in the *de novo* formation of caveolae in lymphocytes (Fra et al., 1995). This indicates that CAV1 and CAV3, but not CAV2, are essential for caveolar formation.

Caveolin-1 over expression is common in prostate cancer

CAV1 expression is associated with aggressive, late stage prostate cancer (Nassar et al., 2013). Increased CAV1 expression has been shown to impact prostate tumour growth, angiogenesis and androgen insensitivity *in vitro* (Li et al., 2009; Li et al., 2001; Nasu et al., 1998; Tahir et al., 2009; Timme et al., 2000). In support of these findings, *in vivo* studies have shown reduced metastasis, tumour burden and increased androgen sensitivity with decreased expression of CAV1 (Watanabe et al., 2009; Williams et al., 2005). CAV1 has been suggested as a biomarker and indicator of therapy efficacy for prostate cancer (Tahir et al., 2013; Yang et al., 1999). Additionally, antibodies against CAV1 have suppressed prostate cancer growth in model systems and have been suggested as a therapy in humans (Kuo et al., 2012). The role of other caveolin family members in prostate cancer is not well studied however evidence suggests that CAV2 is up-regulated in tumour development (Gould et al., 2010).

Cavins

Until recently, caveolae research was mostly restricted to caveolin proteins as the only known class of caveolar structural protein. However, a newly identified family, cavins, works cooperatively with caveolins as a caveolae coat (Bastiani et al., 2009; Hill et al., 2008). The four existing members of this family include polymerase transcript release factor (PTRF)/cavin-1, serum deprivation response protein (SDPR)/cavin-2, PKCδ binding protein (PKCDBP)/cavin-3 and muscle-related coiled coil protein (MURC)/cavin-4 (Hansen and Nichols, 2010). Cavins are co-expressed and associate to form an oligomeric cavin complex which is associated with caveolae in a caveolin-1 and cavin-1 dependent manner (Bastiani et al., 2009). The cavin family members found in these heteromeric complexes varies between cell types (Bastiani et al., 2009).

Cavin-1 is essential in caveolae formation

Current literature has established a fundamental role for cavin-1 in caveolae association and formation (Bastiani et al., 2009; Hill et al., 2008). Ectopic expression of cavin-1 in PC3 cells, which express caveolin-1 but no cavins, significantly increases caveolae density (Hill et al., 2008). Similarly, knockdown of cavin-1 causes a dramatic reduction of caveolae in NIH3T3 fibroblasts, zebrafish notochord and in mice (Hill et al., 2008; Liu et al., 2008). In addition, cavin-1 is required for caveolar localisation of the cavin complex (Bastiani et al., 2009).

Other cavins in caveolae formation and function

The roles of other cavin family members in caveolae formation and function are not well characterised. Cavin-2 overexpression causes caveolae deformation and tubulation of the plasma membrane and is thought to induce membrane-curvature in caveolae (Hansen et al., 2009). Cavin-3 is thought to be involved in the trafficking and internalisation of caveolae and cellular signalling (Hernandez et al., 2013; McMahon et al., 2009). Unlike other cavin family members, cavin-4 is only present in cardiac and skeletal muscle caveolae and its localisation is perturbed in muscle disease (Bastiani et al., 2009). In various cancer types, cavins 2 and 3, along with cavin-1 are frequently down regulated by methylation (Bai et al., 2012; Lee et al., 2008; Zochbauer-Muller et al., 2005). Additionally, there is some evidence that expression of all cavins decreases matrix metalloproteinase 9 (MMP-9) transcription levels thereby decreasing matrix degradation and cell migration (Aung et al., 2011). However, this effect was much larger for cavin-1 than other cavins indicating that cavin-1 is the main regulator of this relationship.

Cavin-1 attenuates the tumour promoting effects of non-caveolar caveolin-1

Without cavin-1 expression, caveolin-1 resides on a flat membrane surface and this planar lipid raft organisation is termed non-caveolar caveolin-1. While this expression pattern is rare in normal prostate tissue, it is common in prostate cancer (Moon et al., 2013). In systems that lack cavin-1 expression, such as PC3 cells, introduction of cavin-1 expression is sufficient to concentrate caveolin-1 in caveolae (Hill et al., 2008). In prostate cancer, non-caveolar caveolin-1 has a tumour promoting effect and is associated with advanced stages of the disease (Moon et al., 2013). Excitingly, cavin-1 expression has been shown to attenuate the pro-tumour effects of non-caveolar caveolin-1 (Moon et al., 2013). In PC3 cells, cavin-1 expression reduces migration through regulation of cell polarization and reduced matrix metalloprotease secretion (Aung et al., 2011; Hill et al., 2012). Interestingly, caveolae are polarized at the rear of migrating, cavin-1 expressing cells and caveolin-1 is polarized at cell protrusions (Hill et al., 2012). In a mouse xenograft model, cavin-1 expression reduced PC3 tumour size and metastasis to the lungs when compared to control PC3 cells (Moon et al., 2013). Therefore, the molecular pathways regulated by cavin-1 are attractive candidates for the development of novel therapeutic targets specific for advanced prostate tumours. One such pathway may involve PKCα which binds to cavin proteins, is a known regulator of migration and is frequently implicated in cancer.

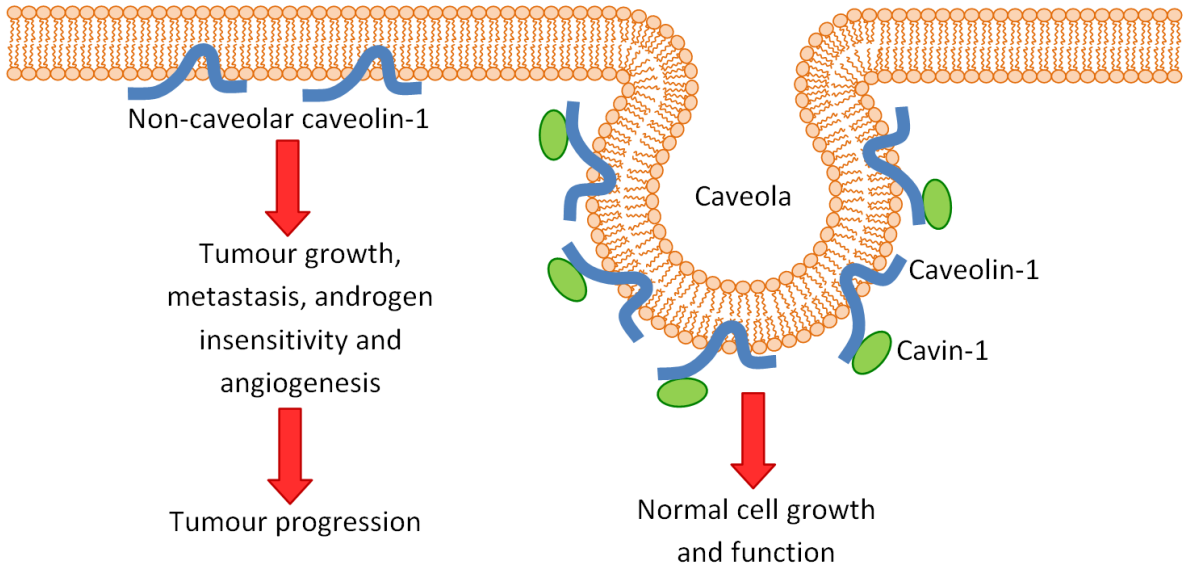

Figure 2: Mechanisms of caveolin-1 and cavins in caveolar formation and tumour progression. Without Cavin-1 expression, caveolin-1 localises on a planar membrane surface and is degraded (left). In prostate cancer, this caveolin-1 organisation, termed non-caveolar caveolin-1, is associated with tumour agressiveness. Introduction of cavin-1 leads to formation of caveolae and attenuates the tumour promoting effects of non-caveolar caveolin-1 (right).

Protein Kinase C α is implicated in cancer development

PKC is a family of serine/threonine kinases important for growth, differentiation and gene expression (Mellor and Parker, 1998). Twelve PKC isozymes have been identified and are grouped into 3 distinct subfamilies based on their enzymatic properties, conventional PKCs (cPKCs), novel PKCs (nPKCs) and atypical PKCs (aPKCs) (Mellor and Parker, 1998). The cPKCs (α, βI, βII and γ isozymes) are activated in a phosphatidylserine (PS), diacylglycerol (DAG) and calcium dependent manner (Kishimoto et al., 1980). Conversely, nPKCs (δ, θ, ε, η and μ isozymes) require PS and DAG, but not calcium, and aPKCs (ι, λ and ζ isozymes) only require PS for activation (Ono et al., 1988, 1989). When inactivated, PKC is auto-inhibited by its regulatory domain which prevents substrate binding in the active site (Pears et al., 1990).

Activation of cPKCs, for example PKCα, occurs at the plasma membrane where conformational changes expose the binding sites for substrates and/or scaffolding proteins to regulate its function (Colon-Gonzalez and Kazanietz, 2006).

There is a plethora of evidence that PKC is involved in human diseases including cancer, diabetes, psychological disorders and cardiovascular and neurological diseases (Ferreira et al., 2011; Garrido et al., 2002; Geraldes and King, 2010; Zarate and Manji, 2009). The role of PKC in cancer appears to be cancer type specific and dependent on the PKC isozyme and tissue type (Konopatskaya and Poole, 2010). Current literature reveals an inconsistent role for various PKC isozymes in both tumour promotion and suppression between and, in some cases, within a tumour type. Although the overall picture is inconsistent, a clear role for PKCα in the regulation cell motility has been established in various cancer models (Koivunen et al., 2004; Masur et al., 2001). Furthermore, the literature on prostate cancer is consistent and indicates that PKCα and other PKC isozymes are over-expressed and that their inhibition can induce apoptosis in prostate cancer cells (Benimetskaya et al., 2001; Koren et al., 2004).

Subcellular localisation of PKC isozymes determines signalling specificity

The regulation and activation of PKC isozymes depends on three mechanisms, phosphorylation, co-factor binding and intracellular localisation (Michie and Nakagawa, 2005; Newton, 2001; Parekh et al., 2000). The specificity of PKC signalling occurs through localised activation of PKC at specific subcellular compartments (Rosse et al., 2010). Targeting of PKC to these specific compartments is dependent upon its conformation and the availability of binding partners (Newton, 2001). Certain membrane-associated proteins, referred to as RACKs (receptors for activated C-kinase), have been identified as PKC scaffolding proteins that tether PKC isozymes to specific subcellular locations (Mochly-Rosen

et al., 1991a; Mochly-Rosen et al., 1991b). RACKs are often implicated in cancer and associated with abnormal PKC activity or localisation. For example RACK1, the first identified RACK protein, is overexpressed in various cancer types including prostate cancer (Adams et al., 2011; Hellberg et al., 2002). In melanoma cells, RACK1 overexpression alters the localisation of PKCα and β isozymes and contributes to metastatic ability (Egidy et al., 2008). Another example of PKC mislocalisation occurs in a subset of highly aggressive pituitary and thyroid tumours in which PKCα is not only over-expressed but also structurally perturbed (Alvaro et al., 1993). A specific mutation (D294G) in these patients results in a defect of PKCα translocation to the plasma membrane (Zhu et al., 2005). The consequences of mislocalised PKC highlight the role of scaffolding proteins which, if altered, can result in aberrant signalling and consequently the deregulation of various cellular processes.

Caveolar localisation of PKCα

Multiple reports indicate that PKCα is localised in lipid rafts and caveolae. Cellular fractionation and immunogold labeling experiments were the first to show that PKCα is localised in caveolae (Mineo et al., 1998; Smart et al., 1995). Furthermore, internalisation of PKCα through caveolae is necessary for trafficking through the endosome (Melnikov and Sagi-Eisenberg, 2009; Prevostel et al., 2000). Some caveolin family members (CAV1 and CAV3) have been found to inhibit PKC kinase activity and phosphorylation and in part, phorbol ester binding to PKCα (Oka et al., 1997). The caveolar localisation of PKCα is likely mediated by cavin proteins, since cavin-1, cavin-2 and cavin-3 have all been identified as binding partners to PKC isozymes. Cavin-2 was identified as a binding partner of PKCα through interaction cloning, immunolocalisation and competitive binding assays (Mineo et al., 1998). A different PKC isozyme, PCKδ, shows binding activity to cavin-3 (Izumi et al., 1997). Despite the evidence for cavins 2 and 3 binding to PKC, data from our lab suggests

that cavin-1 alone is sufficient for the recruitment of PKCα to caveolae (Hill et al., 2012). Upon cavin-1 knockdown in NIH3T3 fibroblasts, which express cavins 1-3, PKCα is lost from the lipid raft fraction of the plasma membrane (Hill et al., 2012). In PC3 cells, which lack cavin expression, cavin-1 expression was sufficient to recruit PKCα to the lipid raft fraction (Hill et al., 2012). In both experiments total cellular levels of PKCα were unchanged (Hill et al., 2012). Therefore, in prostate tumours lacking cavin expression, as in PC3 cells, altered PKCα localisation may result in phenotypic changes.

PKCα regulates cell migration

One potential mechanism by which PKCα mislocalisation could influence prostate cancer progression is by increasing tumour invasiveness and metastasis. PKC family proteins, including PKCα, are known to influence cell morphology through regulation of the cytoskeletal and adhesion proteins (Fogh et al., 2014; Larsson, 2006). PKCα phosphorylates cytoskeletal structural proteins such as actin and α-tubulin which results in altered cell morphology (De et al., 2014; Larsson, 2006). PKCα also interacts with the focal adhesion proteins filamin A, talin and vinculin, which are tethered to the plasma membrane and facilitate attachment to the ECM (Litchfield and Ball, 1986; Muriel et al., 2011; Tigges et al., 2003; Ziegler et al., 2002). Over expression of PKCα increases migration of endothelial, breast cancer and fibrosarcoma cells (Harrington et al., 1997; Ng et al., 2001; Ng et al., 1999). In breast cancer cells, activation of PKCα causes integrin internalisation which stimulates integrin dependent migration (Ng et al., 1999). In colon cancer, PKCα expression levels are associated with migratory capacity (Masur et al., 2001). Moreover, migration in vascular smooth muscle and endothelial cells is decreased by PKCα knockdown (Haller et al., 1998; Wang et al., 2002). Together, this evidence points to a role for PKCα in migration through interactions with the cytoskeleton and cell adhesions.

Hypothesis

From current research, it is known that cavin-1 expression in prostate cancer PC3 cells reduces migration, concomitant with PKCα recruitment to lipid rafts. However, whether other cavin family members recruit PKCα, and the mechanisms behind how PKCα recruitment to caveolae might influence migration, if at all, are not known. Therefore, this project will further investigate the caveolar recruitment of PKCα, the phenotypic changes this may induce in cancer and finally, possible mechanisms behind this interaction. Specifically this project will investigate the hypothesis that direct binding of cavins recruits PKCα to caveolae in cavin-1 expressing cells, leading to reduced migration.

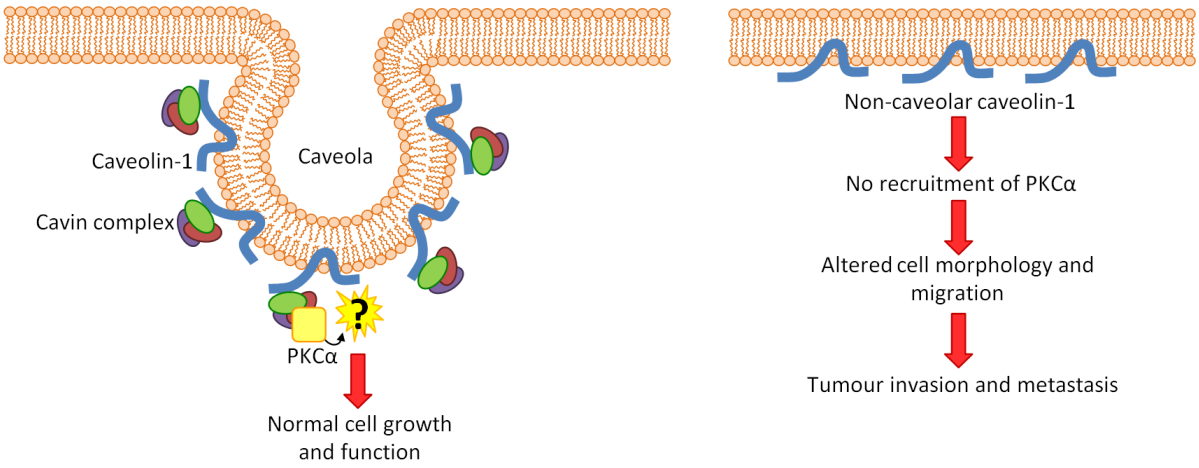

Figure 3: Hypothesised method of PKCα recruitment to caveolae and impact on tumour progression. In normal prostate epithelia, cavin-1 (commonly found in complex with other cavins) and caveolin-1 are required to form caveolae (left). PKCα is recruited to caveolae by cavin-1 and shows binding to cavins 2 and 3 however, the actions of PKCα at caveolae are unclear. Prostate cancer cells that lack cavin-1 expression (ie. lack caveolae) show increased migration (right). It is hypothesized that this partly due to mislocalisation of PKCα, which is a known cytoskeletal regulator.

Aims

1. Determine the relative abilities of cavin proteins to recruit PKCα to caveolae and investigate the mechanism behind this recruitment.
2. Correlate recruitment of PKCα to caveolae with a phenotypic change in migratory ability using functional assays.
3. Investigate the mechanisms by which the caveolar localisation of PKCα may influence other proteins to bring about a phenotypic change.

Methods

Prostate cancer cell line as a model

PC3 cells are an androgen-independent, highly metastatic prostate cancer cell line which expresses caveolin-1 but no cavin proteins (Bastiani et al., 2009; Hill et al., 2008). Therefore, this cell line is ideal for investigating the ability of individual cavins to recruit PKCα to caveolae. PC3 cell lines stably expressing GFP-tagged cavins have already been generated and will be used in this project. Cavin-4 expressing PC3 cells will be excluded as cavin-4 is a muscle specific cavin family member (Bastiani et al., 2009). Transient co-expression of cavin-2 or cavin-3 in cavin-1 expressing PC3 cells will be used to assess the level of PKCα recruited to caveolae containing different cavin complexes. As a control, PC3 cells stably expressing GFP alone will be used. It is expected that PKCα will only be recruited to lipid rafts in cells with caveolae and that additional cavins will enhance this recruitment. Moreover, a decrease in migration will correlate with increased PKCα recruitment to lipid rafts.

Expected phenotypes			
Cavin(s) expressed	**Caveolae present**	**PKCα recruitment to lipid rafts**	**Migration**
None (GFP control)	No	None	↑
Cavin-1	Yes	↑	↓
Cavin-2	No*	None	↑
Cavin-3	No	None	↑
Cavin-1 + Cavin-2	Yes	↑↑	↓↓
Cavin-1 + Cavin-3	Yes	↑↑	↓↓

Table 1: Hypothesised PKCα recruitment and migration phenotype for each PC3 cell line. In cells that contain caveolae, PKCα recruitment and reduced migration is expected. Co-expression of cavin-2 or cavin-3 with cavin-1 is expected to increase PKCα recruitment and in turn, further reduce migration. *minor caveolar formation has been observed (Bastiani et al., 2009)

Aim 1: Which cavins can recruit PKCα to lipid rafts?

Three orthogonal methods will be used to determine the relative ability of cavins to recruit PKCα to lipid rafts. Firstly, co-localisation of PKCα with different cavins and caveolin-1 in the presence or absence of caveolae will be assessed by confocal fluorescence microscopy. Secondly, biochemical fractionation will be used for quantitative assessment of PKCα targeting to the detergent-resistant fraction (DRF). Finally, direct binding of PKCα to cavins will be assessed by co-purification.

Co-localisation by immunofluorescence:

To visualise co-localisation of PKCα and cavins, immunofluorescence and confocal microscopy will be used. Each PC3 cell line will be plated on coverslips and grown to 70%

confluence. After fixation, permeabilising and blocking, cells will be incubated with a PKCα primary antibody and a fluorescent-conjugated secondary antibody. Cells will be co-stained with a caveolin-1 primary and secondary antibody. Cavins are tagged to GFP and therefore can be visualised without antibody staining. In some experiments, cherry-tagged cavin-2 and cavin-3 plasmids will be transfected into cavin-1-GFP cells. Antibody dilutions will be optimised and compared to a negative control using no primary antibody. Co-localisation of caveolin-1 and cavins will indicate caveolae and thus co-localisation of all three proteins will indicate PKCα recruitment to caveolae. All slides will be visualised by confocal microscopy. Significance of co-localisation will be assessed by Pearson correlation.

Co-fractionation:

To confirm the co-localisation of PKCα and cavin protein(s) demonstrated by immunofluorescence, the amount of PKCα in lipid raft fractions prepared from different cell lines will be compared by western immunoblotting. Caveolae reside in lipid rafts, which are resistant to detergents. Extraction of lipid rafts will be performed by isolating the detergent-resistant fraction (DRF) using an adaption from previously described methods (Lingwood and Simons, 2007). It should be noted that although the DRF will isolate caveolae in cavin-1 expressing PC3 cells, it will also contain planar lipid rafts and using this method cannot separate the two. Formation of caveolae in the cavin-expressing PC3 cell lines has been characterised and will be used to correlate PKCa levels with caveolae status (Bastiani et al., 2009). Briefly, to isolate the DRF, cells are collected, lysed in TNE (TRis, NaCl, EDTA and protease inhibitors) with 1% TritonX-100 and centrifuged to produce a DRF pellet and detergent soluble supernatant. The amount of PKCα in each fraction, for each cell line will be compared by western immunoblotting by loading equal amount (20 µg) of protein. Caveolin-1 will be used as a marker for caveolae and should be enriched in the DRF fraction only. At

least three independent experiments will be used as replicates to compare the intensity of PKCα from western blots in each fraction for all cell lines. A T-test will be used to compare significance of PKCα intensity in each cell line compared to the control (pair-wise comparison).

Direct binding measured by co- purification:

To test the hypothesis that cavin protein(s) recruit PKCα to caveolae by direct binding, GFP nanotrap will be used. This versatile technology uses a specifically engineered GFP binding protein to co-purify proteins that bind to the GFP-tagged bait (Rothbauer et al., 2008). This will be used for affinity purification of cavin proteins, as they are tagged with GFP. Interaction of PKCα will be determined by western immunoblotting of the pulldown using a PKCα specific antibody. If PKCα is present, this indicates that cavin(s) bind directly to PKCα and that this is the method of recruitment to caveolae.

Aim 2: Does PKCα recruitment to caveolae correlate with decreased migration?

Existing data correlates cavin-1 expression with reduced PC3 cell migration, and the recruitment of PKCα to caveolae. However, there is no direct evidence that PKCα recruitment to lipid rafts/caveolae is a key step in reduced cell migration. Therefore this aim will investigate the possible link between increased PKCα in caveolae, as identified in aim 1, and reduced migration using a functional migration assay.

Transwell migration assay:

To investigate the effect of PKCα caveolar localisation on migration, transwell migration assays will be used as previously performed for cavin-1 PC3 cells (Hill et al., 2012). Briefly, this assay measures the chemotactic migration of cells through porous polycarbonate filter inserts. $1x10^6$ cells will be prepared in serum free media and added to the upper chamber

and serum-containing media will be in the lower chamber. After 24 hours incubation at 37°c, the filters are washed with cold PBS and fixed. The filters are removed and stained with DAPI to be visualised by fluorescence microscopy and the number of cells are quantified using Image J. To compare significance of each cell line compared to the control a T-test will be used (pair-wise comparison).

Aim 3: How does PKCα recruitment to caveolae influence migration?

While PKCα has been implicated in the regulation of cell migration, it is not clear how the specific localisation to caveolae and/or lipid rafts can alter the action of PKCα on cell migration. To shed light on potential mediators, a discovery experiment from the lab used proteomics to identify putative PKC substrates in lipid rafts. The lipid rafts of cavin-1 expressing and control PC3 cells were isolated and putative PKCα substrates were immunoprecipitated by a PKC substrate antibody and identified using mass spectrometry. Three replicates were performed for each condition. In total, 152 proteins were identified and preliminary analysis of the results presented 4 candidate proteins consistently elevated in cavin-1 expressing cells, all of which have functions in cytoskeletal-membrane linking and have been implicated in various cancer types (Bonkhoff et al., 1993; Clucas and Valderrama, 2014; Estecha et al., 2009; Hynes, 1987; Li et al., 2013; Vedrenne et al., 2005). These proteins are moesin, integrin α6, integrin β4 and cytoskeletal associated protein 4 (CKAP4). In this aim, I will perform further analysis on this dataset to delineate any known modules, and to begin experimental validation of selected candidates.

PKC substrates in lipid rafts as downstream effectors:

To investigate the pathways impacted by PKCα recruitment to caveolae, bioinformatics analysis will be performed on the proteomics results. Specifically, protein-protein interaction

(PPI) networks will be constructed to determine if the putative substrates interact with molecules known to participate in cell migration. Protein Interaction Network Analysis (PINA), a web-based platform for PPI network construction will be used to generate PPIs (Cowley et al., 2012). Results from PINA will give an indication of the PPI's of PKCα substrates in the lipid rafts which will ideally provide some explanation of the phenotypes found from aim 2. In addition, this bioinformatics analysis may provide promising directions for future experiments.

Co-localisation of PKCα substrate Moesin:

To begin validation of the PKCα candidates in lipid rafts, co-localisation by immunofluorescence of phopho-moesin and caveolae will be performed on cavin-1 expressing and control PC3 cells. Moesin is one of three ERM (ezrin, radixin and moesin) proteins which are F-actin binding proteins involved in actin turnover (Clucas and Valderrama, 2014). ERM proteins have very similar protein sequences, therefore a phospho-specific antibody for moesin will also bind to phosphorylated ezrin and radixin. Residue 558 is the only curated threonine phosphorylation site in moesin on Uniprot (Uniprot ID: P26038) and is known to be phosphorylated by PKC (Pietromonaco et al., 1998). However, the proteomics experiment did not identify any specific phosphorylation sites since only non-phosphorylated peptides belonging to phospho-PKC bound proteins were identified. Therefore, I will perform pilot experiments to confirm the phosphorylation of ERM proteins on the putative PKC site in lipid rafts of cavin-1 expressing PC3 cells. To achieve this, immunofluorescence will be performed using similar procedures as outlined from aim 1. A phospho-ezrin(Thr567)/radixin(Thr564)/moesin(Thr558) primary antibody will be used along with a fluorescent conjugated secondary antibody. Cavin-1-GFP PC3 cells will be co-stained with caveolin-1 and cavin-1 can be visualised by its GFP tag. In separate experiments, cells

will be co-stained for cholesterol using filipin III to visualise lipid rafts, and anti-moesin antibody to determine the localisation of non-phosphorylated moesin. Slides will be visualised by confocal microscopy and significance of co-localisation will be assessed by Pearson correlation. Significant co-localisation of phospho-ERM with cavin-1 and caveolin-1 and non-phosphorylated moesin with filipin III in cavin-1 expressing cells will support the proteomics results.

Timeline

Honours Timeline	Apr	May	Jun	Jul	Aug	Sep	Oct
Aim 1							
Immunofluorescence	■	■					
Co-fractionation			■	■			
GFP nanotrap					■	■	
Aim 2							
Migration assay	■	■	■				
Aim 3							
Bioinformatics analysis				■	■		
PKC substrate co-localisation					■	■	
Thesis writing							■

Table 2: Honours timeline. Estimated time for the completion of each aim. Aim 1 has multiple components and will be undertaken throughout the year. Aim 2 will be completed alongside aim 1. Aim 3 will also be completed alongside aim 1 and after aim 2. Ample time has been allocated for thesis writing however some sections (eg. introduction, methods and figures) will be written throughout the year.

Significance

Prostate cancer is of considerable burden in the male population worldwide (Jemal et al., 2011). As a prostate tumour progresses, it can eventually become metastatic which makes it significantly harder to treat (Kirby et al., 2011). Therefore, therapeutic strategies for

advanced tumours are needed in order to combat prostate cancer. This project will hopefully uncover additional mechanisms behind prostate cancer aggressiveness. By correlating PKCα recruitment to caveolae with decreased migration and investigating the protein networks involved in this relationship, novel therapeutic strategies specific for advanced prostate tumours may be presented. Overall, revealing such strategies may contribute to reducing the number of deaths from prostate cancer.

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