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CRISPR Screen for Identification of Kinases that Mediate Prostate Cancer Cell Invasion

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Graduate Program in Pathology

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Abstract

Metastasis is the primary cause of mortality in cancer patients. Inhibition of proteins that are involved in the regulation of metastasis are expected to suppress metastasis and represent treatment targets. Our focus is on prostate cancer metastasis and we have developed a novel high-throughput means of performing in vitro screens for regulators of prostate cancer metastasis. We propose to use a focused CRISPR library screen that will “knock out” all human kinases to determine which ones are responsible for prostate cancer metastasis. CRISPR is a potent genetic editing tool and was used to silence all kinases in prostate cells (BPH cells). These cells form spheroid colonies in 3D culture and were subjected to a CRISPR kinase screen. We observed a morphological change in some colonies to a stellate morphology, which represents aggressive behavior. These stellate colonies were then isolated to identify the responsible kinase. We identified Glycogen synthase kinase 3 beta (GSK3β) as a potential regulator for prostate cancer cell morphology and possible, metastasis.

Keywords

Prostate cancer, DU145, CAM, BPH, Metastasis, CRISPR-Cas9, Kinases, Round, Stellate, Colony, GSK3β
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Dedication

To my mom Lulu Alfrhoud

To my wife Wejdan Almousa

To my daughter Lulu
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<tbody>
<tr>
<td>ADT</td>
<td>Androgen deprivation therapy</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
</tr>
<tr>
<td>BM</td>
<td>Basement membrane</td>
</tr>
<tr>
<td>BMPs</td>
<td>Bone morphogenetic proteins</td>
</tr>
<tr>
<td>BPH</td>
<td>Benign prostate hyperplasia</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine albumin serum</td>
</tr>
<tr>
<td>CAM</td>
<td>Chorioallantoic membrane</td>
</tr>
<tr>
<td>Cas9</td>
<td>CRISPR associated protein 9</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-dependent kinase</td>
</tr>
<tr>
<td>CML</td>
<td>Chronic myeloid leukemia</td>
</tr>
<tr>
<td>CRISPR</td>
<td>Cluster regularly interspaced short palindromic repeat</td>
</tr>
<tr>
<td>crRNA</td>
<td>CRISPR RNA</td>
</tr>
<tr>
<td>CTC</td>
<td>Circulating tumor cells</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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<tr>
<td>dH₂O</td>
<td>Distilled water</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle's Medium</td>
</tr>
<tr>
<td>DSBs</td>
<td>Double-strand breaks</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial to mesenchymal transition</td>
</tr>
<tr>
<td>ET-1</td>
<td>Endothelin-1</td>
</tr>
<tr>
<td>EtBr</td>
<td>Ethidium bromide</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factors</td>
</tr>
<tr>
<td>GEM</td>
<td>Genetically engineered mouse</td>
</tr>
<tr>
<td>GOF</td>
<td>Gain of function</td>
</tr>
<tr>
<td>GPCRs</td>
<td>G protein-coupled receptors</td>
</tr>
<tr>
<td>GSK3β</td>
<td>Glycogen synthase kinase 3 beta</td>
</tr>
<tr>
<td>HDR</td>
<td>Homology-directed repair</td>
</tr>
<tr>
<td>IF</td>
<td>Immunofluorescence</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like growth factors</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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</tr>
<tr>
<td>LB</td>
<td>Lysogeny Broth</td>
</tr>
<tr>
<td>LOS</td>
<td>Loss of function</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>NF1</td>
<td>Neurofibomin</td>
</tr>
<tr>
<td>NHEJ</td>
<td>Nonhomologous end-joining</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer cells</td>
</tr>
<tr>
<td>NRTK</td>
<td>Non-receptor tyrosine kinases</td>
</tr>
<tr>
<td>NSCLC</td>
<td>Non-small cell lung cancer</td>
</tr>
<tr>
<td>PAM</td>
<td>Protospacer Adjacent Motif</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostate-specific antigen</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation assay</td>
</tr>
<tr>
<td>RKIP</td>
<td>Raf kinase inhibitor protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>RPMI</td>
<td>Roswell park memorial medium</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RTKs</td>
<td>Receptor tyrosine kinases</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe combined immune deficiency</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl (lauryl) sulfate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>sgRNA</td>
<td>Small guide RNA</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short hairpin</td>
</tr>
<tr>
<td>siRNA</td>
<td>Interference RNA</td>
</tr>
<tr>
<td>TALEN</td>
<td>Transcriptor activator-like effector nuclease</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris/Borate/EDTA</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline and Tween 20</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>trasrRNA</td>
<td>Transactivating CRISPR RNA</td>
</tr>
<tr>
<td>uPA</td>
<td>Urokinase-type plasminogen activator</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VEGFR</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>ZsGreen</td>
<td>Zoanthus species green</td>
</tr>
</tbody>
</table>
Chapter 1

1 Introduction

1.1 Cancer

Cancer is a group of diseases that involve abnormal cell behaviour, such as uncontrolled cell proliferation and resistance to apoptosis. Tumors regularly evolve from a single transformed cell with few mutations. Clonal evolution of the initiator cell will acquire additional characteristics under the selective stresses of an environment, such as invasiveness and aggressiveness [1]. Malignant cells within a tumor can vary in terms of the quality and quantity of mutations leading to a biologically important property known as tumor heterogeneity. This heterogeneity underlies the difficulty in identifying and neutralizing therapeutic/molecular targets [2].

Cancer and the severity of side effects and complications caused by its treatment significantly shorten a patient’s lifespan. In addition, cancer and its management pose a significant economic burden. In the United States, neoplastic diseases cost $124 billion in 2010, and are projected to increase to $157 billion in 2020 [3]. A number of risk factors are associated with the development of various cancers. These include age, ethnicity, diet, immune system, and family history [4]. Interestingly, Asian populations are less susceptible to certain types of cancer than other ethnicities; however, in the second generation of Asian immigrants who lived in western societies, the susceptibility to developing cancer increased. This observation suggested environmental factors and a diet switch from vegetables and seafood to red meat and beer as potentially being involved [5].
1.2 Prostate Cancer

Prostate cancer (PCa) is the most diagnosed disease in the developed world. It is more likely to affect elderly people compared to other age groups [6]. One in seven Canadian men will develop PCa throughout his lifespan. In 2016, approximately 21,600 Canadian men were diagnosed with PCa and almost 4000 died from it. There are multiple factors that contribute to PCa formation, such as nutrition factors, habit-related factors, androgens, and ethnicity [6]. The Multiethnic Cohort Study (MCS) for statistics revealed that PCa is largely diagnosed in men of African descent compared to their ethnic counterparts in the United States [7], [8]. Patients with prostate cancer are treated with hormone therapy called androgen deprivation therapy (ADT). ADT inhibits androgen production from its main source or blocks its cellular receptor-mediating signaling cascade [6]. In addition, blocking critical pathways for PCa such as Hedgehog, fibroblast growth factor (FGF), transforming growth factor beta (TGF-β), Src, and integrins has been used for PCa patients [9]. Some patients cease to respond to this therapy and develop resistance. This resistance allows PCa to progress in an androgen-independent manner. The second line of treatment is chemotherapy, which is typically docetaxel and cabazitaxel. These treatments stabilize microtubules of rapidly dividing cells [10] and can extend the survival rate by 2-4 months [11], [12]. Other drugs can be used as a second line treatment are based on the individual patient characteristics such as sipuleucel-T (immunotherapy) and radium-233 dichloride (²²³R) (radiotherapy) [13], [14]. Resistance to ADT is also associated with migration of PCa to distant sites such as the bone, brain, liver, and lymph nodes, by a process called metastasis.
1.3 Cancer Metastasis

Cancer metastasis is the leading cause of cancer related-death [15]. Metastasis consists of four key steps: 1) intravasation, when a cancer cell detaches from the primary tumor and enters into the blood circulation, 2) extravasation, when cancer cells exit the blood circulation; 3) micrometastases formation in the new environment, and 4) colonization, when these micrometastases progress into a macroscopic tumor [4], [16]. To initiate this cascade, the cancer cells must first acquire invasive characteristics [17]. One key process in acquiring an invasion capacity is the degradation of the surrounding extracellular matrix and cell protrusion formation [15]. This morphological change is followed by a phenotypic switch in cancer cells from an epithelial to mesenchymal transition (EMT). This mechanism converts the epithelial cells from its polar state into migrating, fibroblast-like mesenchymal cells. Under physiological conditions, EMT phenomenon is a reversible process as shown during embryogenesis [18].

Cancer metastasis, overall, is an inefficient process. Very few cancer cells eventually are able to establish new metastatic colonies [19]. Therefore, metastatic cells which have intravasated into the blood circulation may not successfully go through the extravasation step, which leads to failure in extravasated cell colony formation. In addition, a significant number of extravasated cells are believed to undergo programmed death within 24 hours [20]. The surviving extravasated cells are also not guaranteed to proliferate into a cancer colony and may remain in the dormant state [21], [22], [23]. Therefore, successful metastatic growth requires cancer cells to overcome a number of obstacles, such as the immune system, blood forces, a harsh environment, hypoxia, and acidity [10]. Metastatic cancer cells evade host immunity via several mechanisms. It has been reported that
metastatic cells disguise themselves from natural killer cells (NK) by expressing coagulation factors VIIa and X [24], [25]. Interestingly, treatment with anticoagulants has been shown to attenuate metastatic burden in animal models and in cancer patients [26].

The microenvironment at the secondary site also regulates the metastatic process. The “seed and soil” hypothesis was first put forward by Stephen Paget in 1889 and suggested that metastasis is not a completely random process [27]. This theory suggests that cancer cells from an individual tumor tend to home to a specific secondary site. For example, metastatic melanoma skin cancer predominately homes to liver [28], [29], and other metastatic cancer such as lung, breast, and prostate mainly metastasize to bone [30]. A recent study confirmed the seed-soil hypothesis by implanting tumors in uncommon metastatic sites, wherein the environment was hostile for said tumor; however, tumors were “rescued” when implanted in a favorable metastatic site [31]. Several lines of evidence have discovered that the role of microenvironment at the secondary site manipulates the fate of the metastatic cell by recruiting bone marrow-derived cells [32]. For example, hypoxic conditions in the primary site may favor premetastatic niche formation as a result of three events: 1) production of lysyl oxidase (LOX) that will alter the tumour's microenvironment; 2) production of chronic anhydrase (CAIX) that will mobilize myeloid-derived suppressor cells (MDSCs); and 3) suppression of natural killer (NK) cell activation. Metastatic cells exhibit pre-determined directional behaviour [20].

In terms of bone metastasis, the marrow has a high number of blood vessels and an abundance of growth factors, and an hypoxic environment (1-7% O₂), providing the cancer cells a fertile environment [4]. In addition, osteoblasts highly express adhesive molecules which interact with corresponding receptors on cancer cells [33]. It is not surprising that
bone is a common metastatic site for a number of human cancers as this tissue is one of the first tissues to encounter circulating tumor cells (CTC) [34]. The mutually beneficial relationship between osteoblasts and cancer cells is supported by secretion of growth-promoting factors such as insulin-like growth factors (IGF)-1 and -2, TGF-β, prostate-specific antigen (PSA), urokinase-type plasminogen activator (uPA), fibroblast growth factors (FGF)-1 and -2, bone morphogenetic proteins (BMPs), platelet-derived growth factor (PDGF) and endothelin-1 (ET-1) [35].

The majority of patients (approximately 90%) with metastatic PCa develop metastases in the bone and the rest develop metastases mainly in the brain and lymph nodes. Mortality for prostate cancer metastasis is the highest compared to other bone metastasis associated cancers [11]. Patients with metastatic PCa in the bone exhibit sclerotic bone lesions associated with an increase in osteoblasts [13]. These symptoms can be mitigated at this stage by radiotherapy or osteocyte inhibitors [14]. However, cancer becomes hard to treat when it has metastasized to the bone by affecting bone remodeling processes and leaving patients with severe pain, pathological fractures, hypercalcemia, and spinal and nerve compression [36], [37].

1.4 CRISPR-Cas9 System

A genomic editing tool is a potent approach to delineate which genes or pathways are involved in cellular processes, which can be potentially targeted therapeutically [38]. This emerging approach has been effectively used in different organisms for multiple purposes. Genomic editing tools, in particular the CRISPR-Cas9 system, has been harnessed for
multiple biomedical assay generation, phage-resistant dairy cultures, and phylogenetic classification of bacterial strains [39].

1.4.1 Gene Modification

There are two types of gene dysregulations which can be utilized. The first one is knocking down a gene product. Knockdowns work at the RNA level and generate gene downregulation by using single short hairpins (shRNAs) or interference (siRNA). However, this approach may be unable to provide a genuine phenotypical effect due to targeting the gene of interest [40] as it is only limited to transcribed elements and the effect may be short-lived [41]. The second method is knocking out a gene. Knockouts work at the DNA level to generate a complete loss of gene function. There are multiple techniques for knocking genes out, including meganucleases, Zinc-finger nucleases, transcription activator-like effector nuclease (TALEN), and regularly clustered interspaced short palindromic repeats (CRISPR). These programmable nucleases are subdivided into two groups based on the type of interaction which takes place with the DNA: 1) zinc finger and transcription activator-like effector nucleases (ZFN and TALEN, respectively) which target DNA via a protein; 2) the CRISPR-Cas system, which targets DNA by short guide RNA that base-pairs with DNA.

The CRISPR-Cas system is a unique gene editing tool. It is relatively simple to use and does not require a need to design a cleavage enzyme because of the power of the CRISPR-associated protein (Cas9). Cas9 has also been shown to slice methylated DNA [44]. CRISPR-Cas9 consists of two elements: an oligonucleotide RNA [42] for directing and Cas9 protein which introduces double-strand breaks (DSBs) [43]. The DNA break is then
repaired through nonhomologous end-joining (NHEJ) or homology-directed repair (HDR). NHEJ causes insertions and deletions (indels), and frame shift mutations resulting in a gene knockout. HDR pathway is useful for knocking in genes (inserting) in the presence of a repair template [44], [45]. A range of vectors can be used for introducing genetic material into cells in viral or non-viral formats. For viral vectors, adenoviruses, adeno-associated viruses, and lentiviruses are typically used. An advantage of the lentiviral format is that it is non-replicative, safe, and can incorporate long DNA material (ideal for the CRISPR-Cas9 system) [46]. Lentiviral vectors do present safety concerns but scientists have developed newer generation formats to overcome these concerns. Importantly, these new generation format separates the packaging, transfer, and envelop into separate vectors.

### 1.4.2 CRISPR as a Natural Phenomenon

Microbiologists interested in studying the defense mechanisms of microorganisms against invaders discovered the CRISPR-(Cas)9 system. This system functions as an immune system in bacteria and archaea to fend off viruses, phages, and plasmids. This micro-immune system shares similarities with the mammalian immune system with respect to precision, diversification, and recognition [50]. In contrast to the non-inheritable eukaryotic expression system, the bacterial CRISPR-Cas9 is an inheritable adaptive system [47].

A CRISPR locus is defined as an array of short direct repeats interspersed with spacer sequences. The spacer content of these CRISPR arrays reflects the different phages and plasmids that the host has encountered. Bolotin and his group sequenced the spacers and found a substantial match with the genome of phages and other invaders. They also showed that the high number of spacers is associated with the immune system potency [48]. When
the host encounters an invader, the DNA of invading microorganism is cleaved by the Cas proteins and the sequence is recorded. The sequence is then transcribed, forming small RNA called CRISPR RNA (crRNA). When the host encounters the invader again, the crRNA binds the sequence of invading DNA leading to cleavage [18, 27] (Fig 1). Cas proteins are unable to start cleaving unless the DNA region of interest is followed by a Protospacer Adjacent Motif (PAM). Jink et al. tested the role of PAM by conducting a cleavage assay using wildtype and mutant PAM. They showed that DNA was not cleaved with the mutant PAM but was readily cleaved with the wildtype PAM. This observation is important and supports why protospacers are not cleaved as the PAM sequence is not present at the CRISPR locus of the bacteria [38]. Also, Cas9 preferentially binds sgRNAs containing purines in the last four nucleotides of the spacer sequence, whereas pyrimidines are not favored [41].

1.4.3 CRISPR-Cas9 System Types

Bacteria have a few different CRISPR systems. The Cas structure and its mode of function can be used to broadly divide CRISPR into three different types. Type I is found in Escherichia coli and type III is found in Staphylococcus epidermidis and Pyrococcus furiosus. These two types have multiple Cas proteins that generate genetic modifications. CRISPR type II is the preferred type to be used as a tool and has Cas9 as the only member of that family to mediate cleavage [38]. CRISPR type II system is found in the Streptococcus thermophilus.
Cas9 protein consists of two domains: a HNH domain for cleaving complementary strands and a RuvC domain for cleaving the noncomplementary strands [18]. It has been shown that mutating one of these domains inhibits Cas9 from generating a complete cleavage. A number of engineering advancements have been made in the CRISPR-Cas9 system. Off-target effects in this system have been tested by designing multiple variants of sgRNA with one to four mismatches of complementary DNA. Mismatches near the 5’ end tend to be more tolerated than near the 3’ end [49]. In 2016, Slaymaker and colleagues drastically potentiated Cas9 by manipulating the charges of both Cas9 domains and reduced off-targets [50]. Based on the native Cas9 crystal structure, it was found that the crystal structure of modified Cas9 is highly positively charged, which helped it to facilitate the unwinding of the negatively charged DNA. Thus, lowering the positive charge of Cas9 increases the specificity of sgRNA.

1.4.4 CRISPR Screen In vitro

Genomic screening is of critical importance for identifying the roles of the genes involved in cellular processes associated with pathological disorders. Typically, genomic screens are performed by inducing mutations resulting in either a gain of function (GOF) or a loss of function (LOF). Standard GOF utilizes overexpression of cDNA. The size of cDNA is relatively large compared to the vector making the process difficult. LOF, on the other hand, typically utilizes RNA templates [51]. Genomic screens of CRISPR-Cas9 can provide a positive or negative selection environment. A positive screen allows for the discovery of genes being mutated by sgRNAs which enhance cell proliferation (for example, tumor suppressors). Chen and his colleagues performed a CRISPR screen for the
entire genome by knocking out genes in non-small cell lung cancer (NSCLC). They identified genes responsible for tumor suppression when mutated. These genes included well-known tumor suppressors such as neurofibomin (NF1) and phosphatase and tensin homolog (PTEN) [52]. In contrast to positive screens, negative screens select for genes that are responsible for cell survival (identifying oncogenes) [53].

Figure 1: CRISPR-Cas system naturally requires three phases to take place.
1.5 Kinases

Cells are surrounded and triggered by external stimuli. In response to these stimuli, cells initiate a cascade of intracellular signalling pathways. One of the important mediators for these complicated pathways are kinases, which shuttle the γ-phosphate group from the adenosine triphosphate (ATP) to other molecules with a phenolic group of the tyrosine, or an alcoholic group of the serine, and threonine residue [54], [55]. This process, called phosphorylation, is counter to the process carried out by phosphatases. Phosphorylation is recognized as the predominant post-translational modification compared to methylation, glycosylation, and acetylation, etc. [56]. According to the human genome sequencing project, there are 518 kinases in every 2000 gene-encoded enzymes [57]. When a protein gets phosphorylated, it may start functioning in critical biological processes such as proliferation, migration, differentiation [58], and cytoskeleton arrangement [59]. It is assumed that one-third of cellular proteins are phosphorylatable [56]. The kinase family commonly comprise a catalytic domain of 250-300 amino acids. Tyrosine kinases are divided into two groups based on cellular location; those that exist on the cellular membrane are called receptor tyrosine kinases (RTKs), and cytosolic kinases are called non-receptor
tyrosine kinases (NRTK). All RTKs are monomers with an exception of the insulin receptor [60].

1.5.1 Kinase Involvement in Cancer

Because of the involved on kinases in various cellular processes, it is likely that aberrant expression of kinases will cause a disease or be associated with disease progression. Kinase deregulation has been linked to approximately 400 pathological disorders including cancer [61]. The vast majority of the oncogenes are recognized as kinases. Thus, any dysregulation in kinases is desirable for establishing cancer [62]. It has been reported that most human cancers exhibit an anomalous expression of kinases. For example, kinases have been shown to function aberrantly in breast, lung, prostate, and colorectal cancer [63]–[65]. In the Cancer Gene Census, it was revealed that 10.7% of cancer genes have kinase domains [66]. It is generally assumed that kinases are tumor initiators and phosphatases act as tumor suppressors. However, several lines of evidence reveal that kinases can also function as tumor suppressors [59], [67]. A recent study identified a kinase called Raf kinase inhibitor protein (RKIP), which acts as a metastatic suppressor gene, but behaves differently in prostate cancer. Downregulation of this protein leads to angiogenesis and acquisition of invasive characteristics in cancer cells, as well as resistance to chemotherapy-induced apoptosis [68]. Therefore, aberrant expression of or dysfunction in kinases is a cancer driver whether by conferring oncogenic capacity or tumor suppression capacity.
1.5.2 Kinases as Therapeutic Targets

Chemotherapeutic agents are still the first line of treatment for cancer patients. However, the problem associated with this treatment is its failure to discriminate normal cells from malignant cells, leaving patients with harsh side effects such as the loss of hair/nail and taste sensation. Thus, there is an urgent need to develop drugs with high specificity and minimal side effects. An avenue is targeting cancer cell-associated signaling pathway [69]. As mentioned above, one of the critical mechanisms in signaling transduction is protein phosphorylation [70]. Tyrosine Kinase Inhibitors (TKIs) succeed in diminishing phosphorylation and resulting in suppressing the tumor with minimal cytotoxicity compared to chemotherapy. These inhibitors greatly inhibit some of the hallmarks of cancer such as angiogenesis by vascular endothelial growth factor (VEGFR) inhibition, cell differentiation by mitogen-activated protein kinase (MAPK), and cell proliferation by cyclin-dependent kinase (CDK) [71]. An example of a TKI with high specificity and low side effects is Imatinib which has been used for chronic myeloid leukemia [CML] patients [72], [73]. Kinase activity suppression can be achieved by several ways: blocking the ATP pocket, interrupting protein-protein interactions, and genetic modulations [61]. The next generation of anticancer drugs has focused largely on targeting main cancerous pathways such as TKs by administering consecutive or combined drugs and doses [32]. Within just three years, the pharmaceutical industry has witnessed an astonishing outpouring of drug discovery by approving 15 TK inhibitors between 2012-2015 [74]. The existence of 100-200 protein kinases per cell that reside upstream and downstream of tumor drivers with druggable potential has led investigators to heavily study them [75]. Kinases are considered the second most popular therapeutic target after G protein-coupled receptors (GPCRs) [76].
One advantage of TKs over GPCRs is that an antagonist for a TK leads to the suppression of more than one cellular response, while GPCRs suppression leads only to suppression of one cellular response. This might increase the potency of TK, since the hallmarks of cancer cannot be suppressed by inhibitors that antagonize a single cellular response [4]. Kinase pathways play an essential role in tumorigenesis and inhibiting one may be compensated by another pathway [32]. Suppressing multiple pathways simultaneously might be useful for efficient destruction of the cancer cells. It can, however, be problematic as a result of drug metabolism. For example, the metabolism of one of the two inhibitors can interfere with the other one. Thus, a single multi-kinase inhibitor may potentially be robust and safe [78].

Kinase activity can be suppressed by two approaches: 1) a monoclonal antibody such as trastuzumab and cetuximab, or 2) a small-molecular inhibitor such as Imatinib or Sunitinib. The former approach is commonly used for receptor tyrosine kinase (RTK) inhibition, and these agents are typically administered intravenously due to their large size (150 kDa). Small molecule inhibitors (approximately 500 Da) are used for non-receptor tyrosine kinases (NRTK) and are administered orally [85]. KIs are less specific compared to monoclonal antibodies (mAbs) as KIs may suppress several signalling pathways. mAbs can activate immune responses such as antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). Antibody-mediated therapy has been shown to be more effective compared to KIs in vivo compared to in vitro settings. For example, gefitinib was more effective than cetixiumab in killing small-cell lung cancer (NSCLC) in vivo; however, these different drugs showed the opposite in the in vitro model, possibly due to mAB-induced immune responses.
1.6 Screening Approach

Different screening methods can be applied to the whole genome or select libraries using gene deletion or gene silencing strategies. Downstream analyses of these screens vary based on the nature of study. In order for cells expressing sgRNA/RNAi/shRNA to be considered a ‘hit’, the cells must exhibit a phenotypic change which can be tumor formation, apoptosis, dormancy, or differentiation [77]. For example, cells introduced to RNA-mediated mutations were selected with a BRAF inhibitor (vemurafenib) and the surviving clones revealed genes whose loss conferred resistance to this drug [78]. Another study also used a similar approach with an Akt inhibitor to reveal genes conferring vulnerability to this inhibitor [79]. The kinase family is the most widely screened library aside from the whole genome. A recent screen for kinases was performed to identify genes that are required for the proliferation of cells that overexpress the KRAS gene. Several hits were identified, such as PLK1, STK33, and TBK1 [80]–[82]. There have been several attempts to screen for kinases that are indispensable for cancer cell propagation and survival [83]. A genome-wide screen for different pathological arrays was conducted in different model systems, e.g., yeast [84], worms[85], Drosophila [86], mouse cells [52], [87], and human cells[88], [89]. Wang and colleagues conducted a whole-genome screen to identify genes essential for cellular proliferation, selecting genes whose mutation exerted a selective disadvantage for the cells.
1.7 Models for Studying Cancer Metastasis

There are multiple approaches to study cancer metastasis, whether *in vitro* or *in vivo*. Angiogenesis, cell migration, and cell invasion are of particular importance for metastatic dissemination, and these features can be recapitulated *in vitro* through the tube formation, scratch wound, gelatin degradation, and Boyden chamber assays [90]. On the other hand, for an *in vivo* study, mouse models of different cancer types are widely used [91]. There are multiple ways of establishing cancer in mice, including administering the cancer into immunocompromised mice, spontaneous formation by a genetically engineered mouse (GEM), subcutaneous injection to establish a primary tumor, and tail vein injection for monitoring extravasation and colonization. These mice are typically further treated to evaluate efficacy of chemotherapeutic drugs or small-molecule inhibitors. GEM models allow precise molecular specificity to study the effects of the gene of interest on cancer and its resistance mechanisms to different therapies [92]. Tumors developed in the GEM model can be dissected and transplanted into immunocompetent mice to assess immunotherapy [93]. Another mouse model is the severe combined immune deficiency (SCID) mouse, which is used to study the tolerance of foreign tissue transplants (xenografts). Furthermore, it is well known that it takes a long time to acquire results from cancer mouse models; therefore, we utilize the chorioallantoic membrane (CAM) model.

1.8 Chorioallantoic Membrane (CAM)

Animal models are the gold-standard for preclinical studies, especially for cancer research [94]. However, animal models are expensive and time-consuming with respect to
regulatory guidelines as well as disease recapitulation. Moreover, with the exception of the genetic murine model, there is a lack of a model which mimics all metastatic cascades, including extravasation, intravasation, and colonization [95]. The avian model is an excellent option to circumvent some of these difficulties. The CAM of the chick embryo is a very thin organ composed of three layers: ectoderm, mesoderm, and endoderm [96]. The CAM functions as a respiratory system for the chick embryo since it is aligned through the porous eggshell. It enables the embryo to exchange gases through these pores, and helps transport electrolytes from allantoic sacs and calcium from the shell.

This model is naturally immunodeficient in early stages, cost-effective, amenable to real-time intravital imaging, and highly vascularized with good lymphatic drainage [94], [97]. With the advancement of imaging technology, the CAM model allows tracking injected dyes and cells to illuminate the capillary network, and both extravasated and intravasated cells [19]. As mentioned earlier, the main mediator of cancer cell metastasis is the blood canal [16]. Two out of three key steps of the metastatic cascade take place in the blood vessels. Therefore, the CAM model can be used as a window for monitoring the cancer metastasis cascade. For example, we can assess micrometastases formation, extravasation efficiency, cancer cell survival, and dormancy. In addition, intravasation efficiency can be performed via bolus injection, which takes place by injecting cells in the stroma of the CAM [96]. On-planting a Patient Derived Xenograft (PDX) on top of the CAM after slightly tearing it allows for assessment of angiogenesis..

There are limitations associated with this CAM model. Timing of this experiment is relatively short, which means macrometastases cannot be observed [98]. The survival rate for chicks after the experiment is low at 65-70% even under ideal experimental conditions.
Dynamic angiogenic responses are also difficult to observe [99]. Lastly, shell traces left following cracking of the eggs may induce inflammation and angiogenesis [99].

In my studies, we performed an intravenous injection of cells to assess tumor colony formation. In these studies, cells intravasate the blood vessels at day 9. It has been shown that there is a substantial increase in endothelial cell number at this age [94] (Fig 2).
Figure 2: Chorioallantoic Membrane (CAM) of the Chick Embryo Model after Cracking.

Fertilized eggs cracked three days after incubation were placed in a blue container. At day 9, an expansion of blood vessels seems to appear for performing an IV injection and other assays.
1.9 Cell morphology as a readout of metastasis and invasive behavior

The change in cell shape is of critical importance for metastatic cancer cells and allows movement of cells from one site to another. Metastatic cells have the capacity to adapt to each new site by manipulating cell shape in order to proceed with the metastatic steps. One study did a pairwise comparison between two sets of cells with different metastatic potentials (high and low). They found significant differences between the two sets in terms of cell volume and elongation. *In vitro* studies such as three-dimensional (3D) culture assays which allow individual cells to expand clonally and form colonies. Colonies from metastatic cell lines exhibit two morphologies, round and stellate. Stellate morphology is considered to be illustrative of aggressiveness. For example, in some triple negative breast cancers (TNBC), which is a highly aggressive stage in breast cancer, cell lines formed a stellate morphology in 3D Matrigel cultures [100]. In addition, when they were treated with enzalutamide, an androgen receptor (AR) antagonist, there was a significant shift into round morphology. They also conducted several assays in order to assess critical steps in metastasis and found that AR inhibition reduced migration and invasion. Thus, migration and invasion results were aligned with the phenotypic shift in 3D cultures after treatment.

Another group conducted a study with a panel of 25 cell lines using 3D cultures in order to observe colony morphology [101]. They subdivided colonies based on morphology: round, mass, grape-like, and stellate. They concluded that stellate morphology is correlated with aggressiveness and invasiveness. These stellate colonies predominately lacked the expression of E-cadherin (cell adhesion molecule) expression, which is considered to be a tumour suppressor in solid tumours. Another group performed a similar study in a panel of
24 cell lines to find the best genes for predicting stellate morphology in 3D cultures [115]. They found genes that genes known to regulate cancer hallmarks (such as proliferation) in different cell lines are highly expressed in stellate colonies. Another predicted gene for stellate morphology is paralemmin-2 (PALM2), which has been found to be responsible for filopodia and spine formation during dendritic branching. In PCa, some cell lines consist of heterogeneous morphology with stellate and round colonies [102]. Gene profiling for the stellate colonies revealed genes enriched in cell adhesion, cell-to-cell contact, invasion/metastasis, and extracellular matrix (ECM) turnover [102]. Genes involved in epithelial mesenchymal transition (EMT), a critical mechanism used by metastatic cells for migration, were highly enriched in cell lines that form stellate morphology in 3D cultures that showed highly expressing mesenchymal markers such as vimentin, fibronectin, and N-cadherin [102].

### 1.10 Rationale

Metastatic cells have the capacity to alter their morphology to achieve the key steps involved in the metastatic cascade. PCa cell lines commonly used in studies are quite heterogenous in their morphology. As will be evident from my studies, DU145 and PC3-M-LN4 PCa (cells with high metastatic potential when compared with other classical PCa cell lines) formed metastatic colonies that exhibited relatively higher stellate morphology versus round morphology in the CAM model. As this is not a robust readout (heterogeneity in cell and colony morphology), I proposed using benign prostatic hyperplasia (BPH) cells to assess which kinases alter the morphology of these benign cells. In 3D cultures, BPH
form homogenous colonies with round morphology. Therefore, any alteration in BPH morphology can be reliably measured and may identify kinases which may make PCa cells aggressive.

1.11 Hypothesis

I hypothesized that several kinases that are altered by the CRISPR-Cas9 system will produce a phenotype shift in the morphology of benign prostatic hyperplasia cells when grown in 3D culture, revealing candidate kinases as putative regulators for prostate cancer invasion.

1.12 Research Objectives

To test my hypothesis, I have devised a set of four objectives. These are divided into in vitro (3D cultures) and in vivo (CAM) segments of my studies:

In vivo studies: 1) To identify a metastatic prostate cancer cell line that will form stellate colonies in vivo; 2) To generate a DU145 subline that is capable of forming 100% colonies with a stellate morphology in vivo

In vitro studies: 1) To optimize plating conditions of Cas9 overexpressing BPH clones as grown in Matrigel; 2) To perform a CRISPR-Cas9 kinase screen to identify sgRNAs that induce a stellate morphology in vitro
Chapter 2

2 Materials and Methods

2.1 Cell Culture (2D)

DU145 cells are metastatic PCa cells derived from the brain [103]. These cells were maintained in DMEM. PC3-M-LN4 was initially derived from the bone [104] and was maintained in RPMI media. BPH cells, an inflammatory cell line extracted from a 68-yr-old patient undergoing transurethral resection of the prostate for urinary obstruction [105], were maintained in RPMI. All media were supplemented with 10% FBS, 100 IU/mL Penicillin, 100 µg/mL streptomycin and incubated at 37°C and 5% CO₂. DU145 and PC3-M-LN4 cell lines were lentivirally infected with a ZsGreen vector to intravitaly visualize the cells in the CAM model.

2.2 Cell Culture (3D)

Cells were embedded between two layers of Matrigel. For the bottom layer, 175 µL of 100% Matrigel was added into one well of the 24 well/plate and incubated at 37°C (5% CO₂) for 30 minutes to be polymerized. Cells were next counted and seeded at $1 \times 10^4$ cells/mL density (~1500 cells/ well) on top of the Matrigel layer. Cells were left for 30 minutes to allow attachment. For the upper layer, 150 µL of 20% Matrigel/culture medium was added to cover the cells. Fresh medium was added every two days. After 7-10 days, colonies were counted using the inverted microscope and/or used for cytochemistry.
2.3 CAM Injection

DU145-zsGreen and PC3-M-LN4-zsGreen cells were counted using the hemocytometer chamber and injected at $1 \times 10^6$ cells/mL density (~$1 \times 10^5$ cells/CAM). The cell suspension was kept on ice prior to and during the injection period to maintain cell viability. Afterwards, the cells were injected under a dissecting microscope via a microinjector which consists of four parts: a syringe, a needle, tubing, and micropipette-pulled needle. During and after the injection, the CAM model was immediately visualized under the fluorescent microscope to confirm cell injection. After 7 days, micrometastases were either counted for colony formation efficiency or extracted for passaging and injection.

2.4 Extracting micrometastases out of the CAM

After 7 days post injection into the CAM and under the fluorescent microscope, colonies of interest were picked up by fine forceps and cut out by fine scissors. Then, the extracted colonies were individually dissected from any associated extra tissue by a sterile scalpel. Each colony was sub-cultured in one well of the 24 well/plate and fed with the preferred medium supplemented with (10X) hyaluronidase, (10X) collagenase, and a potent antibiotic (100X) normocin.
2.5 Extracting Hits out of Matrigel

We extracted hit colonies that exhibited a phenotype shift in 24 well/plate using a 1 mL syringe, tubing, and a fine borosilicate needle after assembling them. We then used a microscope with a screen to follow the fine needle, point it to a colony of interest, and then aspirate it with the syringe. The extracted hit was then subcultured in one well of a 24 well plate. Extracted colonies were individually left to expand and proliferate for 3–4 weeks before DNA extraction and sgRNA identification.

2.6 Bacterial transformation

Plasmid DNA was transformed as stated in the manufacturer’s protocol of the DH5α Competent Cells (ThermoFisher Cat. No. 18265017). Five µL (1 ng) of plasmid DNA was added to 1 µL of the competent cells in a centrifuge tube and gently mixed. The mixture was incubated on ice for 20 minutes. In order for the bacteria to absorb the plasmid, the mixture was heat shocked at 42°C for 45 seconds and returned to ice for 2 minutes. Two hundred µL of LB medium without antibiotic was added to the mixture and incubated at 37°C for 40 minutes at 225 rpm. Fifty µL of the bacterial cells were then streaked on LB agar plate with an appropriate antibiotic and incubated at 37°C for overnight. Another plate was used for growing only the competent cells as a negative control for the experiment. One day later, an individual colony from the agar plate was inoculated into 5 mL of LB medium supplemented with an appropriate antibiotic (Ampicillin) and incubated, grown at 37°C for overnight at 225 rpm. Plasmid DNA was purified from the bacteria for downstream experiments.
2.7 DNA purification

2.7.1 Bacterial Plasmid Purification

DNA purification was performed as per the manufacturer’s protocol (Geneaid Cat. No. PD100). In brief, bacterial culture was spun down at 10,000 x g for 10 minutes, and the supernatant was discarded, leaving behind the bacterial pellet. A number of different buffers were subsequently added to the bacterial pellet as follows: 200 µL of suspension buffer, 200 µL of lysate buffer, and 300 µL of neutralization buffer. Then, the mixture was centrifuged at 16,000 x g for 3 minutes. The supernatant was transferred to the binding membrane column and centrifuged for 30 seconds. The flow-through was discarded. The binding membrane was washed with 700 µL of washing buffer and centrifuged for 3 minutes, and the flow-through was discarded. The column was placed in a new centrifuge tube, and 30 µL of the elution buffer was added and centrifuged for 2 minutes to get the eluted DNA.

2.7.2 DNA Extraction from Mammalian Cells

Total DNA was purified from the transduced cells using Puregene Core Kit A (Qiagen) following the manufacturer’s instructions. Three hundred µL of cell lysis solution was added to the cell pellet and vigorously agitated at high speed for 10 seconds. Ten µL of protein precipitation solution was added and vigorously agitated at high speed for 20 seconds. Subsequent centrifugation for the mixture was performed for 1 minute at 16,000 x g. The supernatant was gently poured out into 300 µL of isopropanol in a centrifuge tube. The mixture solution was manually and gently inverted 50 times. Subsequent centrifugation was performed for 1 minute at 16,000 x g, and the supernatant was discarded leaving behind
a white DNA pellet. The DNA pellet was washed with 300 µL of 70% ethanol and spun down for 1 minute at 16,000 x g. The supernatant was discarded, and the DNA pellet was left to air dry for 5 minutes. One hundred µL DNA hydration solution was added and incubated at 65°C for 1 hour to dissolve the DNA. DNA was quantified using the NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific). DNA samples were then amplified using standard PCR for sequencing.

2.8 Conventional PCR

PCR was conducted based on the manufacturer’s instructions (NEB Cat. No. M0270L). In a centrifuge tube, 1 µL of 10 µM forward/reverse primers were mixed with 1 µg of DNA template, 12.5 µL of Taq 2X Master Mix, and Nuclease-free water to a total of 25 µL. The PCR reaction was processed in 96-well Bio-Rad MyCycler Thermal Cycler for 30 cycles (95°C for 15-30 seconds, 45-68°C for 15-30 seconds, and 68°C for 1 minute per kilo base). After incubation, 4 µL of DNA loading dye was added to the reaction tube and loaded subsequently into agarose gel wells. The PCR product was extracted by DNA Gel Extraction Kit (NEB, Cat. No. T1020L), and the PCR product was used to generate genomic products for cloning or sequencing. A list of primers used is presented in Table 1.
Table 1: List of PCR primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cas9</td>
<td>F: CAAGTTTCATCAAGCCCATCC</td>
</tr>
<tr>
<td></td>
<td>R: ATGTCCAGTTCTGGTCCAC</td>
</tr>
<tr>
<td>Tks4</td>
<td>F: GCGTCGAGACCCAACTTTCT</td>
</tr>
<tr>
<td></td>
<td>R: TCTTTAGACCATGGCAACCCC</td>
</tr>
<tr>
<td>pLX-sgRNA</td>
<td>F: AGCGCTAGCTAATGCCAACTT</td>
</tr>
<tr>
<td></td>
<td>R: GCCGGCTCGAGTGTAACAAA</td>
</tr>
<tr>
<td>β-Actin</td>
<td>F: AGAGCTACGAGCTGCTGAC</td>
</tr>
<tr>
<td></td>
<td>R: AGCACTGTGTGGCGGTACAG</td>
</tr>
</tbody>
</table>

2.9 Gel electrophoresis

One % of the agarose powder was melted in Tris/Borate/EDTA (TBE) buffer (0.5 g/50 mL) in an Erlenmeyer flask using the microwave. One µL of the ethidium bromide (EtBr) was subsequently added into the agarose liquid. At moderate temperature, agarose was poured
out into the gel tank (Bio-Rad Cat.1704467), and a comb was placed at the (-) electrode for creating the wells for loading the samples. After 15 minutes, the samples were run at 120 V for an hour. The gel was then visualized and captured using Gel Doc™ XR+ Gel Documentation System (BioRad).

2.10 DNA gel extraction

This technique was accomplished following the manufacturer’s protocol (NEB Cat. T1020L). DNA bands were visualized on top of a UV light box. Bands of interests were excised and trimmed from any gel excesses using a new and clean blade. Each band was put into an individual centrifuge tube, and four volumes of the dissolving gel buffer was added to the gel inside the tube (400 µL/100 mg) and incubated at 50°C for 10 minutes. The melted gel slice was allowed to go through the binding membrane of the column and centrifuged for 30 seconds. The binding membrane was then washed with 700 µL of the washing buffer and centrifuged for 3 minutes. The elution buffer was preheated at 50°C, and 6 µL was added to increase the DNA yield.

2.11 Immunofluorescence (2D)

Eight mm glass coverslips were placed in 24 well/plate prior to cell culture. The cells were grown on top of these coverslips overnight. Cells were then fixed using 10% formalin for 15 minutes. Cells were washed three times and then blocked and permeabilized by a mixture of 0.01% Triton™ X-100 and 1% BSA. Primary antibody was diluted in 1% BSA
at appropriate dilution (1:1000) and incubated at 4°C overnight. The primary antibody solution was aspirated, and the coverslips were then washed three times with PBS. The secondary antibody was diluted in 1% BSA at appropriate dilution (1:2000) and incubated at room temperature for an hour. Prolong mounting medium with DAPI (Invitrogen Cat. P36931), was dropped on top of the slides, and the coverslips were placed upside down on the slide. The cells were then visualized under the confocal microscope. A list of antibodies used is given in Table 2.

2.12 Immunofluorescence (3D)

Small dishes with glass at the bottom (MatTek Cat. No. P35G-0-10-C) were used to enhance imaging resolution for this assay. Prior to staining, cells were immersed in Matrigel (Corning® Cat. No. CACB354234) as previously demonstrated in Section (2.2). Cells were fixed by adding a mixture of 20% acetone and 80% methanol and incubated for 20 minutes at 4°C. After aspirating the fixative, cells were gently washed 3X with PBS to protect the Matrigel, and 3% BSA was subsequently used for blocking for 1 hour. Primary antibody was diluted in 3% BSA at low dilution (1:200) and incubated for one hour. Secondary antibody, diluted as above, was added for one hour. Prolong mounting medium with DAPI was gently dropped on top of cell matrix and a square cover slip was placed on top of it. The colonies were then visualized under the confocal microscope.
### Table 2: List of Fluorescent Dyes and Antibodies

<table>
<thead>
<tr>
<th>Fluorescent Materials</th>
<th>Source (Cat. Number)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-CRISPR-Cas9 antibody</td>
<td>Abcam (ab191468)</td>
</tr>
<tr>
<td>Anti-human Tks4 Antibody</td>
<td>Milliporesigma (09-267)</td>
</tr>
<tr>
<td>Anti-β-Actin Antibody</td>
<td>abm (G043)</td>
</tr>
<tr>
<td>Alexa Fluor™ 488 Phalloidin</td>
<td>ThermoFisher (A22287)</td>
</tr>
<tr>
<td>ProLong™ Gold Antifade Mountant with DAPI</td>
<td>ThermoFisher (P36931)</td>
</tr>
<tr>
<td>Dextran</td>
<td>ThermoFisher (D1868)</td>
</tr>
<tr>
<td>DyLight 649 labeled Lens Culinaris Agglutinin (LCA) (Lectin)</td>
<td>Vector Laboratories (DL-1048)</td>
</tr>
</tbody>
</table>
2.13 qRT-PCR

Total RNA was isolated from cells using RNAeasy Mini kit (Qiagen). In brief, the cells were lysed with 300 µL of cell lysis buffer and subsequently agitated at high speed and centrifuged for 3 minutes at maximum speed. The supernatant was gently aspirated and mixed with 300 µL of 70% ethanol. The total volume was then transferred onto RNeasy Mini spin column in a 2-mL collection tube and centrifuged for 15 s at ≥8000 x g. The flow-through was discarded. The spin column was washed with 700 µL of washing buffer and centrifuged for 3 minutes. The column was then placed in a new centrifuge tube, and 30 µL of RNase-free water was added and centrifuged for 2 minutes. RNA was quantified using the NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific). One µg of RNA was reverse transcribed to cDNA using qScript™ cDNA Synthesis Kit (QuantaBiosciences). One hundred ng of cDNA was amplified with primers listed in Table 1. Actin was used as the housekeeping gene. Samples were run on QuantStudio 5 qPCR (Thermofisher Scientific US). For each reaction, 1 µL of 10 µM forward/reverse primers, 1 µL of 100 ng DNA template, 5 µL SYBR Green qPCR Mastermix, and Nuclease-free water was added to yield a 10 µL reaction mixture. The PCR conditions were 40 cycles of denaturation at 95°C for 15s and annealing at 60°C for 1 min followed by a melt curve stage.
2.14 Western blotting

Lysate mixture consisting of RIPA buffer (Sigma-Aldrich Cat. No. R0278-50ML) and protease inhibitor cocktail was added to the cells, and cells were lysed using a cell scraper. Proteins from different cell lines were then quantified by the Bradford assay. Ten-times Reducing agent and 4X sodium dodecyl sulfate (SDS) were added based on protein quantity. Samples were then boiled for 10 minutes for protein denaturation. Samples were run on a gel at 120 V for an hour. Polyvinylidene difluoride (PVDF) membrane was immersed in methanol to be activated and placed within sponges, filter papers as well as the gel and transferred at 30 V for 2 hours. PVDF membrane was then blocked with 1-3% skim milk in TBS-T and incubated in 1 hour at RT. Primary antibody was diluted at appropriate concentration (1:1000) in 1-3% skim milk/TBS-T and added to the membrane and incubated overnight at 4°C. The membrane was then washed three times with TBS-T for 15 minutes, and secondary antibody was subsequently added after being diluted in 1-3% skim milk/TBS-T (1:5000) and incubated in an hour at RT. One mL of Luminata Crescendo Western HRP substrate (Millipore_Sigma Cat. WBLUR0100) was added on top of the PVDF membrane, and protein bands were developed and visualized using the ChemiDoc XRS System.
2.15 Confocal microscopy

Intravital and confocal images were captured using a Nikon Fast A1R Resonance Confocal Microscope as described previously [106]. To capture circulating cancer cells and/or established colonies in the CAM, Lectin (binds to glycocalyx on the endothelial cells) and Dextran dyes were injected into the CAM to illuminate the vessel lumen and the luminal surface of endothelial cells. The model was then placed in a special chamber with a special lid with a centered hole for placing coverslip to maintain position.

2.16 Lentiviral production and transduction

For preparing the virus, we used the human embryonic kidney (HEK)-293T cell line. Two µL of the transfection agent (X-tremeGENE) was mixed with 250 µL of an Opti-MEM medium and incubated at RT for 5 minutes. The second mixture was packaging (2.25 µg): envelope (0.25 µg): and the Cas9 (2.5 µg) plasmids together. The two mixtures were mixed and incubated at RT for 20-30 minutes and then were added drop-wise to the HEK293 cells. After 16 hours, the medium was removed and fresh medium with high serum was then added. One day later, conditioned medium was aspirated and viruses aliquoted and stored at -80°C for downstream experiments.

For transducing viruses into the cells, old medium was removed from the cells and 2 mL fresh medium supplemented with 4 uL of polybrene (at a stock of 4ug/uL) was added.
Viruses were then added drop-wise into the cells. A day later, the medium was removed and replaced by media with an appropriate antibiotic for selecting only the transduced cells.

A list of vectors used is presented in Table 3.

Table 3: List of used Vectors

<table>
<thead>
<tr>
<th>Vector Name</th>
<th>Source (Cat. Number)</th>
</tr>
</thead>
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<tr>
<td>LentiCRISPR v2 (Cas9)</td>
<td>Addgene (52961)</td>
</tr>
<tr>
<td>pLX-sgRNA (Kinase Library)</td>
<td>Addgene (50662, 51045)</td>
</tr>
<tr>
<td>psPAX2 (Packaging)</td>
<td>Addgene (12260)</td>
</tr>
<tr>
<td>pCMV-VSV-G (Envelop)</td>
<td>Addgene (8454)</td>
</tr>
</tbody>
</table>

2.17 Kill Curve Assay

Approximately 5000 cells/well were seeded in a 96 well/plate and incubated overnight. A range of eight different concentrations of Puromycin (0-10 µg/µL) and Blastcidin (0-20 µg/µL) were added in triplicate. After three days, 100 µL of the AlamarBlue dye, which produces a fluorescent signal in response to metabolic activity, was added for 2 hours. The optical density of the plate was measured by microplate reader at 655 nm.
2.18 MOI Determination of Kinase Library

Cells at $3 \times 10^5$ density were plated per well into a 6-well plate. Then, cells were infected with different amount of virus: Untreated cells (control), 63 µL, 125 µL, 250 µL, and 500 µL. After 24 hours, the media was replaced with the selection media containing 25µg/mL blasticidin. After selection, cells were harvested and counted.

2.19 Sequencing and sgRNA Kinase identification

DNA sequencing was performed at the London Regional Genomics Centre, Robarts Research Institute, London, Ontario. Each sample consisted of 10 µL of 3–5 ng PCR product and 5 µL of 2 µM forward or reverse primers. We used SnapGene Viewer software to analyze the sequencing results. Once the sequencing results were obtained, we matched the sgRNA amplicon sequence of the hits with the kinase library by comparing to the file deposited by Dr. David Sabatini and Dr. Eric Lander on the Addgene website.

2.20 Statistical Analysis

GraphPad prism software was used for all tests. A one-tailed unpaired student t-test was used for analysis. Data are presented as mean ± standard deviation (SD). A probability value $P < 0.05$ was considered statistically significant.
Chapter 3

3 Results

3.1 DU145 PCa cells are more effective at metastatic formation exhibiting a higher percentage of stellate morphology

There are many metastatic PCa lines and it is not clear which are more metastatic or aggressive [19], [34]. Thus, we utilized two cell lines to assess the suitability for our studies. For conducting the CRISPR screen, an aberrant colony morphology among homogenous ones is needed for sgRNA identification. Therefore, the cell line forming more colonies with homogenous morphology would be more favorable for the CRISPR screen. Two metastatic PCa cell lines were evaluated in vivo for their morphology and ability to form metastases. DU145 cells and PC-3M-LN4 cells were injected at $1 \times 10^6$ cells/mL (~$1 \times 10^5$ cells/CAM) into embryonic Day 9 chicken embryos. These cells were permanently expressing ZsGreen by viral transduction for visualization under fluorescence stereoscopy. At 7-days post-injection, we counted the number of cancer colonies using fluorescence stereoscopy and enumerated stellate-shaped colonies versus non-stellate colonies. In an in vivo model, metastatic cells usually form more stellate colonies in the CAM model compared to round colonies seven days post-injection. Thus, our readout in vivo was a phenotype shift into round. Our results showed that DU145 PCa cell line is more effective at metastatic colony formation and that the majority of these exhibit a stellate morphology (Fig 3)
Figure 3: DU145 cells exhibited higher metastatic efficiency and a higher percentage of stellate morphology relative to PC-3M-LN4 cells

A) A bar graph showing that DU145 cells form more colonies (170.1 ± 13.5 colonies/embryo) than the PC-3M-LN4 cells (24.4 ± 4.5). Ninety percent of DU145 colonies exhibited a stellate morphology compared to only 35% for PC-3M-LN4. B) Representative image for a DU145 stellate colony established in the CAM stroma at 7-days post-injection. C) Representative image for a DU145 round colony established in the CAM stroma at 7-days post-injection, Lectin and Dextran dyes were used to illuminate blood vessels. Results as a mean +/- SD.
3.2 Isolation of stellate colonies from the CAM model and subculture

The cell lines used in our study did not form homogenous ‘stellate’ morphology. Therefore, we sought to establish a cell line that is capable of forming a homogenous stellate colonies. It has been shown that serially culturing metastatic colonies after isolation from the host will enrich the isolated cells with more aggressive characteristics relative to the parental cells. Thus, we isolated a total of 70 CAM-colonies from DU145 that exhibit a stellate phenotype and passaged them in culture for a second-round of CAM injection (Fig 4).
Figure 4: Isolation of stellate colonies from the CAM model and subculture

A) A pie chart demonstrating 40 of the isolated colonies that grew in culture. B) A simple comparison in 2D culture between regular DU145 and an isolated colony. C) Representative images of isolated colonies growing in culture using the bright field with the green channel. DC#: DU145 Colony#.
3.3 DU145 sublines increased the percentage of stellate colonies

Only 40 out of 70 isolated colonies were able to grow in culture. Colonies failed to grow in culture mostly due to technical errors. Other growing colonies grew in culture but divided at a low rate or were contaminated. These were all discarded. A total of 20 colonies were contamination-free and grew in culture. These were injected into the CAM model in order to evaluate whether their stellate phenotype is enhanced. The number of stellate colonies were counted at 7-days post-injection (Fig 5).

![Figure 5](image)

**Figure 5:** DU145 sublines formed higher percentage of stellate colonies compared to the parental cells

A bar graph showing that the second round cancer cell formed relatively higher percentage of stellate colonies (93-98%) compared to parental DU145 (83%) (shown in red) after one round of *in vivo* passaging. Results as a mean +/- SD. DC#: DU145 Colony#.
3.4 DC-1- subline formed invadopodia and expressed Tks4

All sublines, after being injected into the CAM model, produced colonies that have relatively similar stellate morphology compared to the original cell line DU145, except DC-1- subline which produced a distinctive phenotype in its stellate form.

In order to evaluate further, we plated DU145 and DC-1- in Matrigel. This assay closely mimics the in vivo environment by allowing the cells to expand clonally in a 3D environment surrounded by matrix. After 7 days, both cell lines form colonies with a mixture of stellate and round morphology. Interestingly, DC-1- cells formed protrusions mimicking invadopodia (Fig 6A-B). These protrusions were not seen in DU145 cells.

We next examined these cell lines at the molecular level. A body of evidence revealed that Tks4 is responsible for protrusion formation, especially invadopodia [107]. Our results showed that DC-1- robustly express Tks4 at the mRNA and protein level over the regular DU145 (Fig 6C-D).
Figure 6: DC-1- subline form invadopodia and express Tks4

A) Two representative images of DU145 colony in stellate state that did not form any protrusions when plated in 3D culture model (insets show higher magnification images).

B) DC-1- forming protrusions (insets show higher magnification images). Black arrows
indicating protrusions. C) qPCR analysis of Tks4 mRNA revealing that DC-1- expressed Tks4. D) Immunoblotting showing high expression of Tks4 in DC-1- subline relative to the original DU145. β-actin was used as a loading control and a housekeeping gene. *p<0.05.

3.5 A schematic diagram for the in vivo CRISPR screen

A schematic illustrating the in vivo model and hit discovery following CRISPR kinase library screen is shown in Fig 7. This model needs to be optimized before moving to the CRISPR screen as the screen relies on a morphology shift. When a colony has a kinase deficit that causes a stellate colony morphology to shift into a round morphology, this would suggest that this kinase suppresses aggressiveness and eventually metastasis. Having a homogenous phenotype of stellate shaped colonies is required for the screen. However, our model did not produce homogenous stellate morphology (Fig 7). DU145 cell line and its sublines failed to produce 100% of stellate morphology in the vivo model.
Figure 7: Schematic for the *In vivo* CAM model screening

Schematic for the *in vivo* screen. Top panel (A) is depicting what the *in vivo* model should ideally show. Attaining a homogenous “stellate” morphology is critical for the CRISPR kinase library screen. In the middle lower boxes, each colored circle represents a KO kinase. Right lower panel (B) showing a boxed black arrow at a phenotypic shift to round as a putative hit.
3.6 Determining BPH cell plating efficiency in Matrigel.

Since it is challenging and time consuming to establish a cell line that is capable of forming a homogenous “stellate” morphology, we reasoned that a cell system which may shift from a homogenous round to a stellate colony may be more sensitive and specific. We selected BPH cells as an alternative cell line for this purpose. BPH cells form a homogenous round morphology after plating in Matrigel. Therefore, we selected this cell line and assessed plating efficiency before proceeding to the screen. We plated BPH cells at $6 \times 10^3$, $3 \times 10^3$, and $1.5 \times 10^3$ in 24-well plate in triplicate in Matrigel. Growing colonies were monitored and imaged daily (Fig 8). The colonies were then manually counted at 7-days post-plating using an inverted microscope (Fig 9A). For determining the plating efficiency, we divided the overall number of colonies by the number of plated cells and multiplied it by a hundred 

\[
\text{number of colonies formed} \div \text{number of cells plated} \times 100.
\]

Our results revealed that $1.5 \times 10^3$ has the highest plating efficiency relative to others (Fig 9B).
Figure 8: Wild type BPH growing in Matrigel (3D culture) for 7 days

A timeline of normal BPH cells growing in Matrigel throughout the 7 day study. Cells exhibited homogenous round morphology. Inset in all images depicting a higher magnification. At day 4, some BPH colonies start to reach their full size.
Figure 9: Plating efficiency of BPH cells in Matrigel.

Optimization for the plating efficiency for colony formation in Matrigel. A) BPH cells were plated in Matrigel and number of colonies were enumerated at 7 days from plating. B) a density of $1.5 \times 10^3$ showed the highest plating efficiency. Results as shown as a mean +/- SD. **p<0.05.
3.7 Kill Curve Assay for determining appropriate antibiotic selection concentration

Before we transduce BPH cells with Cas9 vector or CRISPR-Kinase library, we performed a kill curve assay to determine the effective concentration of an antibiotic to kill un-transduced cells completely. Here, we used two antibiotics; puromycin and blasticidin in order to utilize them for selection of Cas9 and CRISPR kinase library transduced BPH cells. Our data shows that puromycin is an effective selectable marker for Cas9 at 2µg/mL and blasticidin for kinase sgRNA-infected cells at 25µg/mL concentration (Fig 10).
Figure 10: Kill Curve Assay for selectable markers

Top panel depicting 2\(\mu\)g/mL of Puromycin as the minimal concentration required to completely kill BPH-wildtype cells. Lower panel depicting 25\(\mu\)g/mL of Blasticidin as the minimal concentration required for completely killing BPH-wildtype cells.
3.8 BPH cells expressing Cas9

BPH cells were then lentivirally transduced with LentiCRISPR-V2, a vector which constitutively and stably expresses Cas9. This vector has been shown to have a ~10-fold increase in functional viral titer over other vectors, particularly lentiCRISPR-V1 [108]. Constitutive Cas9 expression is critical for generating an efficient knockout [49], [109]. BPH-Cas9 expression was confirmed by Western blotting and immunofluorescence in 2D and 3D culture (Fig 11).
Figure 11: BPH cell lines stably expressing Cas9

A) Western blot for Cas9 expression in infected BPH cells with Cas9 vector vs BPH-wildtype (WT) cells (two replicates). β-actin was used as a loading control. B) Immunofluorescence for Cas9 expression in 2D culture (purple color represents Cas9, blue color represents DAPI staining). C) Immunofluorescence for Cas9 expression in Matrigel
3D culture (purple color represents Cas9 expression, green represents actin for phalloidin staining, and blue represents DAPI staining).

3.9 Generating BPH-Cas9 clone 122 with high induction of Cas9

We attempted to generate a BPH-Cas9 clone with a high and homogenous expression of Cas9. We conducted single cell expansion to generate clones with distinctive and different expression of Cas9. We performed a serial dilution to have 10 cells in 1 mL of media in 15 mL tube. We then added 100 µL of cell suspension into all wells of a 96 well plate, and we left each cell to proliferate for 2-3 weeks, with media changes every week. We next subcultured the clones for immunostaining to evaluate the level of Cas9 expression. BPH-Cas9 clone number 122 showed the highest expression as well as a relatively homogenous population compared to other clones (Fig 12).
Figure 12: BPH-Cas9 clone 122 has a higher expression of Cas9

BPH-WT was used as a negative control for Cas9. Representative images for different BPH-Cas9 clones. Cas9 number 122 clone has a higher expression for Cas9 relative to other clones. C-#: Clone-#.
3.10 Putative hit for CRISPR-Kinase library

Our study so far has shown that BPH colonies grow with a round morphology (Fig 9, 13A). This represents an excellent model for the screen since false positive hits will be highly minimized.

CRISPR kinase library has 6,012 sgRNA sequences with 8-10 sgRNAs designed for per kinase. Each sgRNA was subcloned into pLX vector with a Blasticidin resistance gene as a selectable marker. Library transformation was slightly different than the standard transformation, as per Section 2.5. Four µL of the library clones were distributed into 8 vials of competent cells (vial/5 µL) and incubated in ice for 20 minutes. Then, cells were heat-shocked by placing them in 42° C water bath for 45 seconds. The tubes were returned to ice and kept for 2 minutes before adding 125 µL of Recovery Medium into each tube. We then pooled all the tubes into one tube and added 50µL in each of the 20 agar plates (150mm). The following day, we scraped all colonies with a cell scraper and pooled them in one 50 mL conical tube. After centrifugation, we proceeded to DNA purification using Maxiprep kit which has the capacity to yield the maximum amount of DNA.

After making the viruses, we determined the Multiplicity of Infection (MOI) of BPH-Cas9 cells. A range of different virus volumes was tested for this assay, and the 30% MOI for BPH-Cas9 cells was 180 µL of virus (Fig 1) [each cell has a 30% chance for viral infection].
Figure 13: Schematic for the *In vitro* Screen (3D culture)

Schematic cartoon for the *in vitro* screen. Top panel is depicting the *in vitro* model without the CRISPR-Cas9 kinase library. BPH colonies are able to produce a homogenous “round” morphology, and this homogeneity is of critical importance prior to the CRISPR screen. In the middle lower panel, each colored circle represents a KO kinase. Right lower panel depicting a boxed black arrow indicating phenotype-shift to a stellate morphology revealing it as a putative hit.
Figure 14: MOI Determination for Kinase Library

A line graph depicting MOI determination for kinase library. One hundred eighty µL of the virus is capable of infecting nearly 30% of cells.
BPH-Cas9 cells were infected with the CRISPR kinase library, and after selection with Blasticidin for two days, cells were immediately plated in Matrigel in three 24 well/plates to ensure a whole coverage of the library (3X). Cells were observed under an inverted microscope daily throughout the experiment. Phenotype-shift into a stellate one was observed (Fig 15).
Figure 15: CRISPR-Kinase hits in BPH cells growing in 3D culture

Putative hits after infection of BPH-Cas9 cells with the CRISPR-Kinase library revealed a phenotype-shift into a stellate one. Top left image: a representative colony of BPH-Cas9 growing in 3D culture. All other images depicting a CRISPR-Kinase hit. Note that some hit colonies exhibited a significant shift in morphology (a complete disruption of the round morphology), and others demonstrated a moderate shift (protrusion formation).
3.11 The identification of hits

Seven days from plating the transduced cells with CRISPR-kinase library in Matrigel, we sought to isolate putative hits shown in (Fig 15). In total, we were able to extract 30 colonies and 6 of these isolated colonies were observed growing in culture afterwards (Fig 16). Hit growing colonies were kept in culture for over a month to yield enough cells for DNA extraction. Only four contamination-free colonies persisted and survived throughout the experimental period. These colonies showed substantial variation in terms of doubling time. Approximately \(7 \times 10^4\) to \(2 \times 10^6\) cells were lysed to get genomic DNA. Subsequently, the sgRNA amplicon was amplified by PCR followed by agarose gel electrophoresis. Expected band size was observed and excised. We next performed sequencing analysis for the sgRNAs. For further validations, extracted amplicons from the gel were run again to ensure the presence of DNA before sequencing (Fig 17).
Figure 16: Proportion for the number of extracted “hits” from Matrigel

We isolated 30 hits from Matrigel. A) 6 colonies were observed from the day following isolation. B) Two representative images for the isolated hits from Matrigel growing in vitro 2D culture.
Figure 17: Hit identification

A) Cartoon depicting the isolated colonies. B) Left: line illustrating DNA and red color indicting the sgRNA amplicon. Right side right showing a white strand in a centrifuge tube and the red\black arrowhead indicating the genomic DNA. C) DNA bands for hits on agarose gel at the indicated size. D) DNA bands after excision. E) Extracted bands were then run again on agarose gel to confirm the presence of DNA.
3.12 Glycogen synthase kinase 3 beta (GSK3\(\beta\)) as a potential regulator for prostate cancer invasion

Once we received the sequencing results, we matched the sgRNA amplicon sequence of the hits with the kinase library (an excel file deposited by Dr. David Sabatini & Dr. Eric Lander through Addgene website). One of the hits revealed Glycogen synthase kinase 3 beta (GSK3\(\beta\)) as a putative candidate for prostate cell morphology change (Fig 18). The sequencing results for two hits did not match the kinase library. We also did not retrieve sequence results from 3rd potential hit as there was not enough DNA for sequencing.
Figure 18: GSK3β as a putative regulator for morphological change and prostate cancer invasion

Top panel illustrating sgRNA sequence using SnapGene Viewer software, and sgRNA-GSK3β is highlighted by the yellow color. Lower panel is the pooled sequences for kinases in an excel format. GSK3β sequence is indicated by the red rectangle. The sequence of sgRNA- GSK3β is AGGAGACAAAGGACGCGCAGCA.
4 Discussion

4.1 *in vivo* CAM Model

The aim of this study was to perform an *in vivo/in vitro* CRISPR screening for kinases that may regulate prostate cancer metastasis.

For the *in vivo* model, we chose the chicken embryo as a screening model for candidate kinases. This animal model is convenient, cost-effective, highly vascularized (*Fig 3*), and amenable to real-time imaging. We injected metastatic PCa cell lines into the CAM model to monitor the morphology of colonies at 7-days post-injection. These colonies were expected to produce a homogenous “stellate” morphology [110] indicating an aggressive cell behaviour. Obtaining homogenous colonies was important so that when an individual kinase within these colonies is knocked out by the CRISPR-Cas9 system, a morphology-shift into a round one will take place.

**DU145 vs PC-3M-LN4 cell lines**

The CAM injection results for both cell lines revealed compatible results with previous work regarding metastasis [111]. Metastasis is an inefficient process, and this has been extensively studied. Luzzi and colleagues have shown that only 0.02% of the melanoma B16F10 cells form metastases in the animal model [112]. The metastatic efficiency for PC-3M-LN4 was found to be 0.03%, and 0.1% for DU145 in the CAM model. Surprisingly, the results for comparing the metastatic potential between both cell lines after injection into the CAM showed that the DU145 cells were three times more likely to be metastatic compared to the PC-3M-LN4 cell line (*Fig 4*). There are three classical PCa cell lines: PC-
3M-LN4, DU145, and LNCaP. The two former cell lines are androgen-independent, and the later one is androgen-dependent [113]. Our group showed that these androgen-independent cells are highly metastatic compared to LNCaP [studies performed in the laboratory by Patrick Telmer, data not shown here]. PC-3M-LN4 is a highly representative of PCa metastatic ability [114], [115], [116] relative to DU145 [117]. Furthermore, it is a subline of PC-3 which is a metastatic PCa cell line derived from the human bone. PC-3 was injected and metastasized to the lymph node of the mice. This cycle has been rounded four times [104]. This number of passages of the cell line is expected to make it more aggressive and metastatic than DU145 cell line. Two explanations may be formulated from these observations. First is the extravasation step at day 9 for the CAM injection. The results from the extravasation assay, which typically starts at day 13 of the chicken embryo, revealed that PC-3M-LN4 extravasates well when compared with day 9 injection (0.03% vs 0.09%) [studies performed in the laboratory by Yohan Kim, data not shown here]. Injection at day 9 for the colony formation assay is of a critical importance because the lifetime for the embryos is limited to 18-20 days [97]. Thus, injection of cells at the early age will allow them to proliferate into a colony. Second, the DU145 cell line may have higher metastatic potential since this line is derived from the brain and would have penetrated the blood-brain barrier (BBB) [118], [119]. Metastatic cancer cells tend to metastasize to the brain at a very late stage compared with other distant sites [120]. Another study showed that DU145 can proliferate in the bone environment after intratibial inoculation into the mouse [121], whereas the LNCaP cell line did not survive or proliferate [122], [123]. These studies suggest that the DU145 cell line may be similar to PC-3, which is a bone-derived cell line.
Colonies Passaging

Multiple lines of evidence have shown that a subline may have distinctive differences from the parental population in melanoma [124], breast cancer [125], [126], and prostate cancer [104]. Serial passages of PCa cells could potentiate the cells' metastatic potential by facilitating genetic alterations and independence from androgen hormones [104]. Therefore, we sought to do the same and isolate a subline with more invasive properties (Fig 5), which eventually will form a homogenous stellate morphology compared to the parental cell line. Overall, most of the generated sublines showed a high degree of invasiveness by forming a greater percentage of stellate colonies relative to the parental DU145 cells after injection into the CAM model (Fig 6).

Interestingly, DC-1- subline showed distinctive properties over the original DU145 cell line. DC-1- formed protrusions after plating in 3D culture Matrigel and significantly expressed Tyrosine kinase substrate with four SH3 domains-4 (Tks4) at mRNA as well as at the protein level (Fig 7). Tks4 is an adapter protein that is well known for protrusion formation, podosome formation and cell adhesion [107]. The status of a related protein, Tks5, in DU145 is unknown. We evaluated the mRNA levels of Tks5 in both cell lines, and neither showed any positivity. This might due to the environment for the cells during harvesting prior RNA extraction. Tks5 is a very well known mediator for invadopodia formation, a critical protrusive structure for facilitating cell extravasation [127]. We also speculate that these are not lamellipodia, because this lamellipodia protrusion appears only in 2D culture [128], [129]. Invadopodia and lamellipodia are hard to distinguish [127], [130]. Podosomes are distinctive from invadopodia since the former are found in normal cells such as macrophages and endothelial cells, and invadopodia are found in neoplastic
cells [131]. However, they share strong similarities that they are both included under the umbrella term invadosome [132]. Other markers can be used to identify protrusion formation, in particular invadopodia, including cortactin and the neural Wiskott-Aldrich Syndrome protein (N-WASP).

The use of PCa cells for the proposed CRISPR kinase screen was found to be limited. Both parental as well as the DC-1- subline did not produce a pure population of colonies with stellate morphology (Fig 8). Thus, it is challenging and time-consuming to establish a cell line that is capable of producing a homogenous set of stellate colonies if this morphology is still the readout of the screen. Thus, we adapted the normal BPH cell line for these studies.

4.3 *In vitro*

The BPH cell line has the capability, along with other cell lines [133], to produce a homogenous round colony morphology in Matrigel [134], [135], [102]. BPH cells were implanted in Matrigel to confirm the homogeneity of the round colonies. My results show that BPH colonies exhibited a round morphology (Fig 9). I then plated different BPH cell densities to determine the plating efficiency for colony formation. The results showed that $1.5 \times 10^3$ produced a higher plating efficiency of colonies relative to densities. Although $3-6 \times 10^3$ cells formed more colonies, its plating efficiency was low (Fig 10). A probable reason for this drastic decrease of the number plated cells is the apoptotic response for these cells due to loss of cell-cell contact [136]. Determining the plating efficiency is of critical important to have an enrichment of the whole kinase library. For example, if $3 \times 10^3$ or $6 \times 10^3$
cells with low plating efficiency were plated, 64-82% of the library clones will be lost after subjecting to CRISPR kinase library (Fig 10).

Lentiviral transduction can generate a stable expression of a recombinant protein and is a widely used platform to introduce genetic material [137]. Since each BPH-Cas9 cell will be individually infected with a sgRNA kinase clone, cells will need to exhibit a high and homogenous expression of Cas9 for effective knockout. Several lines of evidence suggested that attaining a homogenous expression of a protein is not possible [138], [139]. However, we generated BPH-Cas9 clone number 122, which met our expectations (Fig 13). The BPH-Cas9-122 cells were subjected to the CRISPR kinase library and treated with Blasticidin antibiotic for selecting only infected cells. The MOI was determined with unmodified BPH cells, so cells were infected at low MOI (0.3) to ensure at least one cell will take up only one viral construct [88]. Another approach we used to minimize off targets events and MOI was that we maximized the number cells relative to the library clones prior transduction [140].

After plating the transduced cells in Matrigel, some colonies exhibit a morphology shift [141], and these colonies were isolated for sgRNA identification. Some exhibited a significant shift as a result of complete disruption of the round morphology, and others exhibited a moderate shift by forming protrusions. This protrusion formation may be due to invadopodia or podosome formation. Podosomes are exclusively formed in non-malignant cells, and these include BPH cells. We also noticed that during incubation and throughout the experiment, a considerable number of cells appeared dormant in contrast to the BPH-WT cells plated in Matrigel. This observation was not surprising since the kinase
family occupies the majority of oncogenes in cancer [62]. In addition, the plating efficiency was greatly reduced with kinase KO (10.6%) compared to typical BPH cells (45%).

Hit colonies were picked using a microneedle under the microscope and transferred to fresh media. Thirty colonies with potential hits were isolated and sub-cultured. The following day, only 6-8 colonies were observed to be growing in culture (Fig 16). These clones exhibited a substantial variation in terms of doubling time. Beaver and his group divided this kind of cell behavior for such clones into three groups: holoclones, showing extensive proliferation and self-renewal; meroclones, having limited proliferative capacity and cannot self-renew, and lastly paraclones, which are incapable of additional proliferation [142]. Eventually, only four isolated colonies persisted in culture. These cells were lysed for DNA isolation, and sgRNA amplicons were amplified. The sequencing results revealed that GSK3ß is potentially involved in mediating morphological changes and possibly PCa metastasis (Fig 18).

Glycogen synthase kinase 3 beta (GSK3ß) is a serine/threonine kinase. It was initially extracted from rat skeletal muscle as a kinase that phosphorylates and inactivates glycogen synthase (GS) to inhibit glycogen synthesis. GS is considered to be the last enzyme involved in glycogen biosynthesis [143]. GSK3 is involved in many pathological disorders [144]. Its role in cancer is not fully established. Some studies show that GSK3ß has a tumor suppressor role by controlling Wnt signaling and ß-catenin degradation [145]. In addition, the absence of GSK3ß expression is correlated with advanced PCa [146]. Other studies revealed that GSK3ß is involved in oncogenesis [147]. GSK3ß has a role in cell cycle regulation through cyclin-dependent kinase (CDK) modulation [145]. Multiple GSK3ß inhibitors have been identified for neurological and diabetes disorders [148]. Type 1
diabetics lack the insulin hormone that is responsible for converting glucose to glycogen; suppression of glycogen synthase kinase 3 (GSK3) promotes conversion of glucose into glycogen [166]. Other GSK3β inhibitors were found to be effective for other types of cancer such as ovarian [149], leukemia [150], pancreatic [151], [152] and colon cancer [153]. A study revealed that the GSK3β protein is an oncogene in PCa, and its suppression leads to decrease in the tumor burden [154]. Lithium chloride (LiCl), TDZD-8, and L803-mts are GSK3 inhibitors. LiCl and TDZD-8 suppressed the growth of human prostate tumor xenograft in mice. In another transgenic adenocarcinoma mouse prostate (TRAMP) model, TDZD-8 and L803-mts administration reduced the presence of prostate tumour lesions. Several limitations for GSK3 inhibitor use have been found. Since GSK3 regulates Wnt signaling and β-catenin, some types of cancer have mutations in these proteins. GSK3 is also known to phosphorylate oncogenic transcription factors such as c-MYC and c-JUN [155]. GSK3 inhibition in this case would be anticipated to dephosphorylate and activate these transcription factors. In our in vitro screen, we consider GSK3β as a tumor suppressor as it changed the normal/benign morphology of a colony into one generally associated with an aggressive phenotype [156]. If true, this identification may lead to a novel approach for cancer therapy [157]. This, of course, requires additional studies to confirm the knocking out of GSK3β at the protein level in addition to mRNA level. In addition, studies are needed to directly modulate GSK3β and determine whether morphological changes are indeed present. Conducting cell migration assay/invasion assay to assess its role are also needed.

In our studies, we utilized BPH and PCa to identify kinases which may regulate metastasis. To appreciate the results of this study, it is important to discuss the properties of BPH and PCa cells. The former is recognized to develop from the transitional and central zones (TZ
and CZ, respectively) of the prostate gland. The latter develops from the peripheral zone (PZ). A link between the two disorders has been found based on epidemiological studies, clinical trials, in vitro experiments, and autopsy samples [158]. BPH is representative of an inflammatory disorder, and PCa along with other types of cancer are known to be preceded by chronic inflammation. In the majority of prostate cancer biopsy samples, inflammation is detected either within the tumour or in close proximity to it. A study was conducted on 180 males with suspected PCa who were diagnosed at baseline and after five years of follow-up. After five years, 20% of patients showed inflammatory signs on their biopsies; however, in 6% of the patients, the biopsies did not show any sign of baseline inflammation. Measurements of C-reactive protein (CRP) levels, which is a circulating inflammatory marker, have been correlated with PCa progression. Genetic analyses have shown that mutations in the RNASEL (encodes 2-5A-dependent ribonuclease) and MSR1 (encodes macrophage scavenger receptor types I and II) genes, both of which are involved in the response to infection, are correlated with increased risk of prostate cancer. Proliferative inflammatory atrophy, which is known to precede PCa and intraepithelial neoplasia, has also been shown to arise from areas in the prostate in which cells are actively regenerated following tissue injury caused by various pathological processes such as infection. Since inflammation play an important role in cancer, treating PCa patients with anti-inflammatory agents could minimize the risk of PCa. This association between the two disorders has significant clinical implications because it reveals the question of whether BPH can predict the risk of developing PCa.
4.3 Limitations

We faced a number of limitations in our CRISPR screen for kinases. First, we attempted to use the in vivo CAM model for the screen. However, a lack of homogenous colony formation by PCa cells prevented progression of studies. Measuring changes in the number of metastatic colonies as a readout may be performed in other animal models. Second is the inability to have a homogenous expression for Cas9 cells, which may lead to inefficient kinase KO. Since there were 8-10 sgRNAs for each kinase member, we hoped this number of clones can compensate for the possibility of low Cas9 expression. Third, isolating colonies from the Matrigel medium is inherently difficult. Our successful isolation rate was only 20% and we lost the majority of the hits during the isolation process. This end-point experiment can be improved with more advanced techniques that allow for precisely extracting a colony with a low potential for contamination. Another way to improve colony viability after isolation is to design a special medium, as in the case with stem cells passaging in Matrigel by using E8 medium [159].

4.4 Future Directions

There is need of further studies for screening the kinase library and validating it. This research provides fundamental strategies and methodologies for developing CRISPR-Cas9 KO kinase library using BPH cells in vitro. The kinases that regulate the hallmarks of cancer can be screened for different cancer cells, in particular, metastatic prostate cancer cell lines such as DU145, PC-3, and LNCaP. For the in vitro model using an invasion assay, cells that invade well should be sequenced and selected as targets for the identification of potential invasion suppressors. However, migration assay is particularly difficult because
the whole library will be mixed, and hits and non-hits will be indistinguishable. Identifying kinases whose loss exerted proliferative or metastatic behaviour will be a key for the development of treatments for PCa. Moreover, kinases that confer resistance to current PCa therapy can be identified. By treating the KO kinase cells with these drugs, genes that confer resistance to these drugs will be negatively selected. In addition, an in vivo screen can be performed to uncover kinases involved in intravasation, extravasation, and metastatic formation step whether by administering KO kinase cells intravenously, subcutaneously, or orthotopically in the mouse model. Overall, CRISPR-Cas9 genomic editing system is a novel approach to delineate the function of genes and their involvement in different pathological disorders, including cancer.

GSK3 contributes to many cellular functions. There is a need to test its function in PCa using the three classical PCa cell lines: PC-3, DU145, and LNCaP. Evaluating its expression at the protein and mRNA levels would be useful for determining if these cells have an altered function of this gene product. Knocking this gene out could be used to evaluate its contribution to colony formation or morphological changes in 3D culture. Forming colonies in soft agar has been correlated with tumorigenesis [180]. Performing cell migration and cell invasion assays would help to determine whether silencing this gene would attenuate or potentiate tumorigenesis in these cell lines.
References


Curriculum Vitae

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