SEMAPHORIN 3C IN PROSTATE CANCER TUMOURIGENESIS

by

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Abstract

Prostate cancer (PCa) is among the most commonly-occurring cancers worldwide and a leading cause of cancer-related deaths in men. Local non-invasive PCa is highly treatable but limited treatment options exist for those with locally-advanced and metastatic forms of the disease. This underscores the need to identify mechanisms mediating PCa progression. One well-established driver of PCa progression is the androgen receptor protein whose transcriptional targets include genes related to cell growth and cell cycle progression. Consequently, the androgen receptor axis is the target of many therapies for those with PCa. Another important aspect of disease progression relates to cancer spread or metastasis. Epithelial-to-mesenchymal transition (EMT) is a cellular process executed during embryogenesis and is defined as the transition of cells from an epithelial phenotype to a mesenchymal phenotype. It is suspected that metastasis is, in part, due to inadvertent re-activation of EMT. Another theorized cause of cancer progression is due to the existence of tumour-initiating cells or 'cancer stem cells' which resist conventional radiation- and chemotherapies and seed relapse and metastasis.

The semaphorins are a large grouping of membrane-associated or secreted signalling proteins whose normal roles reside in embryogenesis and neuronal development. During these processes the semaphorins establish chemotactic gradients and direct cell movement. Various semaphorin family members have been found to be up- or downregulated in a number of cancers. One family member, semaphorin 3C (SEMA3C), has been implicated in several types of cancer and its increased expression is correlated with PCa stage. Given SEMA3C's roles in development and its augmented expression in PCa, we hypothesized that SEMA3C promotes cancer progression by driving EMT and stem-like characteristics.

In the present study, we show that SEMA3C is a direct transcriptional target of the androgen receptor and further show that ectopic expression of SEMA3C in RWPE-1, a normal prostate epithelial cell line, leads to an upregulation of EMT and stem markers which is accompanied by acquisition of invasiveness and stem-like phenotypes. The broader impact of this work pertains to the clinical implications of SEMA3C's involvement in PCa and linking SEMA3C and AR to metastatic recurrent PCa.

Preface

The work surrounding this thesis was carried out at the Vancouver Prostate Centre under the supervision of Dr. Christopher Ong. On work presented in this thesis, Dr. Christopher Ong and Kevin J. Tam conceptualized the research aims; Dr. Christopher Ong, Dr. Michael Cox, Dr. Ralph Buttyan, Dr. Alan So, and Kevin J. Tam designed the experiments; Kevin J. Tam, Dr. Kush Dalal, Dr. Michael Hsing, Chi Wing Cheng, Dr. Shahram Khosravi, Parvin Yenki, Charan Tse, James W. Peacock, Aishwariya Sharma, Dr. Yan Ting Chiang, Daniel H. F. Hui, Wilson W. Lee, Mingshu Dong, Ivy Z. F. Jiao, Tabitha Tombe, Dr. Larissa Ivanova, and Winnie Kwan conducted the experiments; Mingshu Dong generated the image of the SEMA3C working model (Figure 1.3); Dr. Christopher Ong, Dr. Michael Cox, Dr. Ralph Buttyan, Dr. Martin Gleave, and Kevin J. Tam analyzed and interpreted the results.

The data presented in this thesis are part of three manuscripts in various stages of preparation for which I am listed as first or co-first author.

Chapter 2: Androgen receptor transcriptionally regulates Semaphorin 3C in a GATA2-dependent manner

A version of Chapter 2 is published as a research article in *Oncotarget*.

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Chapter 3: Semaphorin 3C drives epithelial-to-mesenchymal transition, invasiveness, and stem-like qualities in prostate cells

Components of Chapter 3 make up a manuscript titled "Semaphorin 3C drives epithelial-to-mesenchymal transition, invasiveness, and stem-like qualities in prostate cells" which is currently awaiting submission. Portions of Chapter 3 are also in a manuscript currently in preparation titled "SEMA3C drives stem-like characteristics through TGFβ2 signalling."

All animal experiments were approved by the UBC Animal Care Committee and adhered to guidelines of the Canadian Council on Animal Care. UBC animal protocol number A15-0150, animal project title: "Semaphorin 3C as a novel therapeutic target for treatment of prostate cancer."

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List of abbreviations

AR: Androgen receptor
NR: Nuclear hormone receptor
NTD: N-terminal domain
DBD: DNA-binding domain
LBD: Ligand-binding domain
ARE: Androgen response element
DHT: Dihydrotestosterone
PCa: Prostate cancer
CRPC: Castration-resistant PCa
PSA: Prostate-specific antigen
SEMA3C: Semaphorin 3C
ARBS: Androgen receptor binding site
GEO: Gene Expression Omnibus
CSS: Charcoal-stripped serum
EMSA: Electrophoretic mobility shift assay
ChIP: Chromatin immunoprecipitation
TSS: Transcription start site
BP: Basepair
AR DBD: AR DNA-binding domain
FOX: Forkhead box
CSC: Cancer stem cell
EMT: Epithelial-to-mesenchymal transition
PLXN: Plexin
NRP: Neuropilin
UbC: Ubiquitin C
qPCR: Quantitative polymerase chain reaction
IF: Immunofluorescence
IVIS: *in vivo* imaging system
NSG: NOD scid gamma
CM: Conditioned media
ALDH1: Aldehyde dehydrogenase

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Dedication

To my family and friends, you guys rock.

CHAPTER 1. Introduction

1.1 Introduction

In 2000 Hanahan and Weinberg distilled many decades worth of cancer research down to six characteristics possessed by all solid cancer cells: self-sufficiency in growth signalling, insensitivity to anti-growth signals, evasion of apoptosis, angiogenesis, limitless replicative potential, and tissue invasion/metastasis (1). Phenomena often seen in cancer cells that contribute to Drs. Hanahan and Weinberg's "Hallmarks" include mutations to DNA which impart altered expression of or aberrant function by critical proteins such as oncogenes and tumour suppressors. Problems arise when these aberrations affect growth or apoptotic pathways and programs relating to invasion and angiogenesis. When the highly organized and tightly regulated temporal and spatial expression of the roughly twenty-thousand genes encoded in the human genome is compromised, cancer can arise. Environmental insults such as pollutants, carcinogens, and cosmic radiation can cause mutations and breaks to DNA and lead to the production of proteins with altered function. This in turn can compromise cell integrity and disrupt the cellular mechanics which maintain normal cellular, tissue, and organismal stability. Although great progress has been made in understanding the mechanisms propelling cancer development and progression, an intimate understanding of the pathways involved and their interconnectivity does not currently exist. A more comprehensive understanding for the networks involved will be pivotal to the development of suitable new therapies.

1.2 The prostate

1.2.1 Prostate biology

The prostate is a walnut-sized exocrine gland situated in front of the rectum and below the bladder. Its main role is the production of prostatic fluid, a component of ejaculate, which

generally functions in prolonging the lifespan of the sperm in the vaginal tract (2). During development, the cloaca, a swelling of the foregut, ultimately gives rise to the urogenital sinus. The prostate, in turn, is formed from the budding of the urogenital sinus epithelium (2). The protrusion of cells from the urogenital sinus and subsequent branching morphogenesis of the early prostate is driven by androgen signalling in the urogenital sinus mesenchyme. Androgen signalling during these processes leads to extensive interplay between the cells of the mesenchyme and the epithelium. In the adult male, the vas deferens extends from the testicle to the seminal vesicle where its fusion to the duct of the seminal vesicle forms the ejaculatory duct. Within the prostate the urethra (extending from the bladder) fuses to the ejaculatory duct. The luminal epithelial cells of the prostate secrete prostatic fluid into the seminal fluid produced by the ejaculatory ducts. These secretions then continue through the urethra to the tip of the penis and out of the body. The prostate exhibits bilateral symmetry and has four zones: the peripheral, central, transition, and anterior fibromuscular zone. Of the four zones, cancer occurs most commonly in the peripheral zone. The prostate stroma contains immune, stromal, smooth muscle, nerve, and endothelial cells, as well as pericytes and fibroblasts. Collectively, these cells fill supportive, structural, and homeostatic roles. By comparison, the prostatic parenchyma includes luminal epithelial, basal, intermediate, and neuroendocrine cells. The lumens of the prostatic glands are enclosed by a layer of luminal epithelial cells. This layer of cells forms a tight barrier through which exchange of fluids and solutes is tightly regulated. The luminal epithelial cells are also responsible for secreting prostatic fluid into the seminal fluid. Prostate epithelium exhibits typical epithelial characteristics including tight cell-to-cell junctions, apical-basolateral polarity, and low motility. Markers for luminal epithelial cells of the prostate include expression of the androgen receptor and cytokeratins 8 and 18. Beneath the luminal epithelial

cells are the basal cells which help form the basement membrane upon which the luminal epithelial cells grow. The basal cells provide structural support as well as contribute regenerative and signalling functions. The basal cells do not express the androgen receptor and are characterized by the expression of cytokeratins 5 and 14. Intermediate cells, which express both luminal and basal cytokeratins, reside alongside luminal epithelial cells. This cell type has a differentiation state somewhere between that of basal and luminal epithelial cells. Lastly, neuroendocrine cells integrate signals from the nervous system and release hormones which alter function and activity of cell populations within the prostate (2).

1.2.2 Prostate cancer (PCa)

Prostate adenocarcinoma is the most commonly occurring non-cutaneous cancer in Canadian men and normally arises in the peripheral zone of the prostate (2). **Localized low grade PCa** can be treated by surgical resection and radiation therapy with or without androgen-deprivation therapy (ADT) and is generally met with favourable response. **Locally advanced PCa** is defined as the cancer having spread beyond the confines of the prostate and into nearby structures such as the seminal vesicle. Those with locally advanced PCa typically receive more aggressive treatments than those with localized low grade PCa. These treatments include ADT in combination with radiation therapy. **Metastatic PCa** refers to the clinical situation where the PCa has spread to distant anatomical sites. Although highly situation-dependent, diagnosis at metastatic stage typically involves ADT in combination with other available treatment strategies such as chemotherapies. Despite initial tumour response to ADT, relapse by those with locally advanced and metastatic PCa to so-called stages of castration-resistant PCa (CRPC) invariably occurs. If not previously metastatic, this form of the disease inevitably involves metastasis and treatments become palliative (3). This phase of the disease, called **metastatic CRPC (mCRPC)**,

requires the use of antiandrogens, chemotherapy, or inhibitors of steroidogenic enzymes (4-7). However, by way of mutations to or increased expression of the AR, aberrant AR signalling underpins PCa progression illustrating the importance of the AR axis in this disease and the therapeutic potential in targeting it. Several lines of evidence support the notion that AR signalling remains a key factor in advanced stages of disease and that PCa remains 'addicted' to AR signalling throughout its course (8). For example, AR transcriptional targets include genes related to cell proliferation and survival such as M phase cell cycle progression genes (9-11). In addition, CRPC is often marked by: retention or amplification of the AR, biochemical recurrence of AR target genes such as prostate-specific antigen (PSA), and mutations to the AR rendering it constitutively active and refractory to the actions of antiandrogens (12). Collectively, these observations indicate a causal role for AR in disease progression and underscore the importance of AR blockade in treating PCa. These realizations also indicate the importance in the identification of novel AR targets. It is generally accepted that a more comprehensive understanding of AR gene targets will provide insight in the development of novel therapeutics for PCa.

While the most commonly-diagnosed PCa is adenocarcinoma, another distinct subtype of this disease is neuroendocrine PCa (NEPC) whose defining features include an upregulation of NCAM1, EZH2, PEG10, MYCN, AURKA, ENO2, SYP, CHGA, and CHGB, a downregulation of AR, and loss of TP53 and RB1 (13). NEPC can arise *de novo* or can emerge due to use of hormonal therapies and AR inhibitors. The development of NEPC features likely represents a selective mechanism to circumvent androgen deprivation. Regardless of etiology, the prognosis for this subtype is poor and treatment options are limited. Patients with NEPC exhibit aggressive visceral organ metastasis and rapidly succumb to disease.

1.2.3 The androgen receptor

The androgen receptor (AR) is a 110 kDa member of the nuclear receptor superfamily of transcription factors. Like other nuclear receptors, the AR is a modular protein and regulates gene transcription in a ligand-dependent manner and through affinity for highly conserved DNA consensus sequences. Structurally, AR contains a highly unstructured N-terminal domain (NTD), a DNA-binding domain (DBD), a hinge region, and a C-terminal ligand-binding domain (LBD). The AR NTD is disordered and contains multiple stretches of amino acid repeats (14); these factors have led to our inability to crystallize the full length AR. The NTD contains the AF1 region while the LBD contains the AF2 and BF3 region. Collectively, the AF1, AF2, and BF3 fill conformation and structural roles as well as contribute to the recruitment of ancillary proteins required for transcription initiation (15,16). The C-terminal LBD contains a hydrophobic pocket that binds androgenic ligands and has been heavily exploited in the development of anti-androgen receptor compounds for clinical application (12). The DBD, which is situated between the NTD and LBD, contains a zinc finger motif. This motif is responsible for recognizing and binding inverted repeats of AGAACA that are separated by a 3 basepair linker (16-19). In the absence of ligand, the AR resides within the cytoplasm associated with heat-shock proteins which render it inactive. Upon binding androgenic ligands such as dihydrotestosterone (DHT), the AR dissociates from its chaperone proteins and translocates to the nucleus. Once in the nucleus, the AR recruits co-activators, RNA polymerase II, and other components of the basal transcriptional complex at androgen response elements (AREs) found in the vicinity of androgen-regulated genes or to sites distant to the gene in the case of enhancers (14,20). AR is additionally known to cooperate with pioneering factors like GATA2, FOXA1, and OCT1 which promote gene transcription through chromatin remodeling and DNA looping (21-24). The AR

holds important roles in normal prostate development and physiology in the adult male, in the maintenance of secondary male characteristics, and in sexual function in the adult male. The dysregulation of AR is also largely responsible for PCa progression (25-30). Accordingly, AR is the focus of many translationally-driven investigational studies.

1.2.4 PCa treatments

A number of parameters are taken into consideration in the clinical assessment of PCa. These factors assist in patient diagnosis, stratification, prognostication, and in the provision of treatment options. The D'Amico Classification system takes into account the TNM (tumour, node, metastasis) system, serum prostate specific antigen (PSA) levels, and Gleason score. The TNM system examines the clinical and pathological stage of the cancer using methods such as the digital rectal exam, medical imaging, and histology (2). Metrics include the extent of primary tumour growth, the degree organ confinement, invasion of the seminal vesicle, lymph node involvement, and the spread of the cancer to other organs (metastasis). PSA levels can be measured from the blood and in general, reflect the growth of the PCa. This is due to the fact that PSA is a downstream transcriptional target of the AR which is typically present and active in the cells of the prostate and prostate tumours. Finally, the Gleason score is a pathological assessment of tumour morphology using histological examination of biopsies of the primary tumour. The Gleason score is the sum of the two most common malignant patterns of the primary cancer and assesses the deviation of the cancer tissue from its normal tissue architecture. Gleason scores indicate cancer cell aggressiveness and allow for inferences on prognosis and metastasis. Important when assessing the Gleason score is the extent and manner in which cancer cells form glandular structures.

Taking into account staging, PSA information, Gleason scoring, and additional factors such as the age and general health of the patient, the physician and patient select treatment course. Common treatment modalities for those with localized low-grade PCa include radiation therapy and surgery. Radiation therapy can include external beam radiation where x-rays or gamma rays are directed at the tumour from a source outside of the body. By comparison, another form of radiation therapy called brachytherapy, involves the placement of multiple small ^{125}I, ^{103}Pd, ^{192}Ir, or ^{137}Cs -labelled seeds inside the prostate tumour. Whether external beam or brachytherapy, DNA damage incurred by the radiation initiates tumour cell death and shrinks the tumour size (2). Those with localized cancer are also often good candidates for surgical resection of the tumour; in a procedure called a radical prostatectomy, the entire prostate and the tumour within it, is removed by open or laparoscopic surgery. An alternate approach for PCa patients with predicted slow growth and non-aggressive disease is called active surveillance or watchful waiting. This entails no initial treatment but medical intervention should progression occur. First line therapy for those with locally advanced PCa includes ADT (31) also known as hormone therapy or castration, in combination with surgery or radiation. PCa cells generally respond to androgens, at least initially, by growing. Consequently, androgen-deprivation therapy is the first-line therapy for those with locally-advanced PCa and attenuates tumour growth by reducing the levels of circulating androgens in the body (32). Androgen-deprivation therapy is most commonly accomplished nowadays by administration of luteinizing hormone-releasing hormone (LHRH) superagonists and antagonists. These agents disrupt the endocrine axis responsible for production of testosterone by the testes ultimately leading to decreased levels of testosterone in the body. Similarly to those with locally advanced PCa, patients with metastatic PCa are also treated with ADT. However, in addition to ADT, these patients are also treated with

chemotherapies. Conventional chemotherapies for PCa patients include docetaxel and cabazitaxel in combination with prednisone. These chemotherapies belong to the taxane family of chemotherapies which inhibit the depolymerisation of microtubules to its tubulin monomers. Microtubules are cellular structures that are necessary for cell division, therefore, taxanes are considered to be anti-mitotic since they disrupt a key event in mitosis. Interference in microtubule dynamics in this way is catastrophic to the cell and induces cell death within the tumour as well as in other rapidly dividing cells of the body leading to many unwanted side effects. Progression on ADT to CRPC, necessitates the usage of a class of drugs called antiandrogens. Bypass mechanisms induced by a reduction in androgen levels due to castration, include increased expression of the androgen receptor (33). Elevated AR levels render tumour cells hypersensitive to depleted androgen levels or lowered ligand specificity. Thus therapeutic approaches which antagonize the AR axis which are complementary to castration are essential in the treatment of CRPC. Antiandrogens are agents that act by displacing androgens from the ligand binding domain of AR. Currently, the most prevalent antiandrogens are enzalutamide and bicalutamide which, together, have replaced flutamide (a first generation antiandrogen) due to greater efficacy and tolerability. Enzalutamide is superior to bicalutamide with respect to its affinity for the AR LBD, its ability to prevent translocation of AR to the nucleus, AR-DNA association, and transcription of AR target genes (12). Following progression on antiandrogens, inhibitors of steroidogenesis are commonly deployed. This class of drugs inhibits cancer cell growth by antagonizing the steroidogenic enzymes which produce testosterone and dihydrotestosterone. Examples include abiraterone acetate and ketoconazole which inhibit CYP17A1, an enzyme that catalyzes multiple steps of the steroidogenic pathway.

1.2.5 Novel therapeutic approaches in PCa

In later stages of the disease, PCa cells become refractory to the arsenal of agents used to contain the cancer. As such, refined iterations of existing drugs and conceptually new ones are constantly being developed. Significant strides made by abiraterone in the treatment of PCa precipitated the development of additional steroidogenic antagonists such as VT-464 (34) and galeterone (TOK-001) (35) which are in early-stage clinical trials and orternonel (TAK-700; (36)) which recently completed phase III clinical trials. Concurrent application of inhibitors of steroidogenic enzymes to block multiple junctions of steroidogenesis is also being explored in clinical trials. Development of additional second-generation AR antiandrogens is also an area of intense investigation. Early stage clinical trials involving the novel second-generation antiandrogens ARN-509 and ODM-201 are underway (37,38). Strategies to attenuate AR activity by targeting AR's other domains (such as NTD and DBD) have also been attempted (39,40). A compound called EPI-001, which binds to the NTD of AR, prevents its association with co-activators, reduces AR activity, and inhibits primary and CRPC tumour growth *in vivo*; early clinical trials of a derivative of EPI-001 are ongoing. Disrupting AR's association to DNA by docking small molecules at the DNA-interacting interface of AR would potentially overcome the challenges presented by promiscuous ligand specificity by AR and constitutively active splice variants of AR. One such molecule termed VPC-14449 has been effective in inhibiting the transcriptional activity of wild type AR and AR-v7 and in inhibiting tumour growth *in vivo* in preclinical studies. Agents targeting the AR axis but which do not target AR itself are also being explored. Clinical trials with therapies that antagonize HSP27, an AR chaperone protein, are underway (41) as are trials using inhibitors of FOXM1, a co-activator of AR (42). Targeted therapies that inhibit other PCa-relevant growth or pro-survival pathways which are independent

of AR are also being vigorously explored including inhibition of clusterin and components of the PI3K pathway (43,44). Sipuleucel-T (Provenge) and PROSTVAC are immunotherapies and are the first of their kind in PCa (45,46). Sipuleucel-T has cleared clinical trials and functions by re-introducing the patient's own dendritic cells which have been primed against antigenic prostatic acid phosphatase *ex vivo*. PROSTVAC is in late-stage clinical trials and works by introducing DNA vectors whose gene products include immunogenic PSA. The patient's own immune system then mounts an immune response against PSA-positive cells such as those of the tumour. Due to massive success in other cancers, immune therapies targeting programmed cell death 1 ligand (PD-1) and cytotoxic T lymphocyte-associated antigen 4 (CTLA4) are also under investigation for use in PCa.

1.2.6 PCa genomics

Genomic instability and compromised cell integrity lead to elevated AR levels, AR ligand promiscuity (47,48), or to the production of variants altogether missing the ligand-binding domain (49). These situations pose a threat to maintained efficacy of mainstay therapies for PCa. However, aided by comprehensive genomics studies, it has been noted that emergence of resistance to an AR antagonist after the onset of CRPC does not signify resistance to *all* AR antagonists. This would indicate the potential utility in sequential administration of AR inhibitors guided by genomic revelations tracking mutationally-driven resistance to AR inhibitors (44). Nevertheless, it will have to be taken into account whether the therapy that is 'next in line' will remain efficacious in the landscape that rendered the tumours refractory to all therapies that preceded it. Decreasing costs and rapidly advancing tools in the field of bioinformatics have massively accelerated the discipline of genomics. Coupled to a steadily increasing appreciation for the biological implications of discoveries made through experimental studies, medical teams

now make informed actionable decisions guided by genomic analyses of patient samples. This so-called ‘precision’ or ‘personalized’ medicine now assists clinicians in diagnosis, stratification, and prognostication of patients, as well as in assessing treatment options. While in its relative infancy, it is anticipated that these practices will become mainstay of medicine for locations that are able to support the infrastructure.

The field of PCa is too a beneficiary of genomics and studies have illuminated several candidate targets for therapeutic intervention including the AR, PI3K, and WNT pathways and the population of cells that are defective in DNA repair mechanisms (50,51). A study by Robinson *et al* examined the molecular events frequently observed in advanced PCa (50). Their results stressed actionable disturbances to AR, PI3K, RAF, as well as to WNT, DNA repair, and cell cycle pathways. Also revealed were clinically relevant perturbations to TP53, PTEN, and ETS family members. A similar study by The Cancer Genome Atlas (TCGA) of 333 clinical samples of primary PCa reiterated those of the Robinson *et al*. This report unveiled novel PCa classifications based on molecular signatures involving gene fusions, mutations, or overexpression of ERG, ETV1, ETV4, FLI1, SPOP, FOXA1, or IDH1 (51). Remarkably, 74% of all samples fell into one of these seven categories, in a largely mutually exclusive manner. Although unified by the single common aforementioned molecular anomaly, embedded within each of these categories were distinct subsets distinguished from one another by copy-number alterations (or lack thereof) and a number of other parameters. At the same time, diverse epigenetic patterning within the different categories was also observed illustrating the complexity and heterogeneity of the disease. Their studies also served in reaffirming previously identified genomic and expressional aberrations of cancer-driving genes and tumour suppressors such as the AR, FOXA1, BRCA2 and PTEN, as well as components of the PI3K and MAPK

signalling pathways. Implicit to their findings was support for consideration of the ETS family of transcription factors as targets for PCa treatment development. ETS, and in particular ERG, fusions to androgen-regulated TMPRSS2 are observed in 40-80% of PCas (52,53). The implications of these and similar bioinformatics studies include the realization that a finite number of molecular events are observed in the vast majority of PCa patients. This holds the potential for more efficient approaches in identifying and targeting the disease. In particular, it has been suggested that viable targets for therapeutic intervention include components of the PI3K, MAPK, and AR pathway or selectively targeting the population of cells whose DNA repair machinery have been compromised. Indeed, an example of application of this knowledge is the administration of olaparib specifically in BRCA2- and ATM-mutated subsets of cancer patients for which olaparib is effective. Mutations to DNA repair machinery such as those genes involved in homologous recombination DNA repair (BRCA2, ATM), are a key feature of CRPC (54). As such, cancer cells become heavily dependent on remaining DNA repair mechanisms such as poly (ADP-ribose) polymerase (PARP). In this way, cells treated with PARP inhibitors seem to incur a catastrophic level of stress which triggers synthetic lethality. Phase II clinical trials with the PARP inhibitor Olaparib (Lynparza; AstraZeneca) have been met with favourable response (55) as have those with niraparib (56) and those with veliparib used in combination with abiraterone and prednisone. TCGA also offered that tailored therapies that target driver genes and driver mutations (discovered through sequencing studies) will be a more effective method in PCa treatment. When comparing the mutational landscape of primary versus advanced PCa, the TCGA consortium determined there to be significant overlap in the types of molecular aberrations but that these alterations occurred with greater severity in the advanced PCa samples. It is anticipated that in the future, a sequence-level and ‘omics’-level resolution of the disease

may be used in place or in conjunction with traditional immunohistological and biochemical assays currently employed for PCa diagnosis. Alternatively, bioinformatics analysis of patient samples may help shape novel PCa stratification paradigms which, for example, might expedite identification of suitable treatment options.

For various different reasons, most available PCa therapies have unpleasant side effects associated with them including physical or emotional pain or disruption to various bodily and cognitive functions. Side effects of ADT and antiandrogens include osteoporosis, sexual dysfunction, metabolic syndrome, lethargy, gynecomastia, depression, and decreases in bone and muscle mass. Motivated by undesirable side effects and acquisition of bypass mechanisms to single-agent approaches by cancer cells, combinatorial approaches may achieve greater clinical success. Synergy between two agents would have the added benefit of potentially reducing the dosage required of each agent and any toxicities associated with them.

1.3 Epithelial-to-mesenchymal transition and stemness in cancer

1.3.1 Epithelial-to-mesenchymal transition

Epithelial cells form the tissues that line the inner and outer surfaces of the human body. Layers of epithelium function in compartmentalization and segregation of different body cavities. Epithelial cells grow in juxtapose formation, exhibit cobblestone morphology, demonstrate apical-basolateral polarity, and display tight cell-cell junctions. Collectively, these attributes combine to maintain tissue integrity and impermeability. The mesenchyme is a mesodermal tissue which arises from the epiblast and gives rise to such tissues as connective tissue, blood, and the circulatory system (57). In contrast to epithelial cells, cells of the mesenchyme demonstrate high motility and exhibit minimal cell-cell junctions and cell polarity (58,59). Epithelial-to-mesenchymal transition (EMT) is a cellular process executed during

embryogenesis and morphogenesis. One example of the normal implementation of EMT is during the formation of the primitive streak in the gastrulation step of embryogenesis. The primitive streak, which goes on to form the three germ layers (endoderm, mesoderm, and ectoderm), originates from the alignment of epiblast-derived mesenchymal cells along the midline. This process requires the mobilization of epithelial cells of the inner cell mass (through an EMT) toward the central region of the blastula. Another critical developmental event which requires EMT is during the attachment and invasion of the maternal endometrium by the blastocyst (60). Without proper tethering of the blastocyst to the uterine wall, subsequent nutrients and gas exchange between the placenta and mother cannot take place. Under certain conditions, epithelial cells can acquire the phenotypic characteristics of mesenchymal cells and this is referred to as EMT. There is also strong support for the notion that stem programs like EMT are commandeered by cancer cells to mediate metastasis. This is an attractive theory because an EMT would grant the cancer cells with numerous abilities necessary for the metastatic process. These qualities include the ability to degrade basement membranes and extracellular matrix, migrate away from the primary tumour, survive the dissemination to distant anatomical sites by way of lymphatic or circulatory systems, and extravasation & colonization of foreign microenvironments (59). In cancer cells EMT is characterized by a shift in expression of specific cell-surface and cytoskeletal proteins as well as EMT-driving transcription factors which, collectively, confer the mesenchymal phenotype (typified by invasive and migratory behavior) (59). E-cadherin and N-cadherin are members of the cadherin family of cell adhesion molecules (CAMs). The CAMs are calcium-dependent cell-surface receptors responsible for cell-to-cell and cell-to-matrix adhesion and cellular signalling. E-cadherin is abundant in epithelial cells and functions in maintaining strong cell-to-cell contacts (61). E-cadherin is often

regarded as a key regulator of EMT. N-cadherin is present in nervous tissue, is associated with motility, and plays important roles in development during gastrulation (62). Cells undergoing EMT tend to downregulate E-cadherin and upregulate N-cadherin. Vimentin is an intermediate filament and cytoskeletal protein that becomes widely expressed in mesenchymal cells in place of epithelial cytokeratins. Tumour tissue frequently expresses vimentin (63). Fibronectin is an extracellular matrix protein whose actions pertain to cell adhesion, migration, growth, differentiation, and development (64). Both vimentin and fibronectin are upregulated in cells undergoing EMT. Twist, Snail, Slug, ZEB1, and ZEB2 are transcription factors that regulate expression of mediators of EMT, E-cadherin being among the most important (65). EMT-activating transcription factors can become upregulated through various mechanisms including through Hedgehog, Notch, Wnt, TGFβ, receptor tyrosine kinase, and hormone signalling (66). This event cascades into the upregulation of mesenchymal markers, including N-cadherin, vimentin, fibronectin, in place of epithelial markers such as E-cadherin. This switch in gene expression imparts the cells with mesenchymal characteristics. EMT-activating transcription factors also cross-regulate each other's expression and are also the target of microRNA regulation. Altered expression of these EMT-associated genes can also result from epigenetic regulation and through chromatin remodeling.

Despite an overwhelming amount of corollary data linking the EMT to metastasis, many have challenged the notion that EMT is obligatory to the metastatic process. Anecdotal evidence has shown metastatic tum ours do not necessarily display mesenchymal markers or phenotypes (67) and the fact that suitable models to probe this precise biological question have not yet been adequately realized. In addition recent experimental evidence by lineage tracing of recombinant reporter-expressing cells has demonstrated that metastasis does not require execution of EMT

programs *in vivo* and that metastasis can occur in mice ablated for EMT-inducing transcription factors (68,69). Further confusion lies in semantics. In the strictest sense, EMT refers to the conversion of cell from epithelial phenotype to a mesenchymal phenotype. Numerous examples can be found in literature where the term EMT is used to describe a situation where only subset of mesenchymal genes and phenotypes are present calling into question whether a partial gene expression and phenotypic profile constitutes an EMT.

1.3.2 Stemness

Despite initial tumour response to conventional PCa treatments, relapse invariably occurs in those with locally advanced or metastatic PCa. The precise events leading to stages of treatment resistance and disease progression are the subject of intensive investigation but one line of thought posits the existence of a sub-population of 'tumour-initiating cells' within the larger tumour. These cells are reported to resist conventional radiation- and chemotherapies and subsequently seed relapse and metastasis (70-72). If this were true, strategies targeting the tumour-initiating population of cells or 'cancer stem cells' hold potential utility in the clinic, particularly for those with advanced treatment-refractory stages of the disease. Further emphasizing the relevance and importance of the cancer stem cell is the fact that EMT can induce stem-like characteristics (73).

The discussion surrounding the concept of a cancer stem cell has steadily increased over the past two decades. The cancer stem cell (CSC) hypothesis was first illustrated in AML by Bonnet and Dick where it was shown that the CD34+/CD38- subset of cells exhibits a high efficiency of tumour-formation in mice (70). The ensuing 'cancer stem cell hypothesis' states that a stem-like compartment of cells, constituting only a fraction of the bulk population, is largely responsible for driving cancer progression. Stemness is the term used to characterize stem

cells which in turn are defined as having potency and the capacity to self-renew. Cancer stem cells are a theorized population of cells within a malignancy that exhibits stemness and are functionally characterized by exquisite efficiency in reconstituting tumours upon serial transplantation in mice. The term 'stem-like' refers to cells that exhibit some of the aspects of stem cells or cancer stem cells. Since Bonnet and Dick first demonstrated the existence of a CSC population in AML, others have shown similar sub-populations of cells with high tumour-initiating capacity in other solid cancers (71,74-76). One poorly understood aspect of this theory, however, is the cell of origin that gives rise to this population. One possibility is that normal stem cells undergo neoplastic transformation followed by population expansion. Another possibility is that differentiated cells acquire stem-like phenotypes through a process of dedifferentiation. Dedifferentiation could come at the hands of inadvertent reactivation of dormant stem programs that are normally reserved for embryogenesis. It is possible that a cell that has usurped these programs could be endowed with newfound stem-like phenotypes. Nevertheless, the so-called cancer stem cells share many qualities with normal stem cells such as pluripotency and self-renewal and their existence is supported by tumour heterogeneity, poor differentiation of tumour tissue upon histological examination, and an overlap in gene expression profiles (77). Another parallel can be drawn in that the reconstitution of an organ by normal stem cells somewhat resembles tumour formation by a cancer stem cell in the heterogeneity displayed by the end products and the overall regenerative and prolific nature of the processes.

While the following report heavily discusses cancer stem cells and SEMA3C's potential involvement in their function, it would be amiss to not discuss the confusion and contention surrounding the cancer stem cell theory. In fact, the cancer stem cell hypothesis is topic that has been shrouded in controversy since its inception. There is lingering debate regarding the

existence of the cancer stem cell and even in the very definition of one. The term ‘cancer stem cell’ alludes to neoplastic cells which harbor the qualities seen in normal stem cells: the ability to self-renew and potency. Misinterpretation of the term ‘stem cell’ in this term has led to the misconception that cancer stem cells originate from normal stem cells, which may or may not be the case (78). Cancer stem cells are defined functionally by their ability to self-renew and to recapitulate the heterogeneous features of the original tumour. While extensive literature can be found demonstrating the existence of a population that meets these criteria, opponents of the theory call into question whether or not those studies were carried out in a biological setting that suitably captures that which is occurring inside of a tumour of a human being (79). Studies that have successfully shown that there exists a tumour-initiating population of cells were done in mouse models where the microenvironment is most decidedly different from that of a person. This is compounded by the fact that the models used were within an immunocompromised setting, further distancing the conditions between those for experimentation and those of a human being. Arguments have been made that the conditions that were amenable to satisfying the criteria of a cancer stem cell population were too artificial and led to false positivity. Nevertheless, the information gained from these experiments provides a broader understanding of possible mechanisms of cancer progression and have at the very least stimulated the discussion of the existence of such a population.

The pathways classically associated with stemness include the Hedgehog, Notch, Wnt, and TGFβ pathways. The Wnt family of secreted glycoproteins are critical factors in organ and embryo development where they help establish axes necessary for proper organ and organism body plan. The Wnt proteins are gatekeepers of a variety of genes related to cell proliferation and cell fate (80). Activated Wnt signalling leads to the stabilization of β–catenin which, together

with the TCF/LEF family of transcription factors, drives expression of genes necessary for sustaining stem phenotypes and a stem population (81). Wnt signalling has been shown to play important roles in many cancers and in particular colorectal cancer. Wnt signalling also participates in PCa where it has been shown that β–catenin associate with the androgen receptor (82). Like the Wnt proteins, some of the earliest characterizations of the Notch proteins were from genetic studies in *Drosophila melanogaster* linking major developmental abnormalities to mutations to what are now known to be the Notch genes (83). The Notch proteins are a family of cell-surface receptors whose signalling cascades are important in normal development of various organ systems. The Notch proteins, much like the Wnt proteins, regulate things like cell fate, differentiation, and survival and are implicated in numerous malignancies. Notch signalling is also known to promote the renewal of stem cells and is suspected of contributing to the cancer stem cell population (84,85). The Hedgehog proteins are a group of secreted proteins that play important roles in body plan patterning, polarity, and development. Hedgehog signalling functions predominantly during embryogenesis and its functions wane in the adult. Targets of the Hedgehog proteins include genes related to cell cycle, survival, and differentiation such as Cyclin D1 (CCND1), c-Myc (MYC), and BCL2 (86). Dysregulation of Hedgehog activity has been described across multiple cancers including in PCa where expression of components of the Hedgehog pathway correlate strongly with metastasis and EMT marker expression (87). The Hedgehog proteins are well-documented in their ability to drive stem-phenotypes. TGFβ is a member of the morphogenetic superfamily of proteins. The TGFβ proteins, for which three isoforms exist, TGFβ1, TGFβ2, TGFβ3, are soluble factors which act on TGFβ receptors at the cell surface. TGFβ receptors, or TβRs, are serine/threonine kinases whose auto- and trans-phosphorylation of each other activate cytoplasmic Smad proteins which then translocate to the

nucleus and initiate transcription of genes related to cell growth, differentiation, and development (88). TGFβ is involved in normal development of numerous epithelial tissues including the breast (89), colon (90,91), lung (92), and prostate (93). TGFβ signalling is multifaceted and has, at times, dichotomous roles in cancer development. On the one hand there is extensive information documenting TGFβ's inhibitory effect on cell growth in various cell types (94-98). In contrast there also exists an abundance of information indicating that TGFβ in fact promotes cell growth in cancer cells (99-102). In non-transformed prostate cells, TGFβ signalling has tumour suppressive roles by virtue of its ability to inhibit cell growth, induce apoptosis, and regulate migratory and invasive properties of cells (103-106). However, in more advanced PCa, TGFβ seems to take on a more sinister role where TGFβ levels predict more invasive cancer behaviour, cancer progression, and poor outcome (107-109). TGFB2 has known roles in oncogenic transformation and can trigger EMT (110,111).

1.4 Semaphorins

1.4.1 Structure and function

The semaphorins are a large grouping of signalling proteins originally described for their roles in axon guidance in the developing nervous system. Seminal studies in the discovery of semaphorins showed that treatment of a protein isolated from chicken brain extract was capable of causing the collapse of explanted dorsal route ganglia (112,113). Further work has shown that semaphorins establish molecular gradients that help guide cell movement (114) but also have essential roles in cardiac development and in the immune system (115,116) in addition to several other cellular processes (117). Semaphorins are separated into 8 classes distinguished from one another by the presence of different structural features such as Ig domains, basic domains, thrombospondin repeats, transmembrane domains, or GPI-linkages (Figure 1.1). Classes 1, 2,

and 5 are found in invertebrates, 3 through 7 are found in vertebrates, and the eighth class, designated V, is found in viruses. Classes are further subdivided into one or more members. The nomenclature for the semaphorins consists of 'SEMA' followed by the numerical class and then a letter indicating the member; for example, semaphorin class 3, member C is denoted SEMA3C. Semaphorins undergo post-translational modifications such as glycosylation and proteolytic cleavage, which nuance their activity.

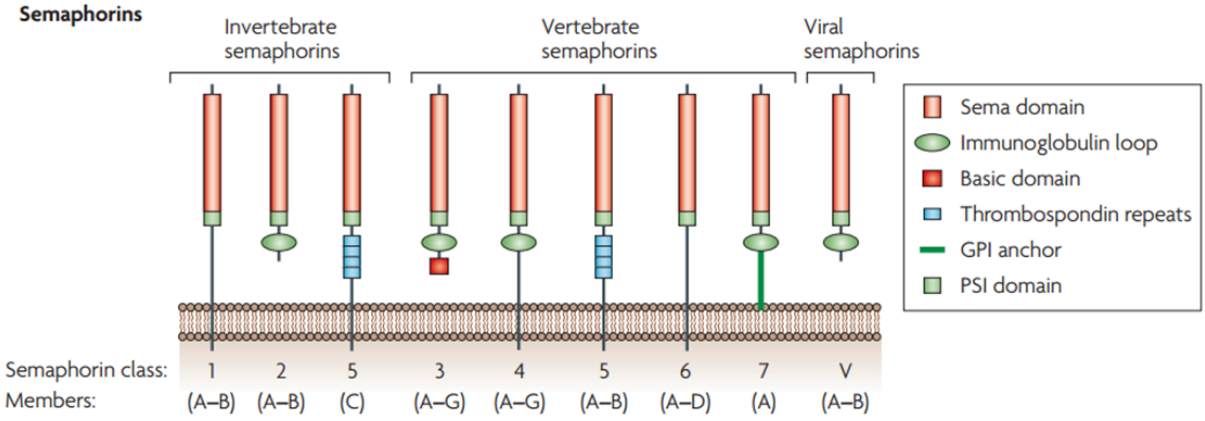

Figure 1.1. The semaphorin classes.
All semaphorins contain a sema and PSI domain but are further separated into eight different classes based on combinations of structural features including an Ig domain, a basic domain, thrombospondin repeats, or GPI-linkages (118). Image used with permission from Nature Publishing Group.

The receptors to the semaphorins are the plexins (PLXNs) and neuropilins (NRPs). Of the vertebrate semaphorins (classes 3 through 7), only the class 3 semaphorins (with the exception of SEMA3E) require NRP co-receptors to bind PLXNs whereas classes 4 through 7 directly associate with PLXNs (114,119). Class 3 semaphorins are secreted by the cell whereas classes 4 through 7 are membrane-associated. Semaphorins are characterized by a highly-conserved N-terminal 500 amino acid sema domain which folds into a seven-bladed β-propeller (120,121) (Figure 1.2). The sema domain is involved in protein-protein interactions with plexin and neuropilin receptors (122-125). In addition, all semaphorins contain a PSI (plexin, semaphorin, integrin) domain c-terminal to the sema domain. In class 3 semaphorins, crystallographic and

biophysical studies involving SEMA3A, NRP1, and PLXNA2 revealed that ternary complexes consisting of one semphorin, one neuropilin, and one plexin dimerize at the cell surface. This is potentially followed by further oligomerization (Figure 1.2), and the initiation of cellular signalling through crosstalk with other cell surface proteins (126). Although not all SEMA-NRP-PLXN crystal structures have been solved, it is presumed that other class 3 semaphorins follow a paradigm similar to SEMA3A.

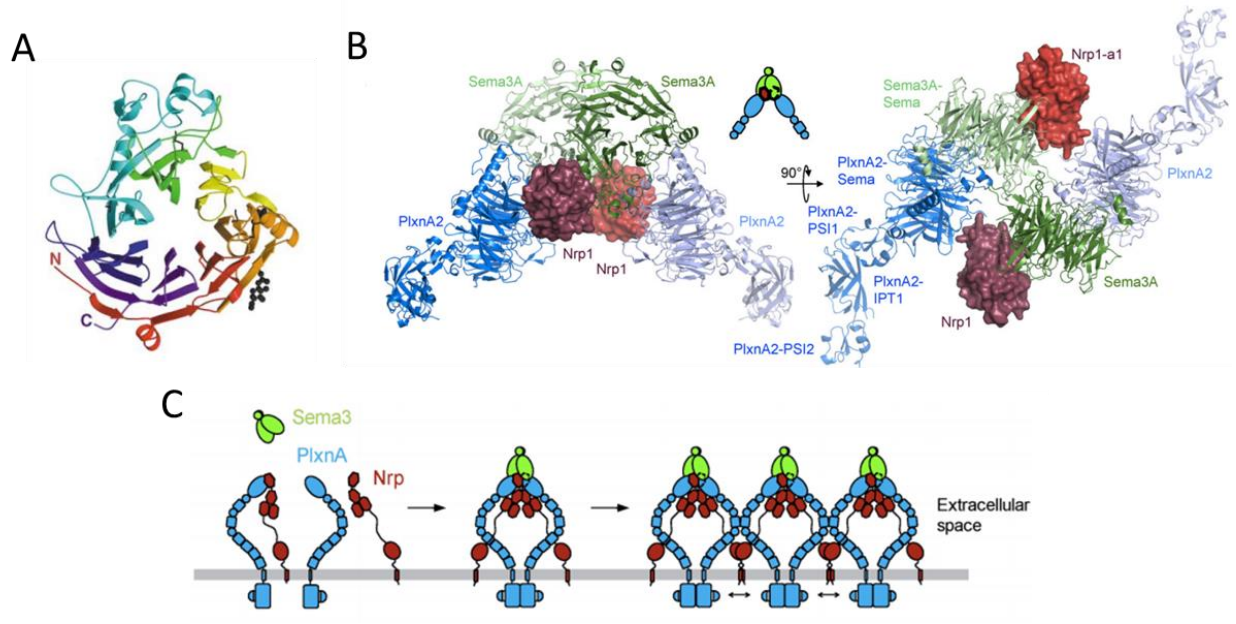

Figure 1.2. Crystal structure of SEMA3A-NRP1-PLXNA2.
(A) Seven-bladed β–propeller topology of SEMA3A sema domain solved to 2.8 Å by Antipenko *et al* (121). Crystal structure of the SEMA3A-NRP1-PLXNA2 ternary complex dimers as described by Janssen *et al* (126). Dimeric SEMA3A associate with two PLXNA2 molecules embedded in the plasma membrane which is structurally reinforced by two NRP1 molecules. Oligomerization of this complex at the cell surface may be required for signal transduction (C). Images used with permission from Elsevier and Nature Publishing Group.

NRPs are believed to stabilize the semaphorin-plexin interaction but emerging evidence suggests that NRPs have roles above and beyond simply stabilization (127). NRPs have separately been described as a co-receptor for VEGF in VEGFR signalling but are also known to play important roles in nervous system development, angiogenesis, and cancer (128). PLXNs are

generally thought to be the signal-transducing molecule for the semaphorins (122,129). This is in large part due to their large cytoplasmic domains which contain GTPase activating protein (GAP) (129,130) and guanine nucleotide exchange factor (GEF) (131,132) activity. In addition to intrinsic catalytic activity, PLXNs also function through crosstalk with receptor tyrosine kinases at the plasma membrane. For example, PLXNB1 and c-Met crosstalk and cooperate in driving invasive growth. In addition, PLXND1 has been shown to activate ErbB2 and EGFR signalling and promote invasiveness and metastatic spread to the lungs *in vivo* (133,134). Two NRPs and seven PLXNs (which fall into one of four different classes) exist where specific PLXNs are discriminately paired with specific semaphorins. A certain degree of redundancy exists within this scheme in that some semaphorins bind to a common plexin receptor and in other cases, one semaphorin can bind multiple plexin receptors (114). Semaphorins act through autocrine, paracrine, and juxtacrine signalling and have been implicated in a broad range of biological functions ranging from tissue morphogenesis to immunity (115,135); altered semaphorin expression has also been observed in various cancers (136).

1.4.2 Semaphorins in cancer

Given the pleiotropic nature of semaphorin function, in particular its roles in cell motility and cytoskeletal rearrangement (137), it is unsurprising that the semaphorins are associated with virtually every hallmark of cancer (136). The semaphorins impinge on proliferative signalling, evasion of apoptosis, angiogenesis, and invasion & metastasis. The following are some examples of this. As alluded to by Hanahan and Weinberg, at some point all cancer cells gain the ability to produce the very growth factors to which they also respond; SEMA5A and SEMA3C, which are overexpressed in various cancer types, have been demonstrated to increase cell proliferation and invasiveness in pancreatic cancers and endothelial cells, respectively (138,139). Cancer cells are

also well-known to disable various mechanisms for programed cell death; SEMA3B is downregulated in a number of cancers and is documented to induce apoptosis in lung cancer cells by suppressing the PI3K signalling and activating casepase-3 (140-142). In order to accommodate increasing demands for oxygen by a growing tumour, cancer cells expand existing vasculature through the process of angiogenesis; SEMA4D and SEMA3C are dysregulated in numerous cancers and have been shown to trigger endothelial cell activation and angiogenesis (139,143). Invasion is the process through which cancer cells dissociate from their positions of origin and disseminate to other anatomical sites to cause metastasis. It involves loss of cell-cell and cell-matrix adhesion, enhanced migratory capabilities, and the production of enzymes that enable proteolysis through basement membranes and extracellular matrices. SEMA3A, which is downregulated in breast cancer, induces integrin activity and cell adhesion in breast cancer cells. Conversely, SEMA3E promotes metastasis of colorectal, breast, and lung cancer cells to the lung (133). Broadly speaking, of the class 3 semaphorins, SEMA3D, SEMA3F, and SEMA3G are thought to have tumour suppressor activity while SEMA3A, SEMA3B, SEMA3C, and SEMA3E have both pro- and anti-tumour activity depending on the context (144-146). As a whole, the roles of semaphorins in cancer are becoming increasingly evident however mechanistic details surrounding their involvement in cancer are incompletely characterized.

1.4.3 Semaphorin 3C

SEMA3C was initially discovered for its roles in neurogenesis and cardiac development (147,148) but has since been implicated in prostate, breast, ovarian, lung, and gastric cancer, as well as glioblastoma (136). The receptors to SEMA3C include NRP1, NRP2, PLXNB1, and PLXND1 (117,149,150) (also unpublished data). SEMA3C is upregulated in response to chemotherapy and radiation treatment (151), promotes metastasis to the lung (152), and

promotes tumourigenicity of glioma cells (153,154). SEMA3C has also been shown to increase cell proliferation and migration, decrease apoptosis, and promote integrin signalling and VEGF secretion in endothelial cells (139). SEMA3C was shown to drive migration in breast cancer and hepatocellular carcinoma cells (155-157) and more recent studies have highlighted the importance and prognostic value of SEMA3C in PCa (158-160). Unpublished work in our lab has shown that SEMA3C expression correlates with intensified PCa treatment regimen (Figure 1.3) and promotes growth signalling in PCa cells. The cellular signalling currently understood in this context is schematically presented in Figure 1.3. Despite compelling evidence implicating SEMA3C in PCa, a complete understanding of its roles in PCa etiology remains to be clearly defined.

Semaphorins are chemotropic agents that trigger cell migration of neural crest cells during neurulation. Akin to its activities in neurogenesis, we postulate that SEMA3C fulfills similar roles during EMT in PCa. Given SEMA3C's amplification in PCa combined with its known roles in development and morphogenesis it is conceivable that SEMA3C promotes PCa through activation of EMT and stem programs. This is further supported by SEMA3C's association with multiple defining features of the recently presented PCa taxonomies by the TCGA and Robinson *et al*. This includes SEMA3C's ability to drive MAPK and Akt signalling and its ties to the androgen receptor - a topic that will be discussed in the work below.

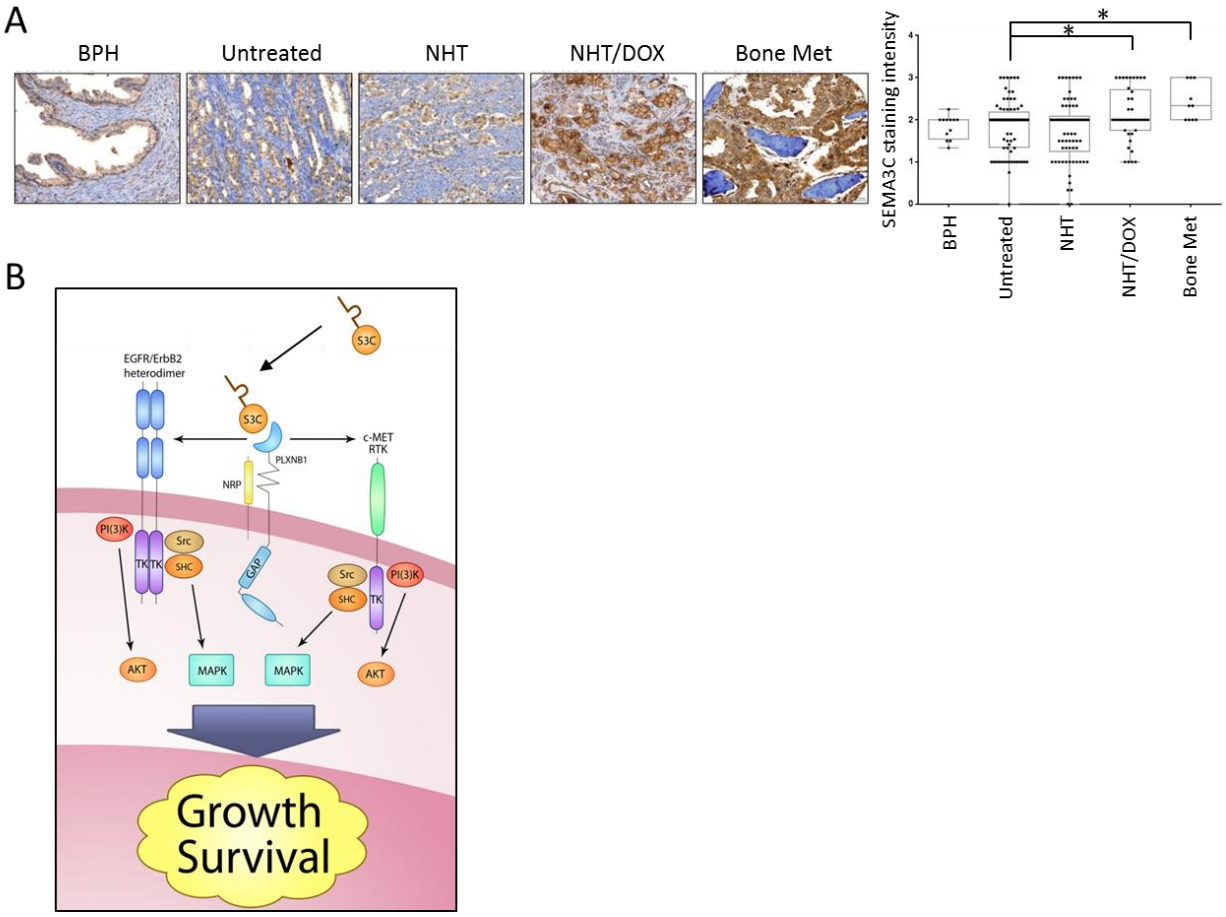

Figure 1.3. SEMA3C expression and cellular signalling in PCa.
Representative SEMA3C immunostaining of BPH, untreated hormone naïve, neo-adjuvant hormone therapy (NHT)-treated, NHT and docetaxel (DOX)-treated radical prostatectomy PCa specimens, and a bone CRPC metastasis specimen (A). Box & whiskers plot of IHC intensity scores of SEMA3C staining from BPH, untreated, NHT-treated, NHT and docetaxel-treated, and CRPC metastatic tissues; mean intensities of 1.8, 1.9, 1.9, 2.2, and 2.4 respectively; *p <0.05. (B) Cell culture studies in LNCaP and DU 145 have shown that recombinant SEMA3C triggers the phosphorylation of EGFR, c-Met, Src, SHC, MAPK, and AKT leading to heightened cell proliferation.

1.5 Hypothesis, objectives, and specific aims

1.5.1 Objectives and hypothesis

The overarching objective of this research aims to identify roles of SEMA3C in the context of PCa. Characterizing SEMA3C in this way would expand on our understanding of cancer biology as well as on SEMA3C itself. Many molecular paradigms currently make up our understanding of the process of PCa oncogenesis; my work sets out to connect those paradigms

to SEMA3C. Since the androgen receptor is a known driver of PCa progression, one portion of my work sought to make the connection between the AR and SEMA3C. I also attempted to establish a link between SEMA3C, EMT, and stem-like characteristics. Success in these objectives would provide evidence that SEMA3C mediates PCa through these well-established pro-cancer pathways. I hypothesize that **SEMA3C is a transcriptional target of the AR and that SEMA3C is able to drive EMT and the acquisition of stem-like characteristics**. In broader terms, I believe that a complex relationship exists between SEMA3C, AR, EMT, and stem-like phenotypes and that, in combination with other events, they cooperate to promote PCa. To prove these hypotheses my specific aims are:

Specific Aim 1: To determine whether SEMA3C is a transcriptional target of AR.

Specific Aim 2: To determine whether SEMA3C can promote EMT and stem-like characteristics in prostate cells.

1.5.2 Experimental plan

To determine if SEMA3C is an AR-regulated gene in specific aim 1, we use the AR-positive LNCaP cell line to test whether *SEMA3C* expression is androgen-inducible. Additionally, we use RSAT DNA analysis software to analyze for the presence of androgen response elements (AREs) in the *SEMA3C* locus. Gel shift assays and chromatin immunoprecipitation assays were used to determine whether AR was recruited to AREs near the SEMA3C gene. Reporter gene assays were used to show transactivation of this ARE by AR.

For specific aim 2, we use gain of function studies in RWPE-1, a normal prostate epithelial cell line, coupled to gene expression (qPCR, Western blot, FACS, immunofluorescence microscopy) and functional studies (migration, invasion, sphere-formation assays, and *in vivo* cell dissemination experiments) to determine if ectopic expression of

SEMA3C can promote EMT and stem-like characteristics. Microarray gene expression analyses and functional annotation were used to identify which stem pathways are contributing to the stem phenotype.

CHAPTER 2. Androgen receptor transcriptionally regulates Semaphorin 3C in a GATA2-dependent manner

2.1 Introduction

First-line therapies for metastatic PCa invariably target the AR axis and are initially met with favourable response. However, restored and aberrant AR signalling fuel disease progression to stages for which treatment is palliative. Transcriptional targets of the AR include genes involved in cell growth and cell fate (26,161-163) but are not completely described, despite the fact that a clear causal relationship exists between the AR and PCa. Similarly, although research has steadily improved our understanding of the normal and pathological roles of semaphorins, documented reports describing their regulation are exceedingly scarce. Given the coincidental implication of both AR and SEMA3C in PCa progression, we hypothesized that *SEMA3C* is a transcriptional target of the androgen receptor. This hypothesis is further supported by a study by Yu *et al* that identified androgen receptor binding sites in the SEMA3C genomic and upstream sequence (164). The concept that semaphorins are hormone-regulated is not unprecedented and it has been reported that SEMA3G is upregulated by R1881 in prostate cells (27) and that SEMA3B and SEMA3F are regulated by estrogens in ovarian cancer cells (165). The broader implications of the research presented here lies in the fact that the AR has fundamental roles in the natural history of PCa and underpins disease progression. A more comprehensive knowledge of the targets of the AR would therefore help in generating a deeper understanding of processes mediating fatal forms of the disease. This chapter provides evidence for the transcriptional regulation of SEMA3C by AR and also explores whether pioneering factors are involved with this process. While GATA2 was seen to be obligatory to AR-mediated expression of SEMA3C, FOXA1 and OCT1 were not.

2.2 Materials and methods

Bioinformatics and data set analysis

Previous ChIP-Seq data from Yu *et al* (164) was extracted from the NCBI Gene Expression Omnibus (GEO) database (166). In particular, the file ('GSM353644_jy10s123.allregions.txt.gz'), which contained enriched DNA regions (i.e. peaks) bound by the AR protein in LNCaP cells treated with R1881 (GEO sample accession: GSM353644), was parsed to a bedGraph format and visualized in the UCSC Genome Browser (167) to identify AR binding sites (ARBS) nearby to the SEMA3C (RefSeq accession number NM_006379) locus on chromosome 7 of the human reference genome (version: hg18). The file contains a total of 44,536 different AR binding sites across the human genome, and each region was annotated for the distance to nearby genes using a ChIP-Seq analysis program, CompleteMOTIFs (168). The actual DNA sequences that compose each AR binding peak region (~ 500 bps) at the SEMA3C locus were extracted and scanned for any presence of the ARE motif (15 bps) and GATA2 motif (14 bps), using a DNA motif scanning program, Patser (169). The DNA frequency matrices that define the ARE motif (ID: MA0007.2) and GATA2 motif (ID: MA0036.2) was obtained from the JASPAR database (170).

DNA sequences

Fifty basepair oligonucleotides centred around the SEMA3C intron 2 ARE were used for electrophoretic mobility shift assay. Oligonucleotides were purchased from Integrated DNA Technologies (Redwood City, CA, USA) and annealed by ramping from 90^{o}C to 25^{o}C at 0.1^{o}C per second; sequences are displayed in Figure 2.3. In reporter gene assays, 150 bases of genomic sequence bearing the ARE were cloned into the luciferase reporter backbone pGL3-Basic to generate the wtARE construct. Six of the basepairs constituting the putative ARE in wtARE

were mutated by transversion mutations to generate the mutARE construct. Truncation mutants were generated by progressive 60 basepair deletions to the 3' end of wtARE insert. Sequences are displayed in Figure 2.4. DNA sequences were cloned in the same reading orientation relative to luciferase as they were found relative to *SEMA3C* in the genome. Ectopic expression of AR or ARv7 was achieved by transient transfection of PC-3, LNCaP, or 293T with overexpression vectors where wild type AR or ARv7 is placed under the control of the CMV promoter in the pcDNA3.1 vector (Invitrogen). Empty pcDNA3.1 vector served as a negative control. Plasmids were transfected using Lipofectamine 2000 (Invitrogen, Cat. No. 111668-027) for 24 hours.

Cell culture

LNCaP (ATCC, CRL-1740), 22Rv1 (ATCC, CRL-2505), and C4-2 cells (kindly provided by Dr. Leland W.K. Chung, MD Anderson Cancer Center, Houston, TX) were cultured in RPMI 1640 supplemented with 10% FBS; VCaP (ATCC, CRL-2876) were cultured in 10% FBS, 1.5 g/L sodium bicarbonate, DMEM; PC-3 (ATCC, CRL-1435), DU 145 cells (ATCC, HTB-81), and HEK/293T cells (ATCC, CRL-11268) were cultured in 10% FBS DMEM. Cells were treated at the indicated concentrations of androgen or 0.05% ethanol as a vehicle control in 0.2% charcoal-stripped serum (CSS) in Opti-MEM (Gibco, Cat. No. 11058-021) for 24 hours for qPCR or 48 hours for Western blot unless otherwise stated. Cells were starved for 24 hours in 0.2% CSS in Opti-MEM prior to treatment with R1881 (Perkin-Elmer, Cat. No. NLP005) or DHT (Sigma-Aldrich, Cat. No. D-073). For inhibition studies involving enzalutamide (MDV3100) or AR DBD inhibitor VPC-14449, LNCaP were co-treated with R1881 and MDV3100, VPC-14449, or DMSO as a vehicle control in 0.2% CSS in Opti-MEM. For LY294002 (EMD /Millipore, Cat. No. 440202) and bpV(HOpic) (Santa Cruz Biotechnology,

Cat. No. sc-221377) treatment studies, cells were treated at the indicated concentrations of inhibitor or vehicle control (DMSO) overnight in serum- and phenol red-free RPMI 1640.

Quantitative polymerase chain reaction

Messenger RNA levels of SEMA3C were measured by qPCR. Total RNA was extracted using TRIzol (Invitrogen, Cat. No. 15596018) and 2 µg of RNA was reverse-transcribed using random hexamers (Roche, Cat. No. R15504) and Superscript II (Invitrogen, Cat. No.18064-014). qPCR was carried out using a ΔΔCt method on an AB ViiA7 real-time PCR machine; reactions were prepared using Platinum SYBR Green (Invitrogen, Cat. No.11744-500) and GAPDH or actin served as an endogenous control. GAPDH primer sequences: 5'- caccagggctgcttttaactc (forward), 5'- gacaagcttcccgttctcag (reverse); actin primer sequences: 5'- gctcttttccagccttcctt (forward), 5'- cggatgtcaacgtcacactt (reverse); SEMA3C primer sequences: 5'- gacaatttgcgtgttggttg (forward), 5'- cggtcctgatcttcatcca (reverse); POU2F1 primer sequences: 5'- atgaacaatccgtcagaaaccag (forward), 5'- gatggagatgtccaaggaaagc (reverse).

Electrophoretic mobility shift assay (EMSA)

Electrophoretic mobility shift assay with the androgen receptor DBD was carried out as described elsewhere (171). Briefly, complementary 50 basepair oligonucleotides centred around the SEMA3C intron 2 ARE were synthesized (Integrated DNA Technologies), annealed, and combined with purified AR DBD. Oligonucleotide at a final concentration of 1.875 µM was mixed with AR DBD at final concentrations of 0.5, 1.0, and 2.0 µM and incubated on ice for 30 minutes in loading buffer. Oligonucleotide alone and oligonucleotide-AR DBD mixtures were run on a 5% native polyacrylamide gel at 125 volts in 1X TBE at 4^{o}C and visualized using SYBR Safe DNA Gel Stain (Invitrogen Cat. No. S33102). See Figure 2.3 for oligonucleotide sequences.

Chromatin immunoprecipitation assay (ChIP)

$2.5x10^6$ LNCaP cells were treated with 0.05% ethanol or 5 nM R1881 overnight and fixed in 1% formaldehyde for chromatin immunoprecipitation using the Millipore EZ-ChIP Chromatin Immunoprecipitation Kit protocol (Cat. No. 17-371). For end-point PCR, 2 µl of purified DNA was used for thermocycling: initial denaturing at 94^{o}C for 3 minutes, followed by 33 cycles of 20 seconds 94^{o}C denaturing, 30 seconds 52^{o}C annealing, 30 seconds 72^{o}C extension, and a single final 72^{o}C 2 minute extension step. Products were run at 90 volts on a 2% agarose TBE gel and stained with SYBR Safe DNA Gel Stain. For qPCR, 1.5 µl of purified DNA was used per reaction. For ChIP qPCR reactions, SEMA3C intron 2 ARE primer sequences: 5'- aaatgccggtactggcctta (forward), 5'- gcttaaaggtcacaagattg (reverse); PCR primers amplify a 150 bp genomic region containing the SEMA3C intron 2 ARE. GAPDH primers were provided with the Millipore EZ-ChIP Chromatin Immunoprecipitation Kit. SEMA3C levels were quantitated using a ΔΔCt method, normalized first to input and then isotype control. Antibodies for immunoprecipitation: Androgen Receptor (Santa Cruz Biotechnology, Cat. No. sc-816), GATA-2 (Santa Cruz Biotechnology, Cat. No. sc-9008), and N-cadherin (isotype control, Santa Cruz Biotechnology, Cat. No. sc-7939).

Luciferase assay

$5x10^5$ LNCaP cells were transiently transfected in triplicate in 12-well format with 1.2 µg of either empty pGL3-Basic (Basic), pGL3-wild type ARE (wtARE), pGL3-mutated ARE (mutARE), or truncated pGL3-wtARE (wtARE-60bp and wtARE-120bp) reporter plasmids and 30 ng of renilla plasmid (phRL-SV40) kindly provided by the Mui lab (Immunity and Infection Research Centre, Vancouver Coastal Health Research Institute, Vancouver, British Columbia). 293T cells were co-transfected with or without AR or ARv7 overexpression plasmids or empty

vector (pcDNA3.1). The following day the cells were treated with EtOH or R1881 in 5% CSS Opti-MEM. 24 hours later, cell extracts were harvested for luciferase assay using the Promega Dual-Luciferase Reporter Assay System (Cat. No. E1960) and read on a TECAN Infinite M200 PRO. For MDV3100 and 14449 dosing studies, $5x10^3$ LNCaP cells were seeded in quadruplicate in 96-well format, transfected with the wtARE construct (50 ng), and co-treated with R1881 (0.1 nM) and one of MDV3100 or VPC-14449 at the indicated concentrations (24 hrs) and read as described above. In all luciferase assays, firefly luciferase luminescence was normalized to renilla luciferase luminescence.

Western blot

Whole cell extracts were prepared in 50 mM Tris-HCl, 150 mM NaCl, 1% NP40, 10 mM NaF, 10% Glycerol, supplemented with protease inhibitor cocktail (Roche, Cat. No. 04693116001) and quantitated using a BCA approach. 60 µg of protein, or 40 µl of conditioned media, was run on 10% acrylamide gels and transferred onto nitrocellulose membrane. Western blots were imaged on radiography film or by a LI-COR Odyssey system. Actin or vinculin served as loading controls. Primary antibodies: phospho-Akt (Ser473; Cell Signalling Technology, Cat. No. 4060S), pan-Akt, (Life Technologies, Cat. No. 44609G), androgen receptor (Santa Cruz Biotechnology, Cat. No. sc-816), SEMA3C (Santa Cruz Biotechnology, Cat. No. sc-27796), GATA2 (Santa Cruz Biotechnology, Cat. No. sc-9008), FOXA1 (Santa Cruz Biotechnology, Cat. No. sc-6553), POU2F1 (Santa Cruz Biotechnology, Cat. No.s sc-232, sc-8024; Cell Signalling Technology, Cat. No. 4428S), actin (Sigma-Aldrich, Cat. No. A2066), and vinculin (Sigma-Aldrich, Cat. No. V4505). Secondary antibodies: anti-rabbit alexa fluor 680 (Invitrogen, Cat. No. A21109), anti-mouse alexa fluor 680 (Invitrogen, Cat. No. A21058), anti-

goat alexa fluor 680 (Invitrogen, Cat. No. A21084), anti-rabbit HRP (Dako, Cat. No. P0448), anti-mouse HRP (Dako, Cat. No. P0447), and anti-goat HRP (Dako, Cat. No. P0160).

RNA knockdown

Cells were transfected with siRNA using Lipofectamine RNAiMAX (Invitrogen, Cat. No. 13778-075) for 48 hours at which time cells were either harvested or treated for an additional 24 hours (qPCR) or 48 hours (Western blot) with EtOH or R1881. Small interfering RNA for GATA2 (siGATA2) were purchased from Dharmacon (Cat. No. J009024-17-0005) and Ambion (Cat. No. 4392420, ID s5596); siFOXA1 was purchased from Ambion (Cat. No. 4392420, IDs s6687 and s6688); siPOU2F1 was purchased from Ambion (Cat. No. 4392420, ID s10849); siAR was purchased from Ambion (Cat. No. 4390824); negative control siRNA (siSCX) were purchased from Dharmacon (Cat. No. D001810-10-05) and Ambion (Cat. No. 4390843).

Statistical analyses

Unless otherwise stated, statistical analysis was performed using the Student's two-tailed t-test. Data are represented as mean ± SD unless otherwise stated. Data presented are representative of three biological replicates.

2.3 Results

2.3.1 ARE and GATA2 DNA motifs at the human SEMA3C locus

In a study identifying androgen receptor binding site (ARBS) distribution across the human genome in LNCaP PCa cells, Yu *et al* identified multiple ARBSs in the *SEMA3C* locus (164). The ChIP-Seq study from Yu *et al* (164) reported a total of 44,536 genomic regions (or peaks) bound by the AR protein (ARBSs) in LNCaP cells under treatment with R1881. The average peak height, an indication of the amount of DNA bound by the AR, is 30 units. The

annotation of the ChIP-Seq data by the CompleteMOTIFs program identified 8 peaks that are within 500 kb of the transcription start site (TSS) of the *SEMA3C* gene, five of which are shown in Figure 2.1A. As illustrated in Figure 2.1A, two ChIP-Seq peaks have heights of 111 and 92, both of which are significantly higher than the average value and are in the 95^{th} percentile; the coding sequences are located on the reverse strand of chromosome 7 (hg18). The two ARBSs span 525 and 500 basepairs (bps) and are located in intron 2 (34.5 kb downstream of TSS) and intron 12 (137.7 kb downstream of TSS) of the *SEMA3C* gene, respectively. The DNA sequences at the ARBSs were scanned for the presence of ARE and GATA2 motifs, using a motif scanning program, Patser. An ARE DNA motif was found within the ARBS peak at intron 2 ($p = 6.46 \times 10^{-6}$), while no ARE motif was found within the peak at intron 12. Prospective GATA2 motifs were identified in both intron 2 ($p = 1.02 \times 10^{-4}$) and intron 12 ($p = 1.17 \times 10^{-4}$) which resemble the GATA2 motif as defined by JASPAR motif database (Figure 2.1B). We began our investigation of AR-mediated regulation of *SEMA3C* by examining the putative ARE that spans from 80,352,119 to 80,352,133 in the intron 2 of SEMA3C, which shares strong resemblance to the ARE motif (Figure 2.1C) as defined in the JASPAR motif database (16-19,170).

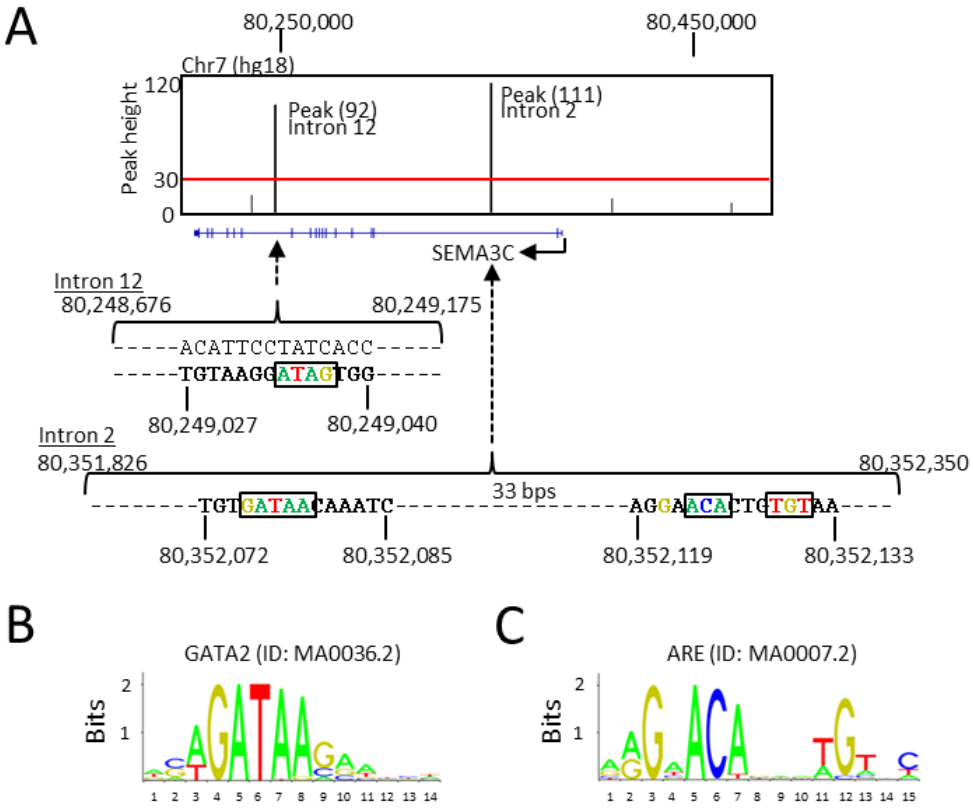

Figure 2.1. ARE and GATA2 DNA motifs at the human SEMA3C locus.
(A) The ChIP-Seq peaks (vertical black bars) from Yu *et al* were overlaid on the SEMA3C locus (blue horizontal line: exons are shown as vertical blue bars) in the UCSC Genome Browser (hg18). A red horizontal line illustrates the average peak height from the ChIP-Seq experiment. There are three other peaks upstream of SEMA3C (not shown), all of which have heights lower than the average value. The DNA sequences containing the ARE and GATA2 motifs, as predicted by the Patser program, are shown within the two peaks that span from 80,351,826 to 80,352,350 in intron 2 and from 80,248,676 to 80,249,175 in intron 12, respectively. (B) A consensus GATA2 motif documented in the JASPAR database is illustrated as a sequence logo. (C) A consensus ARE motif documented in the JASPAR database is illustrated as a sequence logo.

2.3.2 *SEMA3C* is an androgen receptor-regulated gene

Considering that *SEMA3C* contains an ARE in its second intron, *SEMA3C* may be an androgen-regulated gene; androgen receptor is known to act through distantly-located (including intronic) ARBSs through DNA looping (23,29,172,173). To first assess the androgen-responsiveness of *SEMA3C*, AR-positive LNCaP cells were treated with the synthetic androgen R1881 or naturally-occurring ligand, DHT, and tested for SEMA3C expression. LNCaP are prostate cancer cell line derived from a lymph node metastasis of a prostate cancer patient. They

are androgen-responsive and express low endogenous levels of SEMA3C. Consistent with our hypothesis, SEMA3C mRNA levels increased in a dose-dependent manner upon treatment with both R1881 and DHT (Figure 2.2A and 2.2B, respectively). R1881 triggered increases in SEMA3C message levels from 2.2 to 3.1 over that of vehicle control. DHT reached a maximum induction of 1.9-fold over vehicle control at 5 nM. These results are supported by existing microarray datasets (174) which examine the gene expression profiles of LNCaP in response to R1881 over time. Data mining (175) of these datasets (GEO accession number GDS2034) showed that SEMA3C mRNA levels increased in a time-dependent manner (Appendix A). To test if antiandrogens could influence SEMA3C expression, we administered increasing concentrations of MDV3100 (enzalutamide), which competes with androgens for the AR LBD (176), to R1881-stimulated LNCaP cells. MDV3100 inhibited SEMA3C expression by over 50% at all concentrations of MDV3100 examined (Figure 2.2C). A recently-developed small molecule inhibitor ("VPC-14449") with well-characterized AR DBD-interfering activity (171,177) was also capable of inhibiting R1881-mediated induction of SEMA3C expression (Figure 2.2D). VPC-14449 inhibited R1881-induced expression of SEMA3C by 18%, 62%, and 56% at 1, 5, and 10 μM, respectively; inhibition at 20 μM did not reach statistical significance. These findings are consistent with a previously published microarray dataset on MDV3100- and VPC-14449-treated R1881-stimulated LNCaP cells (171) where it was shown that administration of MDV3100 and VPC-14449 decreased SEMA3C expression by 20% ($p = 0.10$) and 25% ($p = 0.04$), respectively. To reinforce the idea that SEMA3C expression requires AR, we also showed that SEMA3C levels were not induced by R1881 in the AR-negative PCa cells lines, PC-3 and DU 145, over the same concentrations (Figure 2.2E & 2.2F). Additionally, ectopic expression of AR in PC-3 and LNCaP led to a 4.6 and 3.1-fold increase in SEMA3C expression, respectively

(Figure 2.2G & 2.2H). In these experiments, cells were transfected with AR-overexpression plasmids in full serum. Thus, the serum contributed androgenic ligands. In the reciprocal experiment, knockdown of AR in LNCaP resulted in a 32% reduction in SEMA3C expression (Figure 2.2I). Collectively, these results demonstrate that steroid-activated AR can trigger upregulation of SEMA3C. The PI3K pathway is frequently mutated in PCa and *PTEN* is mutated in up to 40% of advanced PCa patients. Inhibition of PI3K signalling was shown to upregulate AR-regulated genes (178) therefore we asked whether inhibiting PI3K using LY294002 could trigger upregulation of SEMA3C. In accordance with findings shown by Carver *et al* (178), inhibiting PI3K using LY294002 caused upregulation of SEMA3C (Figure 2.2J) and conversely, inhibiting PTEN using bpV(HOpic) in the PTEN-positive PCa line, 22Rv1, caused a downregulation of SEMA3C (Figure 2.2L). Phospho-Akt levels were used to index the effects of LY294002 and bpV(HOpic) on PI3K activity (Figure 2.2K & 2.2M).

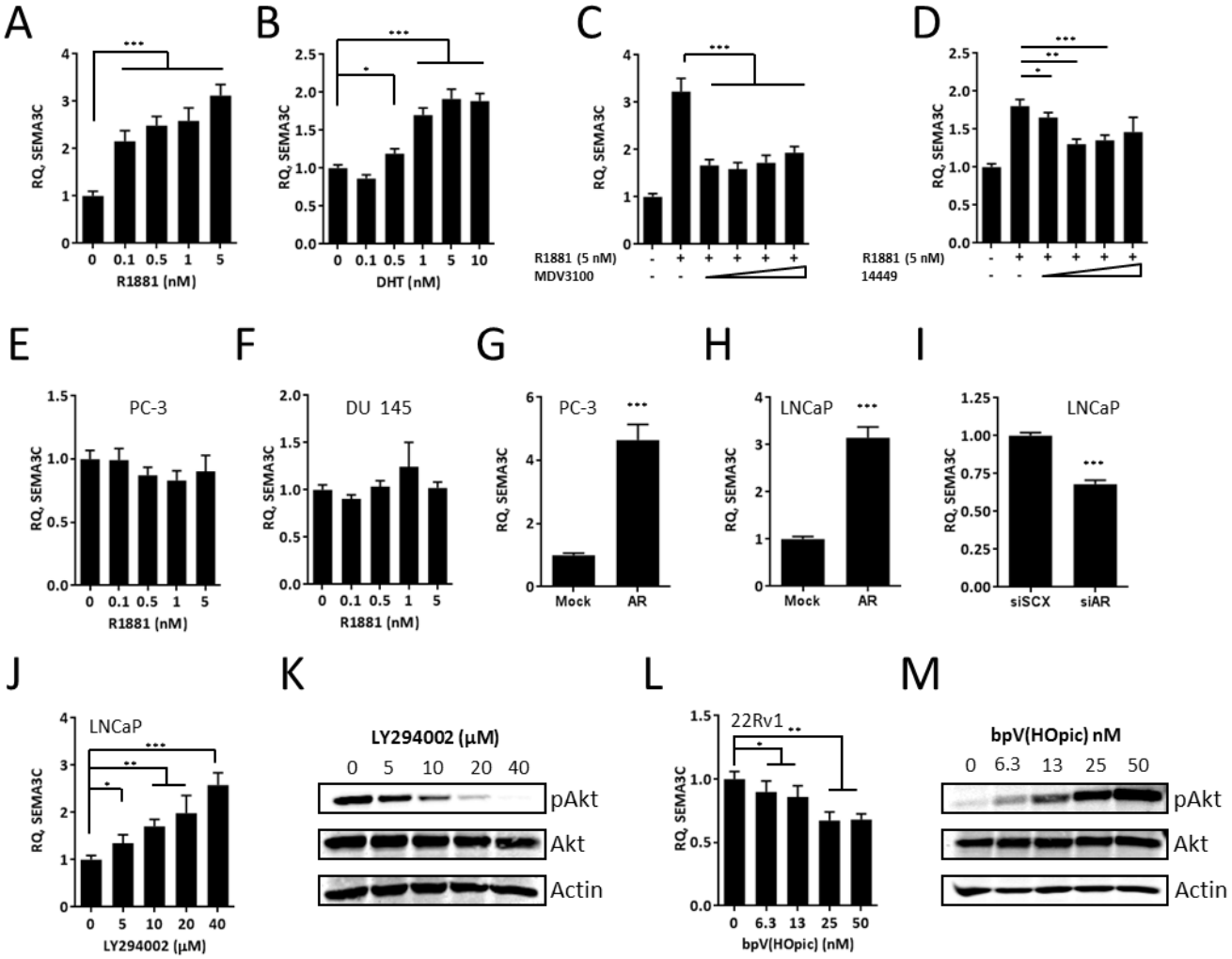

Figure 2.2. SEMA3C is an androgen receptor-regulated gene.
LNCaP were treated with increasing concentrations (0- 5 nM) of the synthetic androgen, R1881 (A), and increasing concentrations (0-10 nM) of dihydrotestosterone (DHT; B) followed by detection of SEMA3C message levels by qPCR; relative quantities (RQ) are presented. R1881-stimulated LNCaP cells were treated with increasing concentrations (6.25, 12.5, 25, and 50 μM) of enzalutamide (MDV3100) (C) and increasing concentrations (1, 5, 10, and 20 μM) of the AR DBD inhibitor VPC-14449 (14449; D) followed by SEMA3C mRNA level detection by qPCR. AR-negative PCa lines PC-3 and DU 145 did not upregulate SEMA3C in response to R1881 (E & F). Overexpression of AR in PC-3 and LNCaP caused upregulating SEMA3C compared to mock transfected cells (Mock) as shown by qPCR (G & H). Knockdown of AR in LNCaP using siRNA (siAR) triggered a decrease in SEMA3C expression compared to LNCaP treated with scrambled siRNA (siSCX; I). LNCaP were treated with PI3K inhibitor LY294002 at the indicated concentrations or DMSO and monitored for SEMA3C message expression (J) and phopho-Akt status (K). 22Rv1 were treated with PTEN inhibitor bpV(HOpic) at the indicated concentrations or DMSO and monitored for SEMA3C message expression (L) and phospho-Akt status (M). Total Akt and actin served as loading controls. Data represent mean, ± SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

2.3.3 The androgen receptor associates with the SEMA3C intron 2 ARE

We next set out to determine if the AR acts in *cis*- at the intron 2 ARE or if the observed SEMA3C induction is the result of upregulation of intermediary factors or pleotropic effects of an activated AR axis. We first tested the capacity of the AR to interact with the SEMA3C intron 2 ARE using an electrophoretic mobility shift assay utilizing purified human AR DNA-binding domain (AR DBD) and a 50 bp double-stranded oligonucleotide centred around the SEMA3C intron 2 ARE (wtARE). Whereas the wtARE oligonucleotide was shifted by the AR DBD, a 50 bp oligonucleotide mapping to an area roughly 200 bps downstream of the endogenous ARE (downARE) did not (Figure 2.3A, compare lanes 2-4 and 6-8). Moreover, incorporation of transversion mutations to six of the core nucleotides constituting the putative ARE (mutARE) abolished the gel-shift observed with the wtARE oligonucleotide (Figure 2.3B, compare lanes 2-4 and 6-8). Thus, the AR DBD is capable of interacting with this putative intragenic ARE.

In canonical NR signalling, NRs are recruited to response elements in the vicinity of target genes in a ligand-inducible manner. To determine if AR is recruited to the SEMA3C locus in this fashion, we used chromatin immunoprecipitation (ChIP) with an AR-specific antibody on lysates from EtOH or R1881-treated LNCaP cells and amplified a 150 bp region mapping to the SEMA3C intronic ARE. R1881 treatment of LNCaP cells resulted in the recruitment of AR to genomic SEMA3C ARE as shown by elevated levels of the SEMA3C ARE amplicon in both end-point (Figure 2.3C) and quantitative PCR (Figure 2.3D). In end-point PCR, inputs contained detectable levels of SEMA3C intronic ARE amplicon but no amplicon was detected in immunoprecipitation with isotype control (Figure 2.3C). GAPDH amplicon was not enriched for by AR immunoprecipitation with R1881 treatment nor was any detectable in the isotype control samples (data not shown). In quantitative PCR, a 3-fold increase in enrichment over isotype

control was observed in the R1881-treated samples whereas no enrichment was observed in the EtOH treatment (Figure 2.3D). Since AR occupancy is a strong indicator of AR-regulation (27), these assays support the notion that SEMA3C is an androgen receptor-regulated gene.

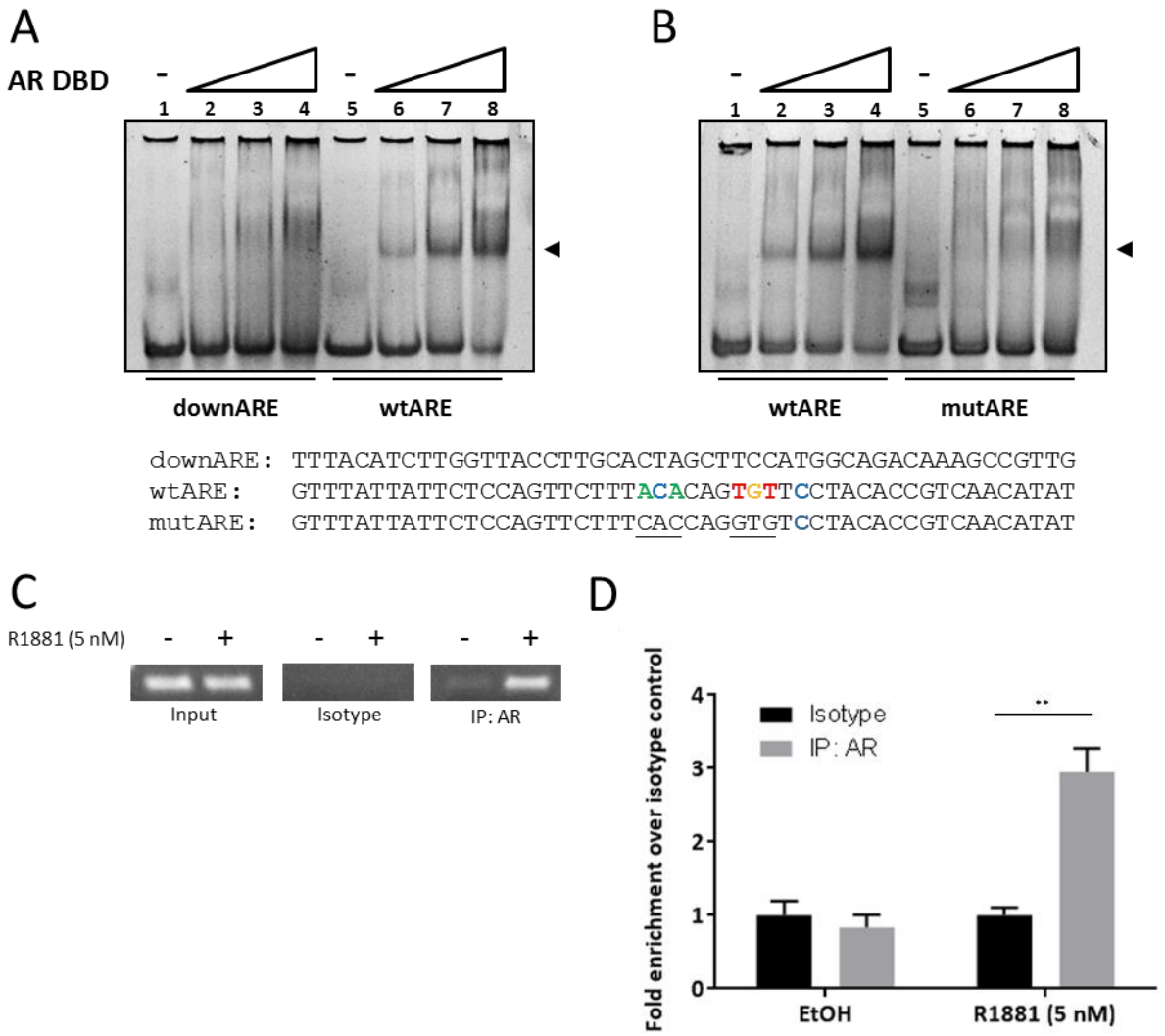

Figure 2.3. The androgen receptor associates with the SEMA3C intron 2 ARE.
In electrophoretic mobility shift assays, 50 basepair oligonucleotides at a final concentration of 1.875 µM were combined with increasing concentrations (0, 0.5, 1.0, 2.0 µM) of purified human androgen receptor DNA-binding domain (AR DBD) and run on a non-denaturing acrylamide gel. A shift (at arrow) was observed when AR DBD was combined with oligonucleotide containing the intron 2 ARE (wtARE) but not when combined with either a 50 bp oligonucleotide mapping to ~200 bp downstream of the intron 2 ARE (downARE) (A) nor with an oligonucleotide with transversion mutations to six bases of the ARE (mutARE) (B). Sequences of the oligonucleotides used for the assay are shown below; sequences shown are complementary to those of Figure 2.1. Bases matching the JASPAR motif are shown in colour; mutations are underlined. ChIP assays were carried out on lysates of LNCaP treated with

0.05% ethanol (vehicle control) or 5 nM R1881. PCR was performed on 1% input (Input), isotype-matched control (Isotype), and AR immunoprecipitates (IP: AR). (C) End-point PCR showed abundant levels of SEMA3C ARE amplicon in input and undetectable levels in isotype control irrespective of R1881 treatment. Ethanol-treated AR immunoprecipitates showed low but detectable levels of SEMA3C ARE amplicon whereas R1881 triggered enriched SEMA3C ARE amplicon in AR immunoprecipitates. Results were confirmed by qPCR (D); values represent fold enrichment over isotype control of the same treatment condition. ± SD; ** $p < 0.01$.

2.3.4 Androgen receptor transactivates the SEMA3C intron 2 ARE

AR-mediated gene transcription culminates in the recruitment of RNA polymerase II, coactivators, and other members of the pre-initiation complex to the promoter region of androgen-responsive genes. This assembly is largely coordinated through AR's AF1, AF2, and BF3 domains (14,179-181). To determine if the isolated SEMA3C ARE is a platform capable of orchestrating these events, we utilized pGL3 reporter constructs bearing a one hundred and fifty bp region surrounding the SEMA3C intron 2 ARE placed upstream of the luciferase gene. Luciferase activity was increased 57 times by R1881 in LNCaP cells transfected with the SEMA3C ARE luciferase reporter construct (wtARE) but not in LNCaP transfected with the empty pGL3-Basic vector (Basic; Figure 2.4A). Luciferase activity was not induced by R1881 in LNCaP transfected with a reporter construct bearing the 6 bp mutant form of the ARE that was described in Figure 2.3 (mutARE; Figure 2.4A). Similar results were obtained when 293T cells were co-transfected with reporter constructs and AR overexpression plasmids. R1881 induced an 8.2-fold increase in luciferase activity in 293T co-transfected with wtARE and AR (Figure 2.4B). In search of additional elements within our insert that are responsible for luciferase induction, a series of constructs with progressive 3' deletions to wtARE insert were generated. Truncation of 60 bp from the full-length insert (wtARE-60bp) resulted in a drastic reduction in R1881 induction of luciferase activity (from 220 to 8.1-fold induction; Figure 2.4C) presumably due to the removal of one or more elements that support AR transcription initiation (discussed further below). The deletion of an additional 60 bp (wtARE-120bp), which removes the putative ARE,

completely abrogated R1881 induction of luciferase activity (from 8.1-fold to no induction; Figure 2.4C).

In PCa, following castration, a restored AR axis is thought to be one of the mechanisms that precipitate the onset of castrate-resistant disease. Restoration of AR activity despite castrate levels of androgens is thought to be through mechanisms that include overexpression of, or mutations to, the AR. The constitutively active AR splice variant, ARv7, which lacks the ligand-binding domain, is one such example. Co-transfection of the ARv7 overexpression plasmid and the wtARE reporter plasmid into 293T cells resulted in luciferase activity comparable to that of ligand-activated wild-type AR (Figure 2.4D) which was irrespective of R1881 treatment. Collectively, these results demonstrate that the identified ARE can coordinate the recruitment of factors necessary for AR-mediated transcription initiation. For biochemical comparison, the luciferase transcriptional output from the SEMA3C intron 2 ARE was titrated against concentrations of MDV3100 and VPC-14449 in R1881-activated LNCaP cells. In these studies, the IC_{50} of MDV3100 and VPC-14449 was discovered to be 1.1 µM and 1.7 µM, respectively (Figure 2.4E & 2.4F). This dose-dependent AR inhibition by MDV3100 and VPC-14449 is consistent with the transcriptional inhibition observed for other ARE-bearing reporter constructs in LNCaP and other cell lines (171,177).

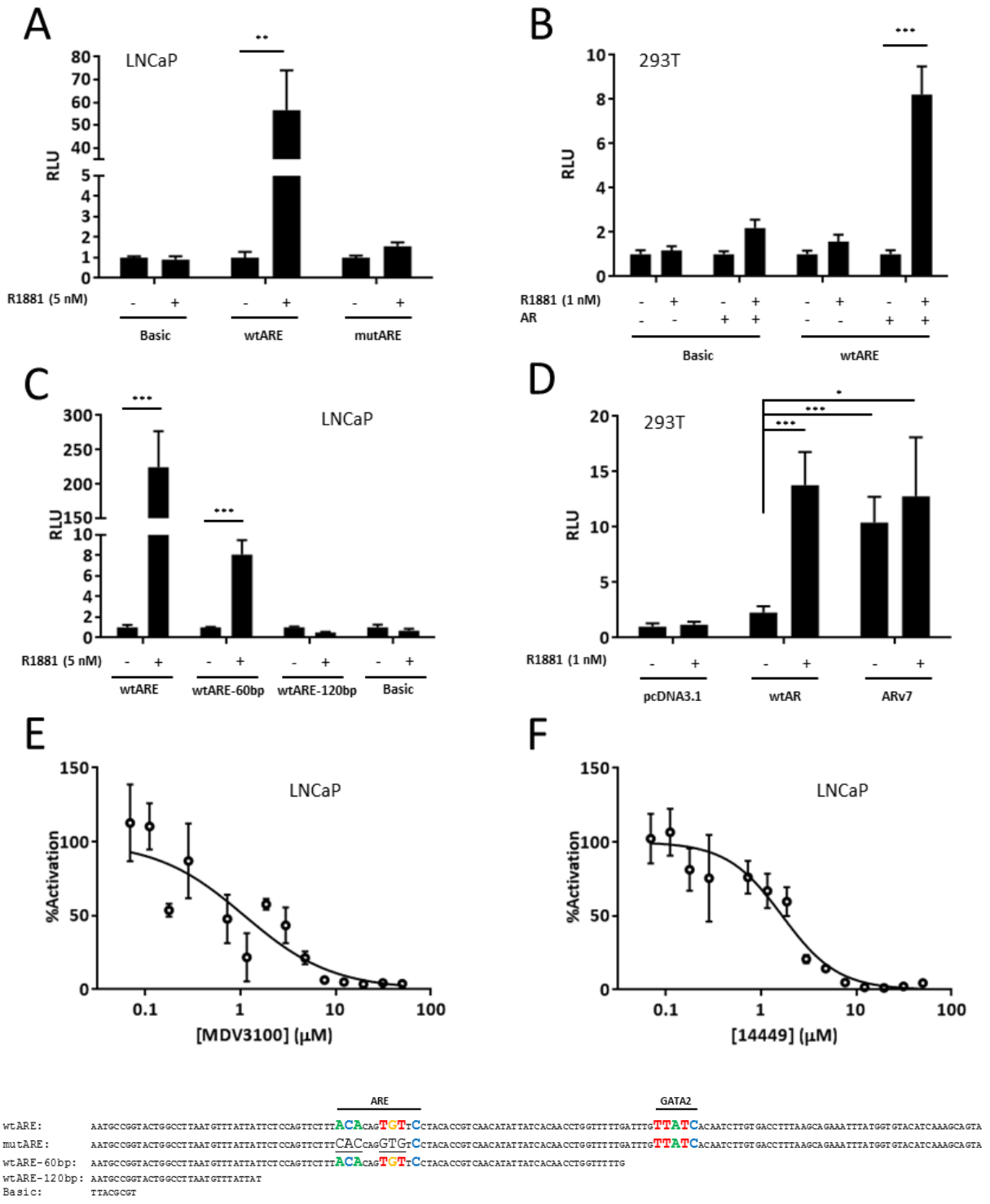

Figure 2.4. Androgen receptor transactivates the SEMA3C intron 2 ARE.
(A) LNCaP cells were transfected with empty pGL3-Basic reporter plasmid (Basic), SEMA3C intron 2 ARE reporter plasmids (wtARE), or reporter plasmids where the ARE was mutated (mutARE). Sequences cloned into reporter plasmids are shown below; sequences shown are complementary to those of Figure 2.1. Bases matching the JASPAR motif are shown in colour and ARE and GATA2 elements are indicated; mutations are underlined. Cells

were then treated with R1881 at 5 nM or vehicle control (0.05% EtOH) and harvested for measurement of relative luminescence (RLU). (B) 293T were co-transfected with reporter plasmids (Basic or wtARE) and AR overexpression plasmids followed by treatment with R1881 at 1 nM or vehicle. (C) LNCaP cells were transfected with wtARE, wtARE with the final 60 basepairs truncated (wtARE-60bp), wtARE with the final 120 basepair truncated (wtARE-120bp), and Basic (sequences are shown below). The wtARE construct contains both the AR and GATA2 motif; the wtARE-60bp construct has the GATA2 motif removed but retains the AR motif; the wtARE-120bp construct contains neither the AR nor the GATA2 motif. Transfected cells were then treated with R1881 at 5 nM or vehicle. (D) 293T cells were transfected with wtARE and either wtAR or ARv7 overexpression plasmid and subsequently treated with R1881 to 1 nM. Transfection with pcDNA3.1 served as a control. Values represent a fold increase over EtOH control (A-C) or fold increase over pcDNA3.1/EtOH treatment (D). Data was analysed using either a Student' t-test (A & B) or one-way ANOVA with Tukey's post hoc test (C & D). Data represent mean, ± SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. LNCaP were transfected with wtARE and co-treated with R1881 (0.1 nM) and dosages of MDV3100 (E) or VPC-14449 (F) ranging from 0.04 to 50 μM. MDV3100 IC_{50} = 1.1 μM and 14449 IC_{50} = 1.7 μM. ± SEM. For all luciferase assays, renilla luciferase was used to normalize readings.

2.3.5 R1881-induction of SEMA3C expression is GATA2-dependent

The GATA family of transcription factors are pioneering factors that control gene expression through epigenetic chromatin remodeling (182,183). One such member, GATA2, has been shown to cooperate with the AR in the regulation of androgen-dependent genes (23,184). GATA2 has roles in development and has recently been ascribed various roles in PCa progression (21,22,185,186). Related family member, GATA6, is known to regulate SEMA3C in cardiac neural crest (187). Inspection of the DNA sequence near the SEMA3C intron 2 ARE using Patser software revealed a GATA2 consensus sequence at the genomic coordinates 80,352,072 to 80,352,085 situated thirty-three bps downstream of the ARE (Figure 2.1A & 2.1B). In reporter gene assays, truncation of this element from the full-length reporter construct corresponded to a nearly 30-fold decrease in R1881-inducibility (Figure 2.4C). In addition to this, in microarray studies we found that GATA2 silencing led to a five-fold decrease in SEMA3C expression in LNCaP (FDR<0.001; NCBI, Gene Expression Omnibus, GEO accession number GSE49342). This data was validated by qPCR where silencing of GATA2 decreased basal levels of SEMA3C by 50% compared to LNCaP treated with negative control scrambled siRNA (Figure 2.5A). Furthermore, knockdown of GATA2 also prevented R1881-induced expression of SEMA3C which was reflected at both the message and protein levels (Figure 2.5A

& 2.5B). These findings are in agreement with reports describing a dependency by androgen-regulated genes on GATA2 (22,23,184). Knockdown of GATA2 was verified by Western blot analysis (Figure 2.5E). We employed ChIP to investigate whether GATA2 is also recruited to the intron 2 ARE region in an androgen-dependent manner. Similar to ChIP results seen with AR (Figure 2.3C & 2.3D), GATA2 was recruited to the SEMA3C intron 2 ARE region in an R1881-dependent manner shown by both end-point PCR (Figure 2.5C) and by qPCR (Figure 2.5D) implying that GATA2 is implicated in AR-driven expression of SEMA3C. Quantitative PCR revealed a 2.3-fold enrichment over isotype control of GATA2 to the intron 2 ARE region upon R1881 treatment whereas no significant enrichment was seen in the EtOH treatment. The presence of a GATA2 motif in such close proximity to the ARE, the dependency on GATA2 in androgen-induced expression of SEMA3C and in reporter gene assays, and the co-recruitment of AR and GATA2 to the intron 2 ARE, together strongly suggest a coordinated effort between AR and GATA2 in the regulation of SEMA3C.

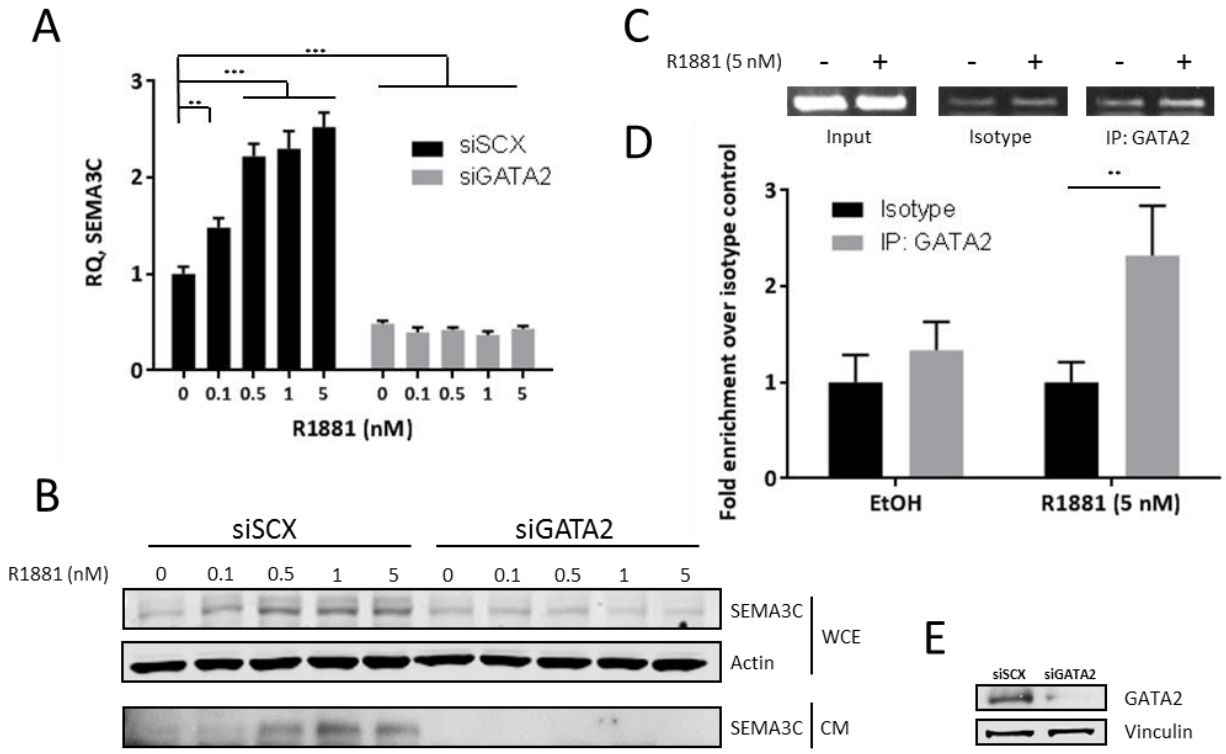

Figure 2.5. R1881-induction of SEMA3C expression is GATA2-dependent.
We examined R1881-induced expression of SEMA3C in the absence of GATA2 to confirm findings from previous microarray studies showing that knockdown of GATA2 decreases SEMA3C expression. When compared to LNCaP treated with scrambled siRNA (siSCX), knockdown of GATA2 (siGATA2) triggered a significant decrease in basal SEMA3C expression and completely attenuated R1881-mediated dose-dependent induction of SEMA3C as shown by qPCR (A). These observations were confirmed at the protein level by Western blot analysis (B) of both conditioned media (CM) and whole cell extract (WCE) where total actin served as loading control. Verification of knockdown was confirmed by Western blot analysis (E) where vinculin served as a loading control. In chromatin immunoprecipitation assays, SEMA3C ARE amplicon was shown be enriched in GATA2 immunoprecipitates of lysates from LNCaP cells treated with R1881 as shown by end-point (C) and qPCR (D) indicating an R1881-dependent recruitment of GATA2 to the SEMA3C intron 2 ARE. Input = 1% input, Isotype = isotype-matched control antibody, IP: GATA2 = GATA2 immunoprecipitates. PCR primers for these experiments were the same as those for Figure 2.3 and map to the SEMA3C intron 2 ARE. ChIP qPCR values represent a fold increase in SEMA3C over isotype control and were quantitated using a ΔΔCt method, normalized to input and isotype control. Data represent mean, ± SD; ** $p < 0.01$, *** $p < 0.001$.

2.3.6 FOXA1 negatively regulates SEMA3C expression

The forkhead box (FOX) and POU-homeodomain family of transcription factors have well-established roles in the expression of genes necessary for development (188,189). Importantly, FOXA1 and POU2F1 (also known as OCT1) are also known to cooperate with GATA2 and AR in the expression of androgen-regulated genes (11,22-24,190,191) and FOXA1

is frequently mutated in advanced PCa patients (53,54,192). This prompted us to explore whether FOXA1 and POU2F1 are also involved in SEMA3C expression. To this end, we examined whether SEMA3C expression was altered following FOXA1 and POU2F1 knockdown. Knockdown of FOXA1 and POU2F1 resulted in an 18 and 1.2-fold induction of SEMA3C expression, respectively, over scrambled siRNA transfection control, strongly indicating that FOXA1 negatively regulates SEMA3C whereas POU2F1 seems not to play a role in SEMA3C expression (Figure 2.6A). Knockdown of POU2F1 and FOXA1 was assessed by qPCR and Western blot, respectively (Figure 2.6Bi & 2.6Bii). Whereas FOXA1 was detectable by Western blot, POU2F1 was not, therefore POU2F1 knockdown was monitored by qPCR. To determine if FOXA1 or POU2F1 has an impact on R1881-induced SEMA3C expression, LNCaP were treated with R1881 in combination with FOXA1 or POU2F1 knockdown. In the absence of FOXA1, basal SEMA3C levels increased substantially and further increased upon treatment with R1881 at both the message and protein level (Figure 2.6C & 2.6D). SEMA3C message levels increased between 47% and 64% with R1881 treatments over vehicle control in the absence of FOXA1 (Figure 2.6C). R1881 induction of SEMA3C in the absence of POU2F1 did not differ significantly from that seen in cells treated with scrambled siRNA control (Figure 2.6C & 2.6D). To clarify if FOXA1 silencing-mediated induction of SEMA3C was dependent on AR, we silenced AR and FOXA1 simultaneously and found that even in the absence of AR, knockdown of FOXA1 triggered induction of SEMA3C, albeit less than in the presence of AR (21 versus 12-fold induction of SEMA3C in siFOXA1 and siFOXA1+siAR, respectively), indicating that the observed induction is not dependent on AR (Figure 2.6E). Efficacy of knockdown of AR and FOXA1 was confirmed by Western blot (Figure 2.6Fi & 2.6Fii). Collectively these results would suggest that while POU2F1 seems not to be involved in SEMA3C regulation, FOXA1 is a

negative regulator of SEMA3C and that this suppression is independent of AR. In light of a propensity for FOXA1 mutations in advanced PCa, aberrant FOXA1 signalling may contribute to elevated SEMA3C expression. The effect of knockdown of each of GATA2, FOXA1, and POU2F1 on SEMA3C expression was repeated in C4-2 cells and results were concordant with those seen in knockdown of the same genes in LNCaP (Appendix B). Knockdown of GATA2 resulted in an 87% reduction of SEMA3C levels while knockdown of FOXA1 and POU2F1 triggered an 8.3 and 1.5-fold induction of SEMA3C, respectively. Knockdown was verified by either Western blot or qPCR (Appendix B).

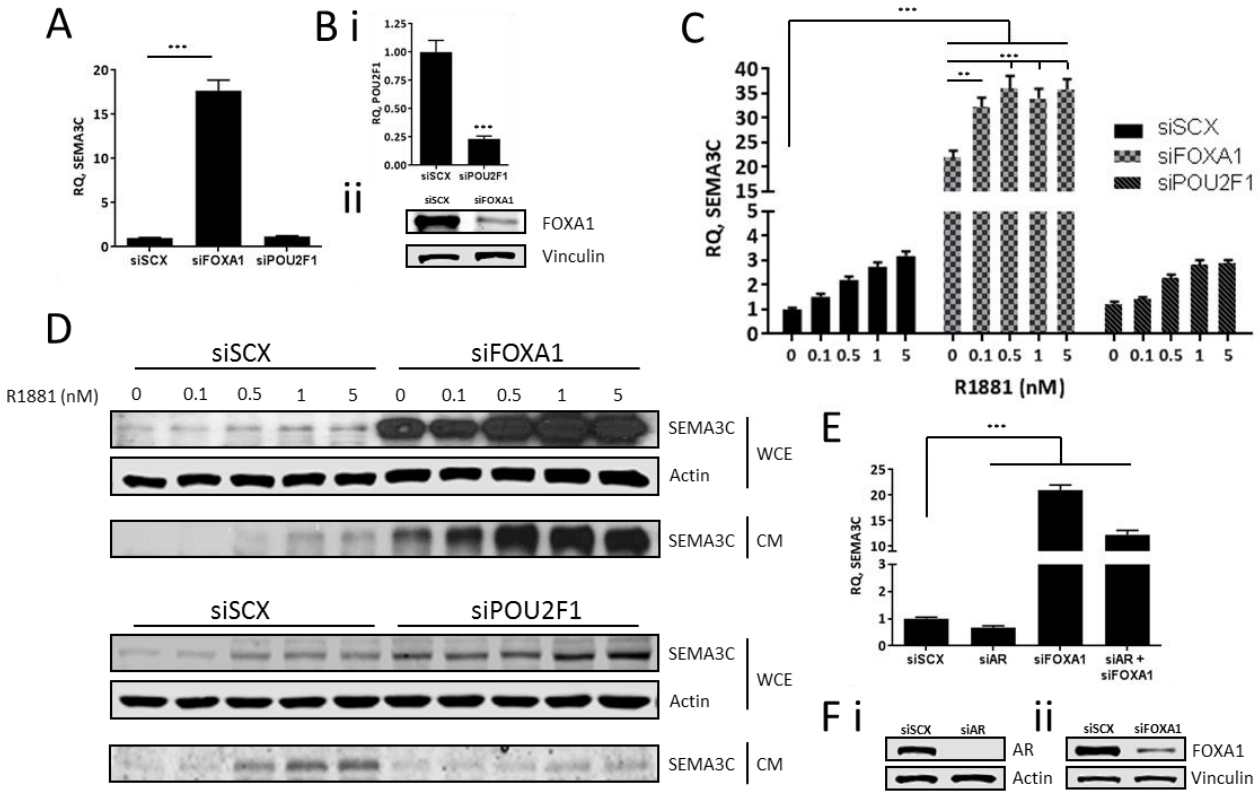

Figure 2.6. FOXA1 negatively regulates SEMA3C expression.
We assessed the effect of silencing of FOXA1 and POU2F1 on SEMA3C message levels. (A) Knockdown of FOXA1 (siFOXA1) triggered an increase in SEMA3C levels when compared to cells treated with scrambled siRNA (siSCX); knockdown of POU2F1 (siPOU2F1) had no effect on SEMA3C expression. Knockdown of these genes was confirmed by qPCR (POU2F1) or Western blot (FOXA1; B). In siFOXA1-treated cells where SEMA3C levels were already elevated, SEMA3C expression was further increased upon R1881 stimulation shown at both the

message (C) and protein (D) level. Knockdown of POU2F1 had little effect on R1881 induction of SEMA3C (C, D). WCE: whole cell extract; CM: conditioned media. (E) LNCaP cells were knocked down with siAR, siFOXA1, or both (siAR+siFOXA1). Compared to LNCaP treated with scrambled siRNA (siSCX), siAR-treated cells had decreased SEMA3C expression while siFOXA1 and siAR+siFOXA1-treated cells had elevated SEMA3C expression. Knockdown of AR and FOXA1 was confirmed by Western blot analysis (F). Data represent mean, ± SD; ** $p < 0.01$, *** $p < 0.001$.

2.4 Discussion

A more complete understanding of the set of androgen receptor-regulated genes will be instrumental to the realization of more efficacious PCa therapies. The ARE in the second intron of SEMA3C identified based on initial studies by Yu *et al*, is located roughly 30 kb downstream of the TSS for this gene. In many AR gene targets, the corresponding AREs are situated downstream of the TSS at distances of tens of kilobases (23,27). Here we demonstrate that SEMA3C expression is regulated by the androgen receptor in a prototypical way. We show that androgens induce SEMA3C expression, that the AR is recruited to the *SEMA3C* locus in an androgen-dependent fashion, and that the intronic ARE can bind to and be transactivated by the AR. We further demonstrate that GATA2 is critical to this process and that FOXA1 is a negative regulator of SEMA3C expression. FOXA1 suppression of SEMA3C expression, although additive in nature with those of AR, is not dependent on AR. Remarkably, the regulatory networks governing semaphorin expression are largely unknown despite the fact that SEMA3C and its kin are heavily implicated in cancer. SOX4, GATA6, and Twist1 are three transcription factors with reported involvement in the regulation of SEMA3C (157,187,193,194). In search of mediators of hepatocellular carcinoma progression, Liao *et al* reveal SOX4 as a candidate driver of hepatocellular carcinoma through its regulation of SEMA3C. The authors support their claim that SOX4 regulates SEMA3C through identification of SOX4 binding motifs in the promoter of SEMA3C, recruitment of SOX4 to the promoter of SEMA3C in chromatin immunoprecipitation assays, transactivation of the SEMA3C promoter by SOX4 in reporter gene assays, and SOX4 loss-of-function studies which result in decreased SEMA3C levels. Interestingly, they also reveal

a similar regulation of the semaphorin receptor, NRP1, by SOX4. In the case of GATA6, Lepore *et al* state that GATA6 is essential for cardiovascular development through a process that likely depends on GATA6's regulation of SEMA3C. Evidence for this was through GATA6 transactivation studies of the SEMA3C promoter region. Lee and Yutzey describe Twist1 regulation of SEMA3C in MC353-E1 preosteoblast cells using microarray and qPCR studies. Specifically, cells treated with Twist1 siRNA resulted in decreased levels of endogenous SEMA3C. This association was further characterized using reporter gene assays and chromatin immunoprecipitation studies which identified an E-box consensus sequence in the first intron of SEMA3C through which Twist1 was likely modulating SEMA3C expression. Identification of SOX4, GATA6, and Twist1 as regulators of SEMA3C is of great importance due to their heavy involvement in development and strong ties to processes also related to functions surrounding SEMA3C. Our characterization of SEMA3C regulation by AR is set apart from these studies, however, because to our knowledge hormone regulation of SEMA3C has not yet been reported. Our success in characterizing the *SEMA3C* gene in this way underscores the need for a more comprehensive understanding of the biological implications of SEMA3C within PCa and particularly in the CRPC landscape.

The co-recruitment of AR and GATA2 to a region well downstream of the transcriptional start site of SEMA3C (Figure 2.3D & 2.5D) would imply involvement of a DNA looping mechanism whereby the ARE and SEMA3C proximal promoter are brought together through a bridge consisting of AR and GATA2 and likely other proteins. AR and GATA2 are known to associate with distally-located ARBS as a form of gene regulation of AR-regulated genes (22,23,172). Techniques such as ChIP-on-chip, ChIP-seq, or ChIP Combined with Chromosome Conformation Capture (5C) will be required to prove that the intron 2 ARE is cooperating with

AR and GATA2 via DNA looping. Despite the known role of GATA2 in chromatin remodelling, our reporter gene assays further suggest that GATA2 also directly supports AR transcription initiation since deletion of the GATA2 motif drastically diminishes R1881-mediated induction of luciferase activity (Figure 2.4C). Nevertheless, the notion that GATA2 is a driver of PCa is seemingly consistent with our findings that GATA2 promotes the expression of oncogenic SEMA3C. FOXA1's inhibitory effect on SEMA3C expression may also be of significance given that FOXA1, which is often found to be mutated in advanced PCa, may account for elevated SEMA3C expression in advanced PCa.

In addition to an ARBS in intron 2, Yu and colleagues also identified an ARBS in intron 12 of SEMA3C (164). Inspection of the ARBS in intron 12 using Patser software did not reveal consensus sequences for the androgen receptor (data not shown) but presumably AR associates with this region of DNA through intermediary transcription factors or coregulators. Patser software analysis did, however, reveal a GATA2 consensus sequence in intron 12 of SEMA3C spanning the genomic coordinates 80,249,027 to 80,249,040 on the reverse strand (Figure 2.1A). It is conceivable that recruitment of AR to this genomic region is GATA2-dependent, further implicating GATA2 in AR-mediated regulation of SEMA3C. The functional significance of the GATA2 element identified in intron 12 and whether the intron 12 ARBS and GATA2 element are acting in concert with the *cis*-acting elements of intron 2 remain to be determined. Our results also raise the possibility that other semaphorins or their receptors fall under AR, GATA2, or FOXA1 regulation, especially given that many semaphorin family members are implicated in cancer. Indeed, Yu *et al*'s results also identify ARBSs in nearly all other class 3 semaphorins, most notably three ARBSs in another class 3 semaphorin with well-documented tumour-promoting activity, SEMA3E. Conversely, receptors to semaphorins have been shown to

promote AR activity (195) thus opening up the possibility that semaphorins or their receptors participate in the regulation of the AR axis in a feed-forward fashion. Future work will need to be undertaken to address the potential involvement of the intron 2 and intron 12 GATA2 motifs in the context of AR regulation of SEMA3C. Additional work will also need to be performed to ascertain whether other semaphorins or semaphorin receptors are regulated by any of AR, GATA2, or FOXA1, and whether a reciprocal relationship exists in the regulation of AR by the semaphorins.

Considering the co-occurrence of aberrant AR and SEMA3C activity in advanced PCa, we speculate that restored AR signalling in advanced PCa drives SEMA3C expression which in turn propels PCa progression. SEMA3C is known to have roles in embryogenesis, therefore, its upregulation might also confer stem-like phenotypes (phenotypes reminiscent of those seen in stem cells) to cancer cells and contribute to tumour heterogeneity frequently observed in tumour histopathology of PCa patients. It is also worth noting that SEMA3C is associated with tumour cell motility (139,158,196,197) and may therefore contribute to cancer cell dissemination. This hypothesis is reinforced by the fact that unpublished work by our lab has shown that SEMA3C can activate receptor tyrosine kinase signalling which is well-known to initiate EMT. This propensity for motility may be especially important in CRPC where elevated SEMA3C expression may confer cancer cells with invasive phenotypes that contribute to metastasis. To pursue this line of questioning and to test whether SEMA3C potentiates invasiveness in this way, we embarked on studies utilizing gain-of-function techniques coupled to gene expression and functional assays to assess the ability of SEMA3C to drive EMT and acquisition of stem-like characteristics which is explored in Chapter 3 below.

CHAPTER 3. Semaphorin 3C drives epithelial-to-mesenchymal transition, invasiveness, and stem-like characteristics in prostate cells

3.1 Introduction

Given the finding that SEMA3C is a transcriptional target of the androgen receptor, we next sought to characterize the nature of its involvement in the disease. High rates of relapse and poor prognosis for those with locally-advanced and metastatic forms of PCa illustrate the importance in identifying mechanisms that drive these fatal forms of the disease. The switch to a mesenchymal phenotype during EMT, which is also often accompanied by the development of cancer stem cell attributes (73), represents one such explanation. The mesenchymal and stem-like population of cells are believed to resist first-line therapies and cause tumour relapse and eventual metastasis. These populations of cells, each exhibiting at least some degree of potency and primitive characteristics, would conveniently explain various observations made during malignancy. For example a stem-like population has the potential to subvert treatments; indeed, stem-like populations have been observed to resist radiation and chemotherapies (198-201) likely through heightened DNA repair, the suppression of apoptotic programs, or elevated ABC transporter activity (200-204). At the same time, a mesenchymal compartment would confer the invasive phenotypes that are often associated with metastasis. Although normally utilized during such developmental stages as gastrulation and organogenesis during embryogenesis, hijacking these stem programs could also lead to dissociation of cells from the primary tumour, invasion and migration through basement membranes and stroma, and dissemination to distant secondary sites through the lymphatic or circulatory systems. The inherent potency of mesenchymal and stem-like populations might also give rise to intratumoural and inter-patient heterogeneity frequently seen in cancer.

Given the known roles of SEMA3C in development and its access to stem-related programs combined with its involvement in numerous cancers, the next logical question is whether SEMA3C is contributing oncogenic phenotypes by way of commissioning EMT and stem programs. Other class 3 semaphorins have been shown to drive EMT and the link between SEMA3C and stem cells has been established in glioblastoma but SEMA3C's precise roles in driving EMT and stemness in PCa have never been discussed. This chapter implicates SEMA3C in the process of EMT in prostate cells and shows that SEMA3C can confer characteristics normally attributed to stem compartments. It is also shown that SEMA3C can promote invasiveness in prostate cells.

3.2 Materials and methods

Cell lines and plasmids

SEMA3C (accession # NM_006379) or luciferase was cloned under the control of a human Ubiquitin C promoter in a modified lentiviral expression vector using Gateway Technology as described previously (205). Cells were transduced with lentivirus generated from this plasmid as described previously (205). RWPE-1 (ATCC, CRL-11609) and RWPE-2 (ATCC, CRL-11610) were cultured in KSFM (Invitrogen, 17005-042) supplemented with bovine pituitary extract to 0.05 mg/ml and 5 ng/ml human recombinant epidermal growth factor and maintained under Blasticidin S selection at 2 μg/ml. BPH-1 (kindly provided by Dr. S. Hayward, Vanderbilt University) and MDA-MB-468 (ATCC, HTB-132) were cultured in 10% FBS, DMEM and maintained under Blasticidin S selection as above. MCF 10A (ATCC, CRL-10317) were cultured in MEBM media + supplements supplied with Lonza CC-3150 and cholera toxin (SIGMA-ALDRICH, C8052) to 100 ng/ml and maintained under Blasticidin S selection as

above. MCF7 (ATCC, HTB-22) and T-47D (ATCC, HTB-133) were cultured in 10% FBS, 0.01 mg/ml insulin, RPMI 1640 and maintained under Blasticidin S selection as above.

Western blot

Conditioned media or whole cell extract were run on 10% acrylamide gels and transferred onto nitrocellulose membrane; lysates were prepared in 50 mM Tris-HCl, 150 mM NaCl, 1% NP40, 10 mM NaF, 10% glycerol, supplemented with protease inhibitor cocktail (Roche, 04693116001); protein concentration were determined using a BCA method (Thermo Scientific, 23228) and 50 µg of total protein was analyzed. Western blots were visualized using radiography film, a Syngene Dyversity, or a LI-COR Odyssey system. Antibodies for Western blot analysis can be found in Appendix C. Actin and vinculin served as loading controls.

Quantitative polymerase chain reaction

Expression of EMT markers was assessed by qPCR. Total RNA was extracted using TRIzol (Invitrogen, 15596018). 2 µg of RNA was reverse-transcribed using random hexamers (Roche, R15504) and Superscript II (Invitrogen, 18064-014). qPCR was carried out using a ΔΔCt method on an AB ViiA7 real-time PCR machine; reactions were prepared using Platinum SYBR Green (Invitrogen, 11744-500) and GAPDH served as an endogenous control. EMT primer sequences were previously described by Zhang *et al* (206). Sequences can be found in Appendix D.

Microscopy

Slides were fixed in 4% paraformaldehyde, PBS for 15 min., rinsed three times with PBS, blocked in 2% BSA, 0.2% Triton X-100, PBS for 30 min., stained with primary antibody diluted at 1/100-1/200 in 1% BSA, 0.2% Triton X-100, PBS for 2 hours or overnight at 4^{o}C, rinsed three times with 0.05% Tween-20, PBS, incubated with secondary antibody diluted at 1/1,000 in 1%

BSA, 0.2% Triton X-100, PBS for 1 hour, rinsed three times with 0.05% Tween-20, PBS, and finally fixed and DAPI-stained using ProLong Gold (Life Technologies, P36935). Bright-field and immunofluorescent images were captured on a Zeiss Axioplan 2 and Zeiss AxioObserver.Z1 using AxioVision LE and ZEN Light Blue software, respectively. For double staining experiments, staining for targets occurred sequentially. Antibodies for immunofluorescence are described in Appendix C.

Migration and invasion assay

Cell migration was measured by wound-healing ('scratch') assay. Cells were seeded in 6-well plates and grown to confluency. Cells were pre-treated with mitomycin C at 15 µg/ml for thirty minutes to inhibit cell proliferation. Following treatment with mitomycin C, cells were mechanically scratched, changed to fresh medium, and imaged for the first time point. Cells were imaged at a second time point twenty-four hours later. Percent migration in wound-healing assay was calculated using the formula: [(pixels at T_{0h})-(pixels at T_{24h})] / (pixels at T_{0h}) x 100% across three biological replicates. Migration was also measured using a Boyden chamber transwell migration assay (Costar, 3422) as per manufacturer's instruction. Two-hundred thousand cells were seeded per chamber. Migration was measured using Calcein, AM (Life Technologies, C3099) and read on a TECAN Infinite F500 plate reader using i-control1.7 software. Invasion was measured using a BD Matrigel Invasion Chamber approach (BD Biosciences, 354480) the setup for which paralleled migration assay. For directional migration assays, two-hundred thousand RWPE-1-FUGWBW cells were placed in the upper chamber and allowed to migrate toward PBS as control or recombinant SEMA3C (1 µM) or conditioned media from RWPE-1-FUGWBW (FUGWBW CM) as control or conditioned media from RWPE-1-SEMA3C (SEMA3C CM) which was placed in the well beneath the chamber. Recombinant Fc-tagged

SEMA3C was purified on a mAb select Protein A column. CM was concentrated five times using centrifugal filters (Millipore, UFC901024).

Flow cytometry

CD44 status was monitored using a BD Biosciences FACSCanto II. Cells were collected using trypsin and washed in FACS buffer (2% FBS, PBS) prior to antibody staining (30 min on ice). Antibodies can be found in Appendix C. Cells were washed three times with 0.5 ml FACS buffer after each antibody. Targets were probed sequentially for double staining. For intracellular staining, cells were fixed and permeabilized using Cytofix/Cytoperm (BD Biosciences, 51-2090KZ) and 0.2% Triton X-100, PBS, blocked and stained in 1% BSA, 0.05% Tween-20, PBS, and washed in 0.05% Tween-20, PBS. Stained and washed cells were brought up in 0.5 ml FACS buffer for running on flow cytometer. FSC: 250v, SSC: 375v, PerCP-Cy5 (CD44): 325v, FITC (E-cadherin, N-cadherin, Vimentin): 400v. FlowJo Analysis software was used to analyze data. $CD44^{low}$ and $CD44^{high}$ cells were sorted using a FACSAria IIu.

Sphere forming assay

Ten-thousand cells were seeded in ultra-low attachment surface 6-well dishes (Corning, 3471) and cultured in MammoCult as per manufacturer's instruction (STEMCELL Technologies, 04620). Spheres were allowed to develop for one week and then imaged on a Zeiss AxioObserver.Z1. To count constituent cells, spheres were dissociated by trypsin and counted by Trypan blue staining and hemocytometer.

Proliferation assay

Three thousand (RWPE-2) or six thousand (RWPE-1) cells were plated in black clear-bottom 96-well plates (Corning, 3904) in KSFM media containing supplements. Viability was measured using PrestoBlue Cell Viability Reagent (ThermoFisher Scientific, A-13261) as

per manufacturer's instruction and read on a TECAN Infinite F500 plate reader using i-control1.7 software.

Animal studies

All animal experiments detailed within the manuscript were approved by the UBC Animal Care Committee, conforming to the mandatory guidelines of the Canadian Council on Animal Care. UBC animal protocol number A15-0150. Assessment of *in vivo* tumourigenicity was carried out by ultrasound-guided (Vevo 770, VisualSonics) intracardiac injection of $5x10^5$ cells resuspended in PBS into NOD scid gamma (NSG) mice (The Jackson Laboratory, strain 005557). Tumour formation was monitored by intraperitoneal injection of mice with luciferin (Caliper Life Sciences, 119222) and bioluminescence readings on an In Vivo Imaging System (IVIS Lumina, PerkinElmer) using Living Image 4.2 software.

Microarray

Microarray gene expression studies were carried out by the Laboratory for Advanced Genome Analysis at the Vancouver Prostate Centre, Vancouver, Canada. Samples were prepared following Agilent's One-Color Microarray-Based Gene Expression Analysis Low Input Quick Amp Labeling v6.0. An input of 100 ng of total RNA was used to generate Cyanine-3 labeled cRNA. Samples were hybridized on Agilent SurePrint G3 Human Gene Expression v3 8x60K Microarray (AMDID 072363). Arrays were scanned with the Agilent DNA Microarray Scanner at a 3 μm scan resolution and data was processed with Agilent Feature Extraction 11.0.1.1. Processed signal was quantile normalized with Agilent GeneSpring 12.0. To find significantly regulated genes, fold changes between the compared groups and *p*-values gained from t-test between same groups were calculated. The t-tests were performed on normalized data

that had been log transformed and the variances were not assumed to be equal between sample groups. A Benjamini-Hochberg multiple test correction was applied.

Statistics

Statistical analysis was performed using the Student's two-tailed t-test. Data are represented as mean ± SD. Data presented are representative of three biological replicates.

3.3 Results

3.3.1 Generation of RWPE-1 cells stably overexpressing SEMA3C

We chose the non-transformed prostate epithelial cell line, RWPE-1, for our studies due to their low intrinsic semaphorin 3C (SEMA3C) levels. Furthermore we anticipated that it would be easier to observe an oncogenic shift in RWPE-1 compared to tumourigenic PCa lines since RWPE-1 cells retain many of their epithelial non-transformed characteristics. The RWPE-1 cell line was clonally derived from a histologically normal prostate and was immortalized using the human papilloma virus 18 (163). *SEMA3C* was cloned downstream of the human Ubiquitin C (UbC) promoter (Figure 3.1A) in a modified FUGW lentiviral vector referred to as FUGWBW from this point forward. RWPE-1 cells that were transduced with virus made from SEMA3C overexpression plasmids (referred to as 'SEMA3C') strongly expressed SEMA3C compared to cells transduced with empty parental vector (referred to as 'FUGWBW'). Overexpression was confirmed by Western blot analysis of their conditioned media and whole cell extract (Figure 3.1B). Overexpression of SEMA3C in RWPE-1 was associated with morphological changes as captured by bright-field microscopy (Figure 3.1C). RWPE-1-FUGWBW cells exhibited cobblestone morphology typical of epithelial cells, however, the RWPE-1-SEMA3C cell population also contained a population of cells with spindle-like morphology reminiscent of mesenchymal cells. The changes in morphology are consistent in the semaphorins' known roles

in cellular morphology and cytoskeletal rearrangements (112-114,117,207). Morphological changes became increasingly evident (with respect to percentage of the entire population) with number of passages suggesting enrichment of a population rather than a uniform transition by the entire population or transdifferentiation. The presence of the receptors to SEMA3C (Plexin B1, Plexin D1, Neuropilin 1, and Neuropilin 2) on these cells was also verified by Western blot (Figure 3.1B). Based on these Western blots, the abundance of some of these receptors was seemingly low; nevertheless, their expression is corroborated by reports that show that these receptors are expressed in a wide variety of prostate lines (159).

We examined potential activation of growth-promoting signalling pathways and found that SEMA3C overexpression led to modest increases in phospho-Akt and phospho-EGFR. Interestingly, total EGFR levels dramatically increased. SEMA3C overexpression did not alter levels of phospho-MAPK.

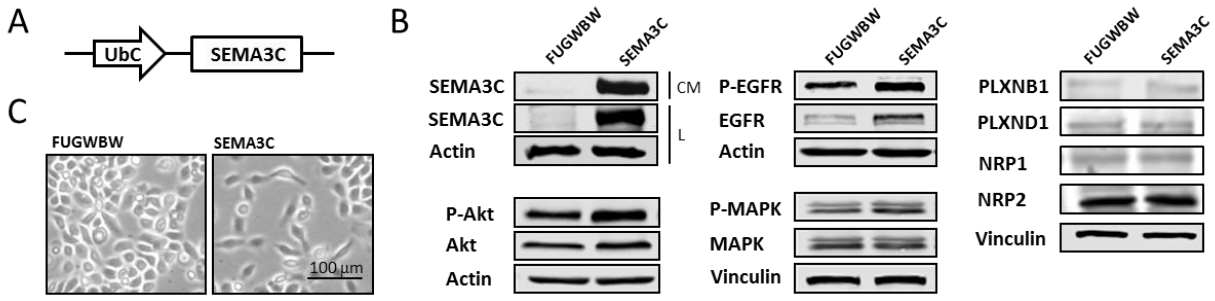

Figure 3.1. Generation of RWPE-1 cells stably overexpressing SEMA3C.
SEMA3C was cloned under the control of a human Ubiquitin C promoter in a modified FUGW lentiviral vector designated FUGWBW using Gateway technology (A) (Invitrogen). Immortalized normal prostate epithelia RWPE-1 cells were transduced with virus made from either a SEMA3C overexpression construct to achieve constitutive expression (SEMA3C) or empty parental vector to serve as a control (FUGWBW). As SEMA3C is a secreted protein, overexpression in cells was confirmed by Western blot analysis of conditioned media (CM) and cell lysate (L) where actin served as loading control (B). Phospho- and total levels of Akt, EGFR, and MAPK were also examined where actin or vinculin served as loading control. Levels of the receptors to SEMA3C were confirmed by Western blot; Plexin B1 (PLXNB1), Plexin D1 (PLXND1), Neuropilin 1 (NRP1), and Neuropilin 2 (NRP2). Control cells showed cobblestone morphology which is characteristic of epithelia while SEMA3C-overexpressing cells showed cobblestone and spindle-like morphologies (C). Bar = 100 µm.

3.3.2 Overexpression of SEMA3C causes an upregulation of EMT markers

The process of metastasis is thought to be a consequence of inappropriate execution of embryonic programs such as EMT. EMT is characterized by diminished cell-to-cell contact, loss of cellular polarity, and increased cell motility. The loss of cobblestone morphology and decreased cell-cell contacts by SEMA3C-overexpressing RWPE-1 cells are indicative of EMT. If EMT were occurring, it would be reflected in altered expression of EMT markers. To examine such a possibility, we compared the expression of a panel of EMT markers between RWPE-1-FUGWBW and RWPE-1-SEMA3C cells. qPCR indicated that cells overexpressing SEMA3C showed a classic mesenchymal profile including an upregulation of vimentin (VIM), fibronectin 1 (FN1), zinc finger E-box binding homeobox 1 (ZEB1), zinc finger E-box binding homeobox 2 (ZEB2), and N-cadherin (CDH2), and a downregulation of E-cadherin (CDH1, Figure 3.2A). Specifically, there was a 3.9, 4.1, 12, 18, and 23-fold induction of vimentin, fibronectin, ZEB1, ZEB2, and N-cadherin, respectively, and a 35% reduction in E-cadherin expression. Other EMT-associated transcription factors such as TWIST1 and SNAI1 did not drastically change. The changes in expression of these genes were confirmed at the protein level by Western blot analysis (Figure 3.2B). We were unable to confirm increases in ZEB2 by Western blot due to the lack of an effective commercially-available antibody. Collectively, these results show that overexpression of SEMA3C promotes an epithelial-to-mesenchymal transition gene expression profile.

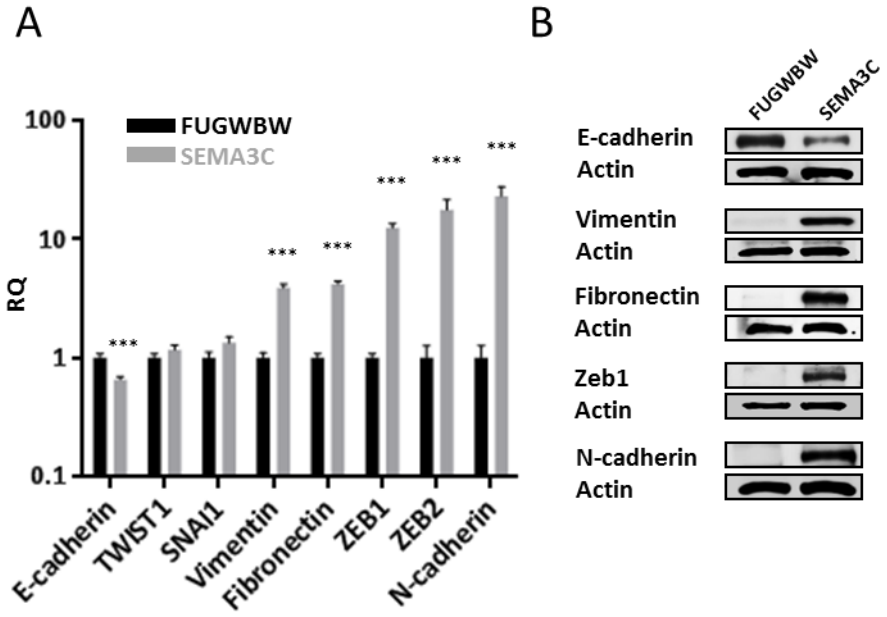

Figure 3.2. Overexpression of SEMA3C causes an upregulation of EMT markers.
Expression levels of a panel of EMT markers were compared between SEMA3C-overexpressing RWPE-1 cells (RWPE-1-SEMA3C) and control cells (RWPE-1-FUGWBW). Significant upregulation of N-cadherin, ZEB2, ZEB1, fibronectin, vimentin, and downregulation of E-cadherin was observed in RWPE-1-SEMA3C compared to control RWPE-1-FUGWBW as shown by qPCR (A). These findings were verified by Western blot analysis (B). Data represent mean, ± SD; *** $p < 0.001$ comparing RWPE-1-SEMA3C to RWPE-1-FUGWBW cells.

3.3.3 SEMA3C increases migration and invasion *in vitro*

Motility and invasiveness are characteristics of mesenchymal cells and serve as *in vitro* measures of metastatic potential. These traits are thought to bestow cancer cells with the ability to disseminate from the primary tumour to mark the onset of metastatic disease. As we found that RWPE-1-SEMA3C cells displayed a typical EMT gene expression signature, we next sought to determine if this was accompanied by acquisition of motile and invasive phenotypes. In these experiments, RWPE-1-SEMA3C cells were seen to rapidly close the scratch in wound-healing assays compared to RWPE-1-FUGWBW (Figure 3.3A). RWPE-1-FUGWBW cells closed the wound by ~20% after a 24 hour period whereas RWPE-1-SEMA3C cells closed the wound an average of 80% over three biological replicates in the same time frame (Figure 3.3B). RWPE-1-SEMA3C cells were also 3.4 times more migratory than RWPE-1-FUGWBW cells when measured by transwell migration assay (Figure 3.3C). To assess the invasiveness of RWPE-1-

SEMA3C cells, we performed a matrigel transwell migration assay and observed that RWPE-1-SEMA3C cells were 2.1 times more invasive than control cells (Figure 3.3D). Consistent with previous reports showing that SEMA3C is a chemo-attractant (139), RWPE-1-FUGWBW cells migrated 8.7 times more strongly toward recombinant SEMA3C than to PBS control (Figure 3.3E). RWPE-1-FUGWBW cells similarly migrated 1.9 times more strongly toward SEMA3C-containing conditioned media than to control conditioned media (Appendix E). Collectively this demonstrates that overexpression of SEMA3C in RWPE-1 cells promotes EMT at the molecular and phenotypic level.

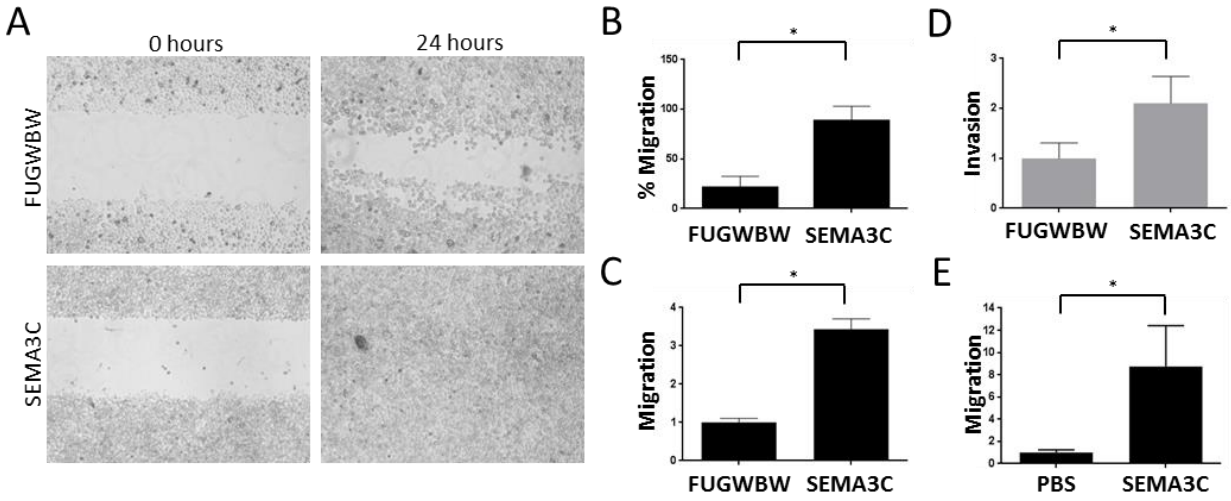

Figure 3.3. SEMA3C increases migration and invasion *in vitro*.
The migration of RWPE-1-SEMA3C was compared to that of control cells by wound-healing assay (A). % Migration in wound-healing assay was quantitated by the formula [(pixels at T_{0h})-(pixels at T_{24h})] / (pixels at T_{0h}) x 100% (B). In transwell migration assay, RWPE-1-SEMA3C cells were roughly 3.5 times more motile than control cells (C); y-axis is fold increase in migration over control cells. In Matrigel Invasion assays, RWPE-1-SEMA3C cells were 2 times more invasive than control cells (D); y-axis is fold increase in invasion over control cells. RWPE-1-FUGWBW cells migrated more strongly toward SEMA3C (1 μM) than to PBS (E); y-axis is fold increase in migration over PBS. Data represent mean, ± SD; * $p < 0.05$ compared to control.

3.3.4 SEMA3C promotes cell dissemination *in vivo*

To determine if the invasiveness we observed in *in vitro* studies translated to more aggressive tumour dynamics *in vivo*, we compared the metastatic potential of RWPE-2 cells overexpressing SEMA3C to mock transduced cells. To overcome the inherently low tumour-

initiating capabilities of RWPE-1 cells, we utilized RWPE-2 cells for these studies which were derived from the RWPE-1 and are transformed by virtue of infection by the Kirsten murine sarcoma virus and Ki-Ras (208). RWPE-2 cells are known to form tumours *in vivo* whereas RWPE-1 cells do not. When SEMA3C was overexpressed in RWPE-2, levels of phospho-Akt, phospho-EGFR, and total EGFR increased whereas levels of phospho-MAPK did not (Figure 3.4A). These findings closely parallel those seen in RWPE-1 (Figure 3.1B) supporting the authenticity of these results. In cell viability assays, RWPE-2-SEMA3C displayed the most aggressive growth kinetics followed by RWPE-2-FUGWBW and finally RWPE-1-FUGWBW and RWPE-1-SEMA3C which grew at approximately the same rate (Figure 3.4B). Given that SEMA3C overexpression promoted cell growth only on the RWPE-2 background and not in the RWPE-1 background combined with the fact that the Ki-Ras oncogene is a feature unique to the RWPE-2 cells, this suggests that SEMA3C may cooperate with Ras during oncogenesis.

For *in vivo* studies SEMA3C overexpressing and control RWPE-2 cells were engineered to also express luciferase. Cells were introduced by ultrasound-guided intracardiac injection of NOD scid gamma mice and monitored for tumour formation by *in vivo* imaging system (IVIS). Seven weeks after injection, three of four mice injected with RWPE-2-SEMA3C cells displayed tumours in the head and groin region by IVIS while zero of four mice injected with RWPE-2-FUGWBW formed tumours (Figure 3.4C) nor did tumours form in mice xenografted with RWPE-1 stable cells (Appendix F). These results would suggest that SEMA3C promotes cell dissemination *in vivo* matching our *in vitro* findings.

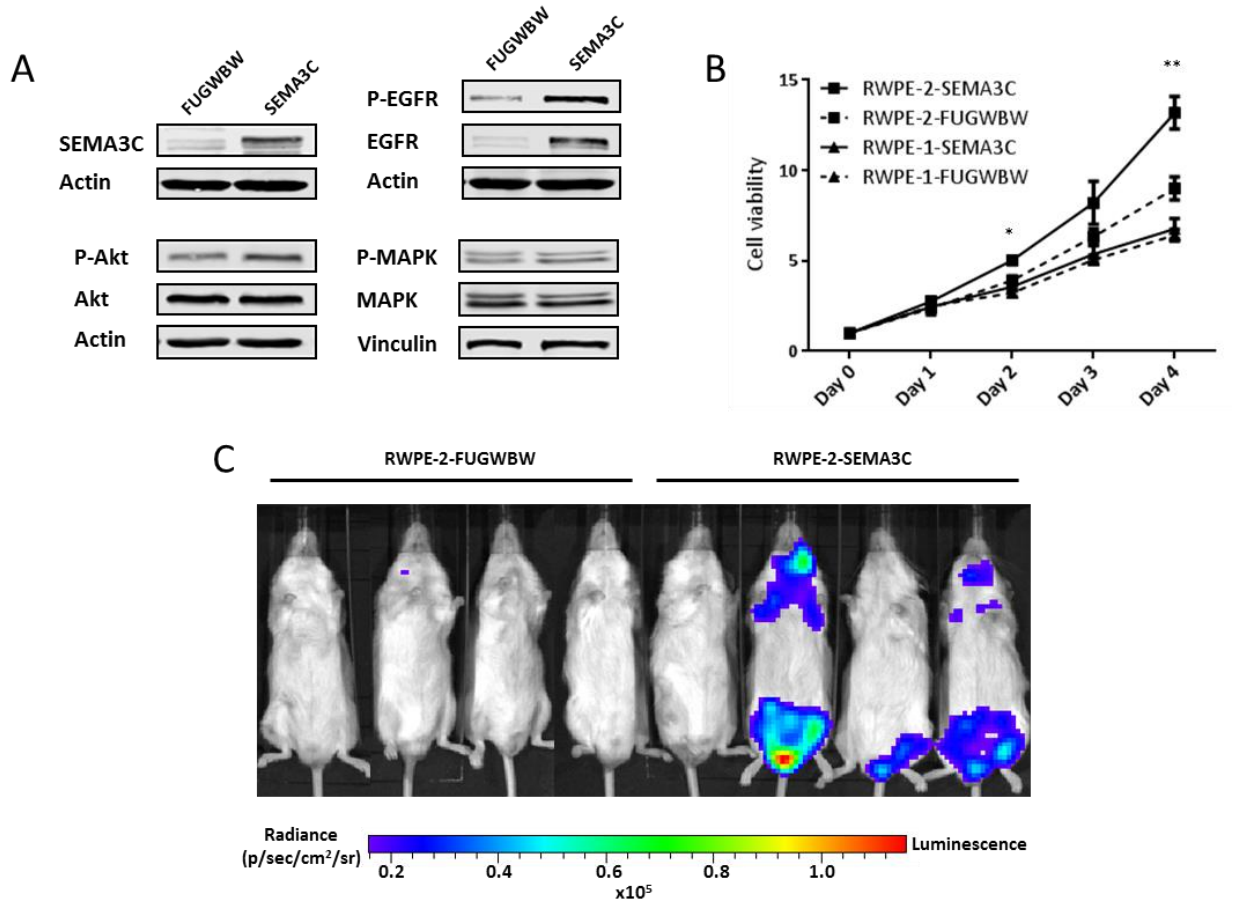

Figure 3.4. SEMA3C promotes cell dissemination *in vivo*.
RWPE-2 cells were made to stably overexpress SEMA3C and firefly luciferase by lentiviral transduction to generate RWPE-2-FUGWBW and RWPE-2-SEMA3C. SEMA3C overexpression and phosphorylated and total levels of Akt, EGFR, and MAPK were examined by Western blot analysis (A). Cell viability of RWPE-1-FUGWBW, RWPE-1-SEMA3C, RWPE-2-FUGWBW, and RWPE-2-SEMA3C was compared (B); data represent mean viability over day 1 ± SD; * $p < 0.05$, ** $p < 0.01$ where significance is measured between RWPE-2-FUGWBW and RWPE-2-SEMA3C. (C) IVIS measurements of NOD scid gamma mice xenografted with RWPE-2-FUGWBW and RWPE-2-SEMA3C by ultrasound-guided intracardiac injection; measurements were taken at 7 weeks post-inoculation.

3.3.5 Overexpression of SEMA3C promotes stem-like characteristics

It has been proposed that a population of cancer stem cells is responsible for causing tumour relapse and subsequent metastatic disease. This sub-population of cells shares many qualities with traditional stem cells and also with cells that have undergone EMT. Among other things, the cancer stem cell or tumour-initiating population would be uniquely capable of self-renewal, would retain the capability to differentiate into multiple cell types and would exhibit

both anchorage-independent growth and highly invasive and motile behaviour. Given SEMA3C's known roles in development and morphogenesis we hypothesized that SEMA3C drives PCa progression by contributing aspects of the cancer stem cell phenotype which has been operationally defined by others (73,206,209-213). CD44 is a cell-surface glycoprotein initially described for its expression on leukocytes and affinity for extracellular matrix proteins such as hyaluronic acid. CD44 can signal intracellularly through several downstream molecules and pathways that culminate in a variety of cellular responses including migration, cell proliferation, and cell survival (214). CD44 has also been extensively used as a marker for cancer stem cells (71,74,215). To test whether RWPE-1-SEMA3C cells displayed elevated CD44 on their cell surface, we used flow cytometry to examine CD44 status. FACS analysis showed that control cells were $CD44^{low}$ while RWPE-1-SEMA3C harboured both $CD44^{low}$ and $CD44^{high}$ populations (Figure 3.5A). To evaluate the plasticity of the $CD44^{high}$ and $CD44^{low}$ cell populations within the RWPE-1-SEMA3C cells, cells were sorted based on their CD44 status. $CD44^{low}$ cells were cobblestone in morphology (Figure 3.5B) and remained low in CD44 expression upon passaging (Figure 3.5C) whereas the $CD44^{high}$ cells were spindle-shaped (Figure 3.5B) and reconstituted the $CD44^{low}$ population over successive passages (Figure 3.5C). While the $CD44^{high}$ population was initially higher in expression for SEMA3C and mesenchymal markers than the $CD44^{low}$ population, the expression of these genes became similar to the $CD44^{low}$ population over time (Figure 3.5D). The two CD44 populations that arose from the $CD44^{high}$-sorted cells were again sorted on CD44 status and similar results were obtained (Appendix G). Sphere-formation *in vitro* is a partial measure of stemness. Sphere-formation takes advantage of the fact that only specialized cell types such as stem cells and cancer stem cells can grow under anchorage-independent conditions; the ability of a gene to drive anchorage-independent growth is

considered to be one of the defining characteristics of an oncogene. In the sphere-forming assays, we observed that RWPE-1-SEMA3C cells displayed superior sphere formation than the control cells with respect to sphere size (Figure 3.5E) and number of constituent cells (Figure 3.5F). The RWPE-1-SEMA3C cells formed large sphere aggregates whereas RWPE-1-FUGWBW cells formed smaller spheres which remained as separate entities that did not amalgamate (Figure 3.5E). This observation might indicate differences in expression of cell-surface proteins between the two recombinant lines which resulted in differing abilities to aggregate. To more accurately measure the cell growth under the anchorage-independent conditions, cells were dissociated and counted by hemocytometer (Figure 3.5F). Enrichment for cells with strong sphere forming abilities was particularly evident when spheres were dissociated to single cells and serially passaged; this was evidenced by retained robust sphere-forming abilities by RWPE-1-SEMA3C and less so by RWPE-1-FUGWBW after three weeks (equalling three serial passages) of culturing under anchorage-independent conditions (Appendix H). Also, parental RWPE-1 cells which were grown as spheres exhibit 2.4 times higher SEMA3C expression compared to their adherent counterparts (Appendix I). The demonstration that SEMA3C promotes elevated CD44 expression on the cell surface, cell potency, and sphere-formation collectively suggests that SEMA3C promotes the formation of a stem-like population of cells.

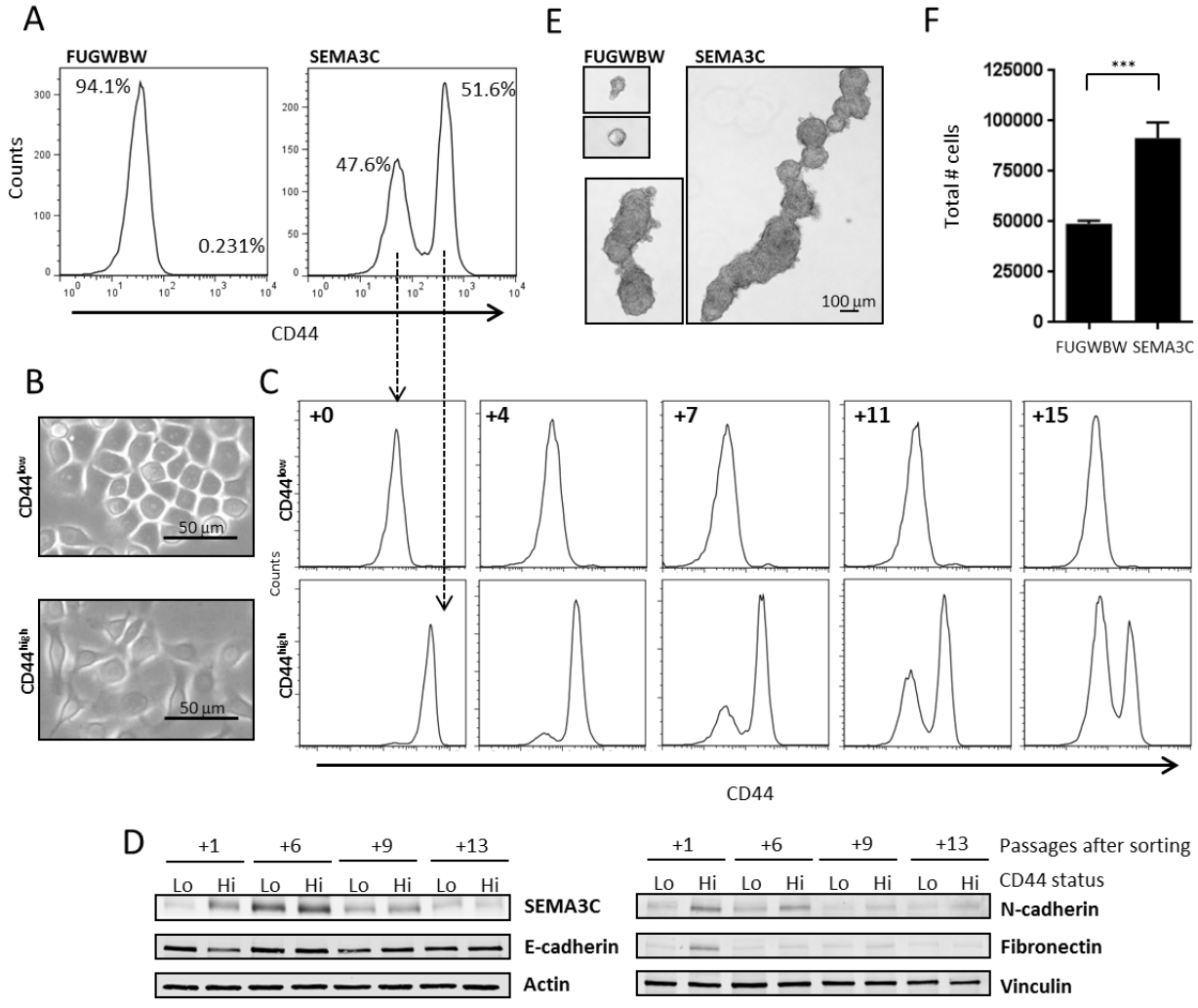

Figure 3.5. Overexpression of SEMA3C promotes stem-like characteristics.
RWPE-1-FUGWBW cells expressed detectable levels of the PCa stem cell marker CD44 as shown by flow cytometry. However, RWPE-1-SEMA3C contained two distinguishable CD44 populations, a $CD44^{low}$ population and a $CD44^{high}$ population (A). The two CD44 cell populations within the RWPE-1-SEMA3C cell population were sorted on CD44 status; $CD44^{low}$ cells were cobblestone in morphology (B) and remained CD44-low (C) whereas $CD44^{high}$ cells were spindle-shaped (B) and reconstituted the $CD44^{low}$ population (C). Bar = 50 µm. Numbers in the top left corner of FACS plots refer to the number of passages following cell sorting. Levels of SEMA3C, E-cadherin, N-cadherin, and fibronectin in $CD44^{low}$ and $CD44^{high}$ cells were examined by Western blot over successive passages following sorting (D). In sphere-forming assays, while RWPE-1-FUGWBW cells were capable of forming modestly sized spheres which existed as solitary or aggregates of three or fewer spheres, RWPE-1-SEMA3C cells formed larger spheres which likely represented the coalescence of many individual cells (E). Bar = 100 µm. To quantitate sphere-forming abilities, spheres were dissociated and constituent cells were counted by hemocytometer. RWPE-1-SEMA3C cells formed spheres roughly two times more efficiently than control cells when evaluated in this way (F); results are representative of three independent experiments. Data represent mean, ± SD; ***$p < 0.001$ compared to RWPE-1-FUGWBW control.

3.3.6 Co-expression of EMT and stem markers on SEMA3C-overexpressing cells

Although traditionally considered separate processes, accumulating evidence links the processes of EMT and stemness (73,206,209-213).. We were curious to know if the correlation between EMT and stem-like characteristics also existed in our system and therefore sought to determine whether RWPE-1-SEMA3C cells concurrently expressed EMT and stem markers. When we co-stained for CD44 and various EMT markers, we observed that cells inversely expressed CD44 and E-cadherin and co-expressed CD44 and N-cadherin and also CD44 and vimentin which was shown by flow cytometry (Figure 3.6A). In the FACS plots of the RWPE-1-SEMA3C cells, two distinct populations can be observed; furthermore, those cells that were $CD44^{high}$ (as indicated on the y-axis) were E-$cadherin^{low}$ (as indicated on the x-axis) and those that were $CD44^{low}$ were E-$cadherin^{high}$. Consistent with this were the findings that the $CD44^{low}$ cells were N-$cadherin^{low}$ and $vimentin^{low}$ while the $CD44^{high}$ cells were N-$cadherin^{high}$ and $vimentin^{high}$. Findings from the FACS co-expression studies were confirmed by immunofluorescence which showed a similar inverse staining relationship between CD44 and E-cadherin and a positive relationship between CD44 and N-cadherin and between CD44 and vimentin within the RWPE-1-SEMA3C population (Figure 3.6B). These findings support the notion that a common pool of cells possess both stem and EMT characteristics and are in agreement with findings by others.

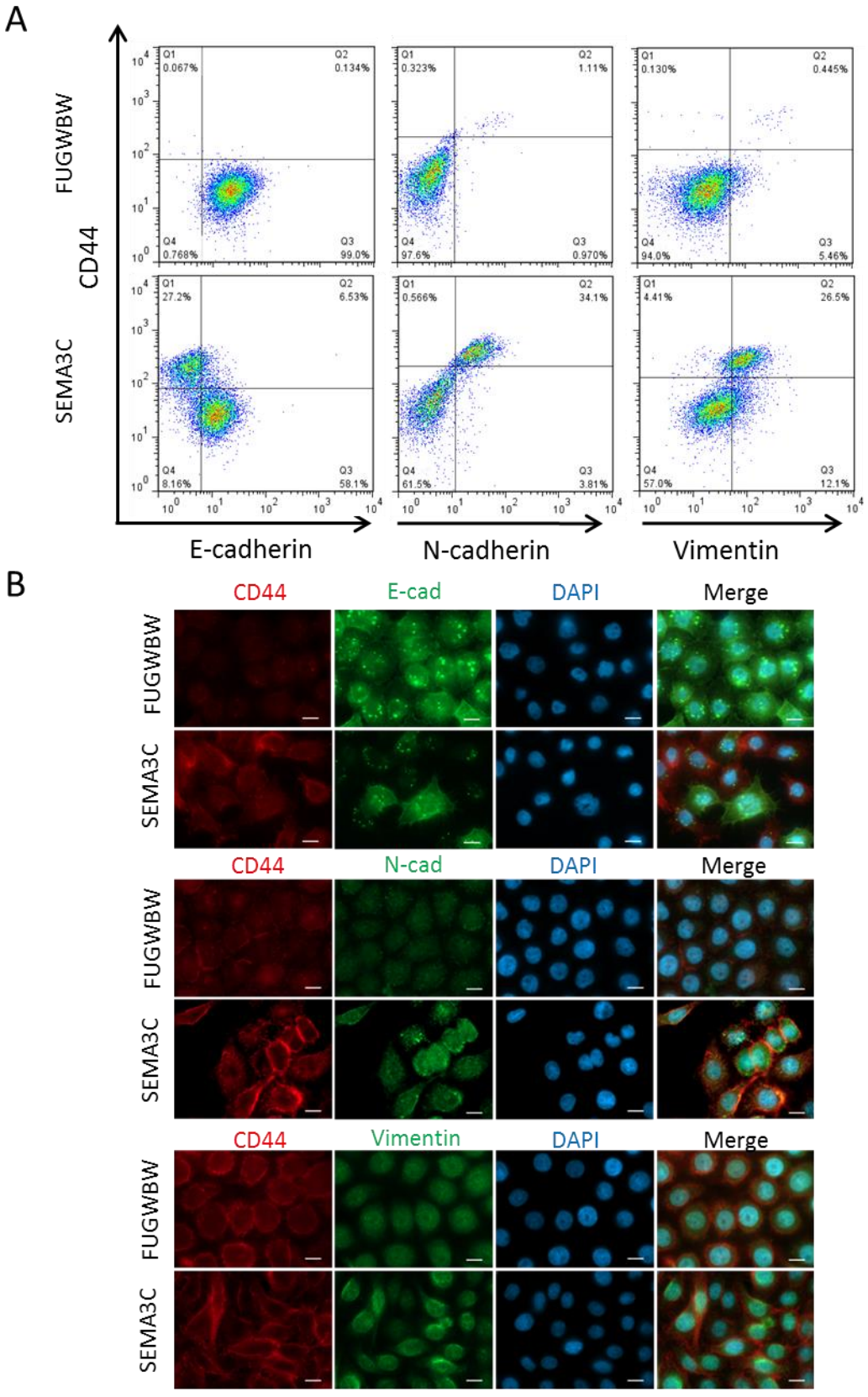
A
FUGWBW
SEMA3C
CD44
E-cadherin
N-cadherin
Vimentin
Q1 0.067%
Q2 0.134%
Q4 0.768%
Q3 99.0%
Q1 0.323%
Q2 1.11%
Q4 97.6%
Q3 0.970%
Q1 0.130%
Q2 0.445%
Q4 94.0%
Q3 5.46%
Q1 27.2%
Q2 6.53%
Q4 8.16%
Q3 58.1%
Q1 0.566%
Q2 34.1%
Q4 61.5%
Q3 3.81%
Q1 4.41%
Q2 26.5%
Q4 57.0%
Q3 12.1%
B
CD44
E-cad
DAPI
Merge
N-cad
Vimentin

Figure 3.6. Co-expression of EMT and stem markers on SEMA3C-overexpressing cells.
Co-expression of E-cadherin and CD44 was determined by co-staining for E-cadherin and CD44 followed by flow cytometry. Within the RWPE-1-SEMA3C cell population, high CD44 expression was associated with low E-cadherin, high N-cadherin, and high vimentin expression while low CD44 expression was associated with high E-cadherin, low N-cadherin, and low vimentin expression (A). Co-expression of mesenchymal and stem markers was confirmed by immunofluorescence microscopy where an inverse staining relationship existed between CD44 and E-cadherin and a positive staining relationship existed between CD44 and N-cadherin and between CD44 and vimentin (B). Bar = 10 μm.

3.3.7 SEMA3C pathway analysis

Given that SEMA3C was shown to drive EMT and numerous aspects of stem cells as functionally defined by others, we next sought to uncover which of the classical stem pathways SEMA3C was eliciting stem-like qualities through. To do this we compared the global gene expression profile of the RWPE-1-FUGWBW to that of RWPE-1-SEMA3C cells using an expression array approach. To reveal cell functions that are potentially implicated in SEMA3C activity, significantly ($p < 0.05$) and differentially (greater than two-fold increase or decrease) expressed genes between the two groups were processed using gene ontology (GO) analysis by DAVID 6.7 software. These analyses identified many cellular functions previously associated with SEMA3C activity (Figure 3.7A) including those related to development and cell motility, adhesion, and proliferation. The top twenty up- and downregulated genes in the SEMA3C-overexpressing cells compared to control cells are presented (Figure 3.7B). Within these top-ranked genes, of particular interest due to their close ties to various aspect of cancer were the genes WNT5A, CXCL8, FLI1, ZEB1, and LEF1. In order to narrow our search to genes giving rise to the stem phenotype, we selected 39 genes representing the Hedgehog, Notch, Wnt, and TGFβ pathways whose expression are commonly associated with cancer and examined their microarray expression data (Figure 3.7C). Of these 39 genes, six were significantly and differentially expressed between the two groups; the fold-change in expression of WNT5A, TGFB2, SMO, DLL1, GLI1, and NOTCH3 in SEMA3C-overexpresing cells compared to that of control cells was 19, 7.3, 4.4, 3.5, 2.9, and -2.8, respectively. Differential expression of the

remaining genes did not meet statistical significance or was less than two fold in change. The expression levels of these 39 genes were then confirmed by qPCR (Figure 3.7D). Upregulation of WNT5A, TGFB2, and DLL1 and downregulation of NOTCH3 in RWPE-1-SEMA3C cells was also observed in qPCR; 3.1, 12, 5.2, and -3.4 fold-change in WNT5A, TGFB2, DLL1, and NOTCH3 expression, respectively, in RWPE-1-SEMA3C compared to control. The consistency in the microarray data and qPCR data gave us confidence that these four genes were indeed differentially expressed between the two recombinant lines. The upregulation of GLI1 was not detected in qPCR. Discrepant results arose when examining whether SMO was upregulated in qPCR. SMO levels were found to be both increased several thousand fold and decreased fifty fold across different biological replicates examined. For this reason, the SMO data-point is left unpopulated. The inconsistency in qPCR results warrants a closer examination of the SMO target in these confirmational qPCRs using different qPCR primers or confirmation at the protein level by Western blot. We next looked for co-expression of SEMA3C and those genes that were found to be significantly and differentially expressed in microarray (WNT5A, TGFB2, SMO, DLL1, GLI1, and NOTCH3) using publically available TCGA datasets of clinical gene expression data of PCa (PRAD). Co-expression of SEMA3C and these stem markers would lend credence to our hypothesis that SEMA3C was driving stem-like characteristics through these stem genes. UCSC Xena analyses (https://xena.ucsc.edu/) showed that SEMA3C and TGFB2 are co-expressed in patients of PCa whereas no correlation was seen between SEMA3C and any of WNT5A, SMO, DLL1, GLI1, and NOTCH3 (Figure 3.7E). The co-expression data was confirmed using cBioPortal analyses (216,217) of the prostate adenocarcinoma TCGA provisional dataset (Figure 3.7F).

A

GO TERM	Gene Numbers	-Log10 (p-value)
cell adhesion	70	11.73659
response to wounding	55	9.768752
regulation of cell proliferation	53	3.683169
cell motion	46	7.250121
ectoderm development	43	18.69621
neuron differentiation	43	6.943087
skeletal system development	36	7.288078
cellular component morphogenesis	33	3.922415
response to endogenous stimulus	32	3.421976

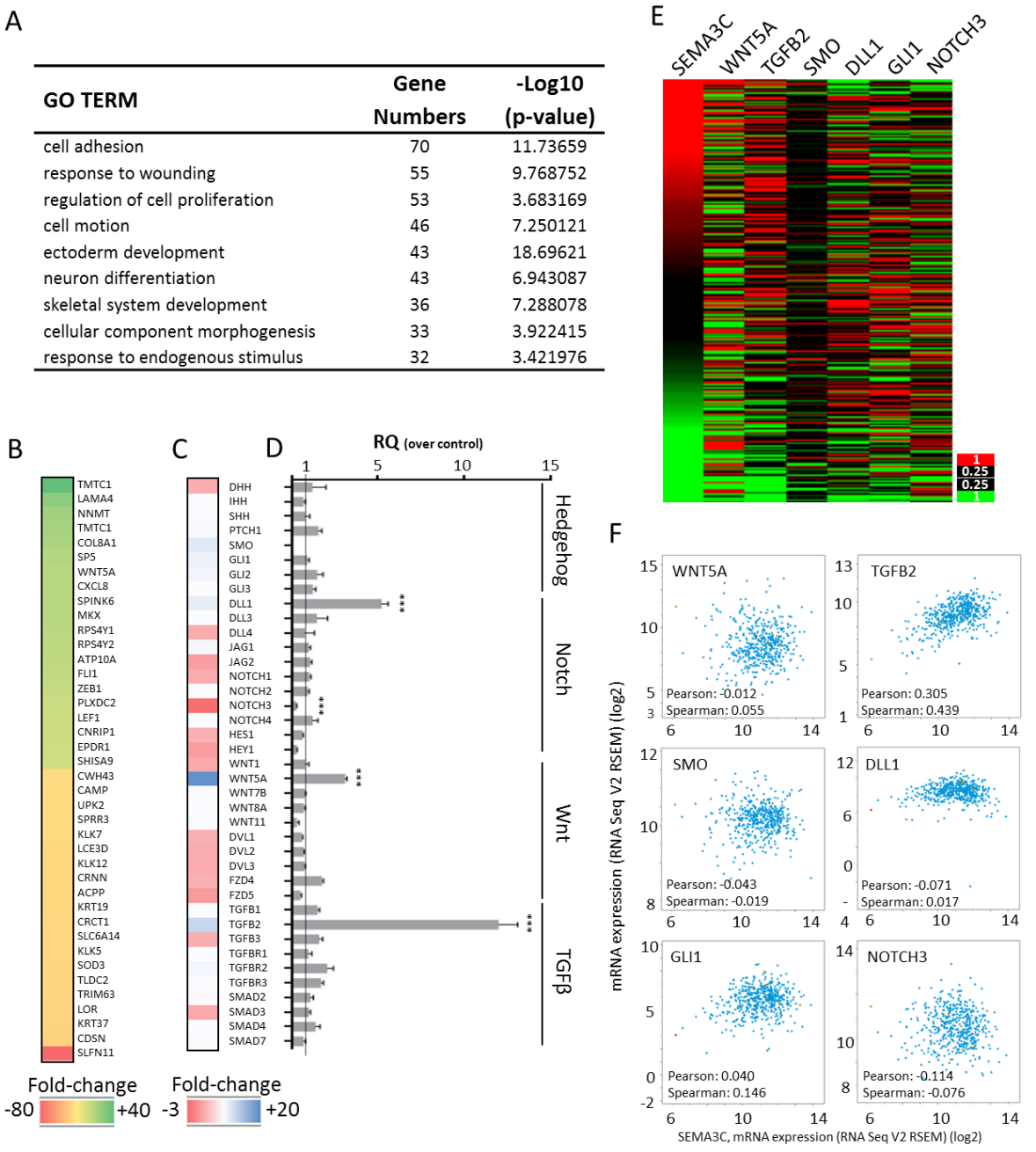

Figure 3.7. SEMA3C pathway analysis.

An Agilent One-Color Microarray-Based Gene Expression assay was performed to examine global gene expression in RWPE-1-FUGWBW and RWPE-1-SEMA3C cells. Gene ontology analyses by DAVID online software were performed on significantly ($p < 0.05$) and differentially (greater than two-fold increase or decrease) expressed genes between the two groups to identify cellular processes most affected by SEMA3C overexpression (A). The number of genes from the microarray that were involved in the gene ontology analyses are shown. The top twenty up- and downregulated genes identified by microarray in RWPE-1-SEMA3C compared to control are listed (B); fold-changes are indicated by colour. The microarray expression of thirty-nine stem-associated genes was examined between the two groups (C); fold-changes (RWPE-1-SEMA3C over control) are indicated by colour. The fold-

change in expression (RWPE-1-SEMA3C over control) of these thirty-nine genes was confirmed by qPCR (D); data represent mean, ± SD; *** $p < 0.001$ comparing RWPE-1-SEMA3C to RWPE-1-FUGWBW cells. UCSC Xena visualization software was used to examine co-expression of SEMA3C and WNT5A, TGFB2, SMO, DLL1, GLI1, and NOTCH3 in the TCGA PRAD PCa dataset (E). Fold-changes are shown in colour, log2 (normalized_count+1) mean is subtracted per column across 550 samples. cBioPortal plots of the TCGA, provisional dataset show the degree of correlation in expression of SEMA3C and each of WNT5A, TGFB2, SMO, DLL1, GLI1, and NOTCH3 (F). Pearson and Spearman correlations are indicated.

3.4 Discussion

Work of Chapter 3 demonstrates that ectopic expression of SEMA3C leads to the development of EMT and stem-like characteristics in the RWPE-1 cell line. In showing this we illuminate potential roles of SEMA3C in PCa carcinogenesis. This knowledge is beneficial since EMT and stemness are factors theorized to fuel elements of disease progression that include metastasis and tumour relapse (59,218). As such, future therapies that selectively target the cell compartment responsible for EMT and stemness would theoretically eliminate the cells responsible for relapse with higher efficiency than current treatment modalities. Work in Chapter 3 shows that overexpression of SEMA3C triggered changes in cell morphology, expression of EMT and stem markers, and acquisition of invasive and stem-like phenotypes. The notion that class 3 semaphorins can drive EMT is not altogether novel (158,219) nor are the links between SEMA3C and the promotion of certain aspects of stem cells (154,220). We predict that amplification of SEMA3C occurs in later stages of PCa, possibly instigated through aberrant AR signalling and subsequently, that vestiges of SEMA3C's embryonic programs are unwittingly unleashed to further drive PCa progression. Due to its roles in cell motility and cytoskeletal rearrangement it is conceivable that SEMA3C could contribute these aspects to the EMT process.

The PCa stem cell is described to be a CD133, α2β1, and CD44 positive cell (71); future studies will therefore need to examine whether SEMA3C is capable of also upregulating expression of CD133 and α2β1. We acknowledge that the upregulation of CD44 alone does not

signify a population of cancer stem cells; instead CD44 in our experiments served as a surrogate for a population enriched for stem-like qualities. Aldehyde dehydrogenase (ALDH1) is an enzyme that is abundant in progenitor populations and which correlates with poor prognosis in breast cancer and is thought to contribute to treatment resistance (221). Exploration of whether SEMA3C overexpression corresponds to increased ALDH1 levels or ALDH1 activity could also be undertaken.

Ectopic SEMA3C overexpression was also concomitant with elevated TGFβ2 expression suggesting that TGFβ2 may mediate SEMA3C-induced stem-like characteristics. Further studies will need to be undertaken to confirm the role of TGFβ2 in this capacity. For instance, the dependency of SEMA3C-induced stem-like characteristics on TGFβ2 should be assessed. It also remains to be seen whether other components of the TGFβ2 signalling cascade become activated upon SEMA3C stimulation. For example, whether TGFβ receptors 1 & 2 and the signal transducing Smads 2 & 3 become phosphorylated. Correlations between SEMA3C treatment and phosphorylation of these substrates could be verified by Western blot analyses or mining of TCGA datasets using the reverse phase protein array application. Alternatively, one could examine whether SEMA3C triggers translocation of Smads 2, 3, and 4 into the nucleus or whether transcriptional targets of the TGFβ2 pathway are upregulated. The former could be examined using immunofluorescence microscopy or cell fractionation experiments while the latter could be tested using qPCR or Western blot analyses.

Still another topic not yet addressed includes the signal transduction mediating the observations made in Chapter 3 but presumably, given existing knowledge of cascades downstream of the semaphorins, this involves the NRPs, PLXNs, and receptor tyrosine kinases at the cell surface followed by intracellular activation of the MAPK and Akt pathways or G protein

signalling. It is known that the PLXNs can activate receptor tyrosine kinases such as c-Met, ErbB2, and VEGFR (133,134,139,224,225) (also unpublished data) and that semaphorins can activate Akt, MAPK, Src, and PI3K signalling pathways (226,227).

The apparent conversion of the $CD44^{high}$ population into a $CD44^{low}$ population with respect to their SEMA3C and EMT marker expression as suggested by the Western blots of Figure 3.5D is perplexing. These observations support a situation where the $CD44^{low}$ population is necessary for maintaining the $CD44^{high}$ population and when the two populations were separated by cell sorting, this disrupted an equilibrium leading to a unidirectional shift from a $CD44^{high}$ profile to a $CD44^{low}$ profile. It was not addressed in this study whether the CD44 expression of the $CD44^{high}$ population would eventually become identical to that of the $CD44^{low}$ population if given enough passages (beyond +15 passages). If the $CD44^{high}$ population was eventually completely lost to the $CD44^{low}$ population with respect to their CD44 expression, this would be consistent with the findings of the Western blots of the two populations. However, this would raise the question of if and how the $CD44^{low}$ population supports the maintenance of the $CD44^{high}$ population. Support may come in the form of juxtacrine or paracrine-acting factors produced by the $CD44^{low}$ population that help sustain the $CD44^{high}$ population in their growth or survival. In the event that the $CD44^{high}$ population was *not* completely lost to the $CD44^{low}$ population then the question becomes why do the two population's SEMA3C and EMT marker profiles seemingly coalesce as shown by Western blot. Alternatively, while it may appear that the $CD44^{high}$ population is shifting to a $CD44^{low}$ population, perhaps this data is only capturing the descending half of an oscillating system which has not yet reached equilibrium. Perhaps, if given enough time, SEMA3C and the EMT marker expression would once again ascend in the $CD44^{high}$ population relative to the $CD44^{low}$ population. Nevertheless, it is well-recognized that

the transition from epithelial phenotype to mesenchymal phenotype is not unidirectional (222,223). Rather, the transition is reversible. Another unanswered question surrounds the expression levels of SEMA3C in the $CD44^{low}$ and $CD44^{high}$ populations. The stable RWPE-1-SEMA3C cell line was a pooled population of lentiviral-transduced cells and therefore heterogeneity in SEMA3C expression is not surprising in the early (+1) passages of CD44-sorted RWPE-1-SEMA3C cells. However, the decrease in SEMA3C overexpression by the $CD44^{high}$ population relative to the $CD44^{low}$ population over time is not expected since cells were continually maintained under Blasticidin S selection (before and after cell sorting). Fluctuations in the expression of SEMA3C may reflect technical limitations to our approach (such as the ejection of SEMA3C overexpression DNA sequences from the genome of the cells) or may reflect a prevailing and concerted effort by the cell to restore homeostasis by decreasing SEMA3C and EMT marker expression through gene silencing. These observations may also be due to stochastic events where unintended amplification or suppression of other genes involved in the overexpression of SEMA3C had the net effect of loss of SEMA3C overexpression. For example, perhaps accrual of a series of genomic or transcriptomic events due to sustained overexpression of SEMA3C led to the loss in expression of transcription factors necessary for transcription from the Ubiquitin C promoter through which SEMA3C was being constitutively expressed. Yet another explanation for loss of SEMA3C overexpression is the methylation of the Ubiquitin C promoter or other epigenetic events which render the local DNA environment non-conducive to SEMA3C overexpression.

Increased levels of total EGFR in response to SEMA3C overexpression and the absence of marked induction of MAPK and Akt signalling was repeatedly observed in these studies. These phenomena warrant further investigation. SEMA3C-induced upregulation of EGFR could

have profound implications in cancer development and could underpin our findings and those of studies involving semaphorins elsewhere. Additional signalling studies involving receptor tyrosine kinases like ErbB2 and c-Met and other substrates such as Src, Shc, and PI3K will shed light on mechanistic details surrounding SEMA3C. Alternatively, as PLXNs are shown to signal through the Rho family of GTPases, which are involved in cytoskeletal rearrangement and cell motility, SEMA3C may initiate cell motility and invasion through these proteins. In any event, we predict these studies will show convergence of SEMA3C signalling networks with other well-characterized stem and EMT pathways.

CHAPTER 4. Discussion

4.1 Limitations of this work

4.1.1 Lentiviral transduction

As lentiviral transduction involves random integration of viral DNA into the genome, it is possible that catastrophic integration events that, for example, inactivated tumour suppressors such as *TP53* or *RB1* or activated oncogenes are what led to disruption of cell homeostasis and transformation. To rule out these chance integration events as the cause for our results, lentiviral transduction of RWPE-1 with SEMA3C-overexpression and control vectors was repeated a second and third time with similar results being observed in one of those repetitions (Appendix J); an upregulation of CD44 with inverse E-cadherin expression served as a surrogate experiment for the broad set of studies carried out by the first biological replicate. It was noted, however, that the same replicate that exhibited greater overexpression of SEMA3C also successfully recapitulated the CD44 upregulation/E-cadherin downregulation of the first biological replicate whereas the replicate with lesser overexpression failed to do so. This would suggest that a minimum threshold in overexpression of SEMA3C must be met in order to elicit these effects. In addition, the abundance of $CD44^{high}$ cells within the RWPE-1-SEMA3C cells increased over successive passages indicating the outgrowth of an initially small population. The identity of the initial population which grew out as well as how and why they expanded in this way is not known. Collectively these observations would suggest that sustained high levels of SEMA3C is required for the emergence of a population of cells with invasive qualities.

4.1.2 Exclusivity in cell lines used

Limitations to this work include the exclusivity in systems utilized to test the stated hypotheses (that AR regulates SEMA3C and that SEMA3C drives EMT and stem-like

characteristics). Experiments testing AR regulation of SEMA3C used only the LNCaP model. Although LNCaP express conveniently low levels of SEMA3C and are the gold-standard for examining AR response in, utilizing a single system for a study carries inherent risk. For instance, the complement of transcriptional coregulators present in the cell, which can vary from cell type to cell type, affects which genes are expressed and their degree of expression. Demonstration of AR regulation of SEMA3C in other cell lines would provide confidence that AR regulation of SEMA3C is not exclusive to the coregulator profile of LNCaP cells. Other AR-positive cell lines include the LNCaP-derived C4-2 cells, 22Rv1, and VCaP cells. C4-2 and 22Rv1 cells express high levels of SEMA3C even under serum-starved conditions (Appendix K) and are therefore not suitable for use in studies examining the induction of SEMA3C. In addition, 22Rv1 contain AR variants which are constitutively active and therefore cannot be relied upon to express AR-regulated genes in an androgen-dependent manner. However, in each of C4-2, 22Rv1, and VCaP cells, silencing of AR using siRNA should theoretically downregulate SEMA3C expression. However, silencing of AR in these cell lines did not lead to a decrease in SEMA3C levels (Appendix L) despite the fact that PSA mRNA levels declined by 46%, 64%, and 74% in C4-2, 22Rv1, and VCaP cells, respectively, upon AR knockdown. In fact, when AR was silenced in 22Rv1 and VCaP cells, SEMA3C mRNA levels increased by 1.5 and 1.3 fold, respectively. Knockdown of AR in C4-2 cells seemed not to affect SEMA3C levels. Knockdown of AR was confirmed by qPCR where levels of AR mRNA decreased by 74%, 47%, and 69% in C4-2, 22Rv1, and VCaP cells, respectively. This would indicate that other factors are contributing to SEMA3C expression in these cells and that those mechanisms either do not involve the AR or somehow compensate for loss of AR when AR is knocked down. A similar drawback exists for the work showing that SEMA3C drives EMT and stem-like phenotypes. The

vast majority of these studies took place in the RWPE-1 cell line and although the RWPE-1 cells represent a suitable model for addressing the aims that we set out to study, it cannot be ruled out that the effects we observed are an artifact of the cell type utilized. To address this, PCa cell lines expressing high levels of SEMA3C, namely 22Rv1 and DU 145 (Appendix K), were silenced for SEMA3C to determine if SEMA3C loss-of-function studies decreased any of the same effects that SEMA3C gain-of-function studies increased. In these experiments, knockdown of SEMA3C in 22Rv1 and DU 145 did not cause a decrease in migration or invasion (Appendix M). In the case of DU 145, knockdown of SEMA3C in fact increased migration and invasion by 1.4 and 1.5 fold, respectively. Knockdown of SEMA3C in these studies was confirmed by qPCR where levels decreased by 77% upon treatment with SEMA3C siRNA in both 22Rv1 and DU 145. These findings would imply that the invasive phenotypes exhibited by these cell lines are not exclusively due to SEMA3C or that SEMA3C inhibition alone is not sufficient to reverse the invasive phenotype. Alternatively, it is possible that a knockdown of 77% was insufficient to inhibit the migration and invasion in these cells. A higher efficiency in knockdown or a different SEMA3C siRNA should be explored.

4.1.3 Context-specific observations

Cell line-specific phenomena are inherent to *in vitro*-based experimentation. In general, results that are consistent across multiple cell lines and tissue types can help discriminate principles that are context-specific from those that can be more universally-applied. Proteins, including the semaphorins, are frequently found to have functions that are highly dependent on the environmental context. For example, SEMA3E simultaneously inhibits growth of primary tumours but exacerbates metastasis of primary cancers to the lungs (133). SEMA3E has been described to promote invasiveness in ovarian and lung cancer (219,224) while inhibiting these

same activities in breast and PCa (159,225). Discordant function across different cell or tissue types can be explained by the fact that cells of different tissues are decorated with different proteins which in turn influence micro-environmental context and function. Although we have observed that SEMA3C induces upregulation of CD44 not only in RWPE-1 cells but also in additional cell lines including BPH-1, MCF 10A, and MDA-MB-468 through similar overexpression studies (Appendix N), recent reports have shown that SEMA3C in fact *inhibits,* rather than promotes, cancer by preventing angiogenesis, lymphangiogenesis, and metastasis (145,146). Some of the studies presented by other research groups were conducted in breast cancer cells; interestingly, we have seen decreased cell migration in breast and breast cancer cell lines overexpressing SEMA3C seemingly consistent with the findings of Mumblat *et al* (Appendix O). Discrepant SEMA3C function between their findings and the RWPE-1 results presented here, may relate to the particular set of receptors, proteolytic enzymes, or modified forms of SEMA3C present in the two systems. The biological activities of semaphorins are refined by proteolytic cleavage and post-translational modifications and are likely further fine-tuned by the repertoire of NRP and PLXN receptors present on the recipient cell. Thus inconsistent or opposing semaphorin actions across different cell types may simply reflect the different proteolytic enzymes or glycosyltransferases that are expressed across the cell types examined. Similarly, different combinations or quantities of PLXN and NRP receptors on different cell types might influence the impact that the semaphorin has on that cell due to the initiation of different signalling cascades. Studies in more diverse cell types will be useful in distinguishing between context-specific versus more widely applicable principles of semaphorin biology. Studies correlating activity of the semaphorin and the receptors and proteolytic enzymes present on and in those cells will be of particular use. From such studies, we will be better

situated to draw conclusions on the functions associated with the different cleaved or glycosylated forms of semaphorins as well as the potential impact that semaphorin-modifying factors have on semaphorin function. Further, these studies will bring to light whether one semaphorin can elicit different functions through different NRP and PLXN members.

4.2 General discussion and conclusion

In summary, our findings identify SEMA3C as a novel target of the androgen receptor and further show that GATA2 is indispensable to AR-mediated expression of SEMA3C. AR propels castration-resistant forms of PCa, therefore, identification of AR-regulated genes may unveil therapeutic targets for castrate and enzalutamide-resistant stages of the disease. We have also shown that ectopic expression of SEMA3C triggers the upregulation of stem and EMT markers in RWPE-1 cells and that this was accompanied by an increase in sphere forming ability, cell plasticity, motility, and invasiveness possibly mediated by the TGFβ2 pathway. Novelty in this work lies in the illumination of numerous previously unappreciated facets of SEMA3C signalling, namely the connection between AR and SEMA3C, and the further association between SEMA3C, EMT, and stemness.

The scarcity in effective treatments for CRPC speaks to our limited understanding of the mechanisms at play in the establishment of castrate-resistant PCa. The broader impact of the work presented here relates to identification of potential mechanisms driving PCa progression and CRPC. Given that SEMA3C was shown to be regulated by AR, one might speculate that abnormal AR signalling, which is characteristic of more advanced stages of PCa including fatal and incurable CRPC, could give rise to elevated SEMA3C levels. Augmented SEMA3C levels might then confer EMT and stem-like characteristics to initiate the metastatic stage of the disease. It remains to be seen whether or not this is the case but the evidence presented here leads

us to propose this scenario. To fit this model, SEMA3C levels would rise at the onset of CRPC and AR amplification but prior to metastasis. Castration resistance, AR amplification, and metastasis tend to occur in rapid succession in PCa, therefore identifying changes in SEMA3C expression with respect to these events will be important in confirming our model. Nevertheless the findings detailed in this report satisfy objectives stated in the **Hypothesis, objectives, and specific aims** section and are consistent with the hypothesis that SEMA3C, AR, EMT, and stemness collaborate at some level in PCa progression.

Identification of SEMA3C as a direct transcriptional target of AR makes SEMA3C one of only a limited number of genes shown to be co-regulated by AR and GATA2 and characterized to the extent that SEMA3C has been characterized (taking into account the body of work done on SEMA3C not just by us but also others). Although numerous targets of AR have been identified in large-scale studies, accompanying work to validate this regulation and to verify associated function does not always occur. The transcription factors documented to regulate SEMA3C include SOX4, GATA6, and Twist1 but these reports are not in the context of PCa and do not involve nuclear receptor regulation which is of particular importance in PCa.

EMT and stemness are two processes that drive the development of many solid cancers. Growth factors and receptor tyrosine kinase signalling are generally thought to be the main initiators of EMT which in turn promotes stemness. To describe SEMA3C as an instigator of EMT adds to a relatively limited list of factors that contribute to the EMT phenotype which are alternative to receptor tyrosine kinase signalling and the classical stem pathways. Despite being heavily involved in stem and developmental pathways, semaphorins are only lightly discussed in having pro-EMT or pro-stem characteristics (154,158,219,220). SEMA3C has been shown to confer a variety of malignant phenotypes but these studies focus mainly on SEMA3C's ability to

drive motility, invasion, and angiogenesis (139,145,146,155,156,226). Furthermore, with the exception of one report, all are documented outside of PCa. Alternatively, PLXNs have been shown to mediate cytoskeletal reorganization during axon extension and collapse through G proteins via interaction with GAPs and GEFs (129-132). Thus, alternatively to initiating EMT, SEMA3C may activate PLXN and G protein signalling to initiate actin dynamics and motility.

The semaphorin family of proteins are implicated in virtually every Hallmark of Cancer (136) and SEMA3C specifically is frequently associated with cancer growth (144). Assuming findings presented here hold true, therapeutic targeting of SEMA3C may hold clinical utility. SEMA3C holds prognostic value in PCa and has been reported to cause metastasis to the lung, a site that PCa frequently metastasizes to. SEMA3C is an attractive target in this regard because SEMA3C's roles lie primarily in development and its functions in adults are diminished (117). SEMA3C knockout mice die shortly after birth due to cardiovascular defects but littermates that survive, likely through compensatory signalling, exhibit normal phenotype indicating non-essential roles for SEMA3C in the adult (227). Furthermore, as the biologically active form of SEMA3C is secreted, the biological fraction of SEMA3C that is accessible for targeting by pharmacological agents is high. Importantly, on the current backdrop of contemporary views that state that the AR, PI3K pathway, DNA repair pathway, cell cycle, RAF, and WNT pathway represent actionable lesions in the treatment of PCa, SEMA3C is situated particularly well for exploitation as a target for therapy. This is rationalized by SEMA3C's centrality within this network. Signalling studies by our laboratory showed that stimulation of PCa cell lines with recombinant SEMA3C led to the phosphorylation of receptor tyrosine kinases and culminated in phosphorylation of Akt and MAPK. Thus, targeting SEMA3C may inhibit growth by attenuating Akt and MAPK in at least some cases of PCa. Secondly, as has been discussed, SEMA3C is a

target of the AR pathway. Implicit to the fact that AR remains relevant even in CRPC is the fact that so too are the gene targets of AR. Thus, designing therapies that target genes downstream of the AR, presumably the mediators of disease progression, may represent an approach which would not be subject to the same shortfalls associated in targeting AR itself. In this way, inhibitors of SEMA3C may still be effective in the CRPC landscape and would seemingly be most effective if administered after the emergence of CRPC and also post-enzalutamide resistance. At the same time, given SEMA3C's stem-promoting capacities, anti-SEMA3C therapies should be deployed relatively early on during treatment to eliminate the cancer stem cell population that is responsible for relapse. Stem cells have devised mechanisms to ensure a longevity that supports a life-time worth of cell turnover, tissue repair, and morphogenesis. The theorized cancer stem cells would share these characteristics in the form of inherent resistance to therapies (77,209). Consequently, when a tumour is irradiated or treated with chemotherapies, the cancer stem cells would persist and potentially give rise to recurrent cancer and eventual metastasis. Targeting the cancer stem cell population through anti-SEMA3C or anti-cancer stem cell therapies would then theoretically mitigate the chance of relapse by diminishing the population that is responsible for persisting following primary treatments. Since fatality is associated with cancer relapse, combining treatments that target the bulk tumour population with treatments that target the smaller tumour-initiation population might be a superior strategy in cancer treatment than current methods. Methodological approaches in antagonizing SEMA3C signalling could include by means of small molecules, monoclonal antibodies, or decoy proteins. Due to their extracellular localization, targeting SEMA3C and semaphorins as a whole would be easier than targeting intracellular or nuclear proteins which, by comparison, necessitate that the drug traverse one or two membranes, respectively, before reaching their intended target.

Inhibition of SEMA3C could also be combined with other pharmacological agents for maximal tumour response. Examples of this include co-administration of AR inhibitors and SEMA3C inhibitors for potential synergy between AR blockade and antagonism of an activator of receptor tyrosine kinases and Akt. This regimen would be suitable at the onset of CRPC since SEMA3C's inhibition at this stage would address: resumed AR activity, cellular growth associated with kinase pathway activation, as well as any metastatic potential that SEMA3C may be conferring. For the same reasons, SEMA3C inhibitors may be effective in the metastatic CRPC landscape as well. Combinations between cabazitaxel or other taxanes with SEMA3C inhibitors could therefore be explored.

A tumour's SEMA3C features may also serve as a means of stratification much like the inferences made about AR, PI3K, and ETS rearrangements in PCa taxonomy. Expressional signatures and copy-number aberrations to SEMA3C could also be used as a means of PCa classification and provide information on the suitability of treatment course. SEMA3C is dysregulated in numerous cancers. TCGA analyses confirm this and show *SEMA3C* upregulation or copy number aberration in 6% of clinical samples of primary PCa. However, mutations to *SEMA3C* were not detected. This is concordant with observations that actionable driver mutations seemingly occur most frequently in genes which encode for proteins with enzymatic activity or those with direct roles in gene regulation or cell cycle.

To summarize, SEMA3C intersects several nodes of PCa signalling: the PI3K and AR signalling pathways, EMT, and stemness. This thesis commenced with a discussion surrounding the importance of proper gene regulation in the homeostasis of cells and of the organism. Results presented thereafter have shown a prime example of the functional consequences of when systems become deregulated in the cell. SEMA3C expression is elevated in advanced PCa and so

too is the AR, a regulator of SEMA3C. Following the rise in SEMA3C levels at the hands of AR or through other mechanisms, SEMA3C-related cascades may then unhinge cell stability by initiating cellular programs that have no place occurring under ordinary physiological circumstances. The prospect that SEMA3C represents a mechanism through which dysregulated AR brings about aggressive disease is a tempting one; however, confirmatory studies will need to be undertaken. In closing, the work presented here showcases several novel aspects of SEMA3C signalling and in doing also uncovers multiple previously unacknowledged and potentially important elements of PCa biology. We are hopeful that this work stimulates discussion surrounding SEMA3C's potential roles in PCa progression and inspires novel strategies that target it.

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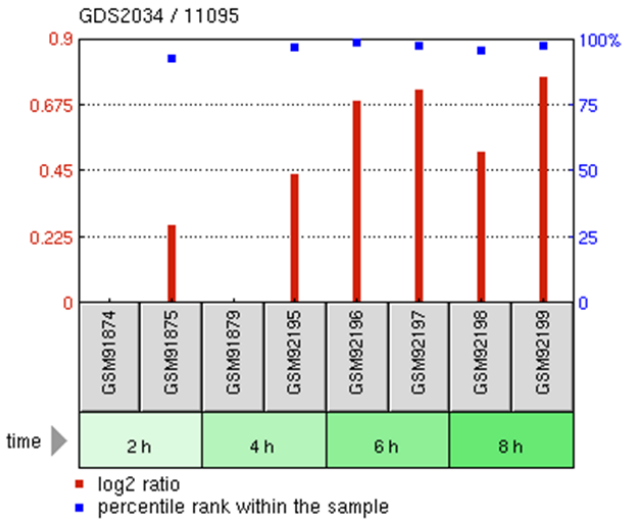
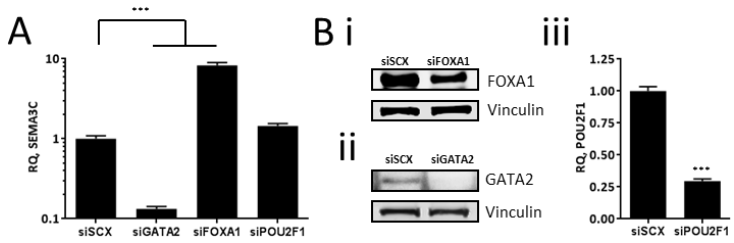
Appendices

Appendix A. Time-dependent increase in SEMA3C expression in response to R1881 in microarray



Appendix A. Time-dependent increase in SEMA3C expression in response to R1881 in microarray. Data mining of GEO accession number GDS2034 show that the levels of SEMA3C mRNA increase over time in LNCaP treated with R1881. To identify targets of the AR, Hendriksen *et al* looked at expression profiles of LNCaP treated with R1881 for up to 8 hours. Samples: GSM91874: LNCaP_2h_R1881_rep1, GSM91875: LNCaP_2h_R1881_rep2, GSM91879: LNCaP_4h_R1881_rep1, GSM92195: LNCaP_4h_R1881_rep2, GSM92196: LNCaP_6h_R1881_rep1, GSM92197: LNCaP_6h_R1881_rep2, GSM92198: LNCaP_8h_R1881_rep1, GSM92199: LNCaP_8h_R1881_rep2.

Appendix B. GATA2 positively regulates SEMA3C expression and FOXA1 negatively regulates SEMA3C expression in C4-2 cells



Appendix B. GATA2 positively regulates SEMA3C expression and FOXA1 negatively regulates SEMA3C expression in C4-2 cells. (A) C4-2 cells were separately knocked down with each of siGATA2, siFOXA1, and siPOU2F1, to confirm findings seen in LNCaP. Compared to C4-2 cells treated with scrambled siRNA (siSCX), C4-2 cells treated with siGATA2 had significantly diminished SEMA3C expression while C4-2 cells treated with siFOXA1 showed an upregulation of SEMA3C; C4-2 cells treated with siPOU2F1 showed a modest induction of SEMA3C. These results mirror those seen in LNCaP. (B) Validation of knockdown was shown by Western blot analysis or qPCR. Data represent mean, ± SD; *** $p < 0.001$.

Appendix C. Antibodies used

	Ab	Company	Catalogue Number
WESTERN BLOT	Actin	SIGMA	A2066
	Vinculin	SIGMA	V4505
	SEMA3C (N-20)	Santa Cruz Biotechnology	sc-27796
	P-ERK	Cell Signaling Technology	4370S
	ERK	Cell Signaling Technology	4696S
	P-Akt	Cell Signaling Technology	4060S
	Akt	Invitrogen	44609G
	P-EGFR	Cell Signaling Technology	3777S
	EGFR	Santa Cruz Biotechnology	sc-377229
	E-cadherin	BD Transduction Laboratories	610181
	N-cadherin	BD Transduction Laboratories	610921
	Vimentin	Cell Signaling Technology	3932S
	Fibronectin	BD Transduction Laboratories	610077
	Zeb1	Cell Signaling Technology	3396S
	Plexin B1	Santa Cruz Biotechnology	sc-28372
	Plexin D1	Santa Cruz Biotechnology	sc-67145
	Neuropilin 1	R & D Systems	AF3870
	Neuropilin 2	R & D Systems	AF2215
	anti-goat HRP	Dako	P0160
	anti-rabbit HRP	Dako	P0448
	anti-mouse HRP	Dako	P0447
	Alexa Fluor 680 anti-rabbit IgG	Invitrogen	A21109
	Alexa Fluor 680 anti-mouse IgG	Invitrogen	A21058
FLOW CYTOMETRY	PE-Cy5 Isotype Control	eBioscience	15-4031-81
	CD44 PE-Cy5	eBioscience	15-0441-82
	E-cadherin (5H9)	Santa Cruz Biotechnology	sc-52327
	N-cadherin	BD Transduction Laboratories	610921
	Vimentin	Cell Signaling Technology	3932S
	Alexa Fluor 488 anti-mouse IgG	Invitrogen	A11059
	Alexa Fluor 488 anti-rabbit IgG	Invitrogen	A11008
IMMUNOFLUORESCENCE			
	CD44 PE-Cy5	eBioscience	15-0441-82
	E-cadherin	BD Transduction Laboratories	610181
	N-cadherin	BD Transduction Laboratories	610921
	Vimentin	Cell Signaling Technology	3932S
	Alexa Fluor 488 anti-mouse IgG	Invitrogen	A11059
	Alexa Fluor 488 anti-rabbit IgG	Invitrogen	A11008

Appendix D. Primer sequences used

E-cadherin-F: 5’-GACAACAAGCCCGAATT-3’
E-cadherin-R: 5’-GGAAACTCTCTCGGTCCA-3’

N-cadherin-F: 5’-CGGGTAATCCTCCCAAATCA-3’
N-cadherin -R: 5’-CTTTATCCCGGCGTTTCATC-3’

Vimentin-F: 5’-GAGAACTTTGCCGTTGAAGC-3’
Vimentin-R: 5’-GCTTCCTGTAGGTGGCAATC-3’

FN1-F: 5’-CAGTGGGAGACCTCGAGAAG-3’
FN1-R: 5’-TCCCTCGGAACATCAGAAAC-3’

Snail1-F: 5’-GCAAATACTGCAACAAGG-3’
Snail1-R: 5’-GCACTGGTACTTCTTGACA -3’

Twist1-F: 5’-GGAGTCCGCAGTCTTACGAG-3’
Twist1-R: 5’-TCTGGAGGACCTGGTAGAGG -3’

ZEB1-F: 5'-TGCACTGAGTGTGGAAAAGC-3'
ZEB1-R: 5'-TGGTGATGCTGAAAGAGACG-3'

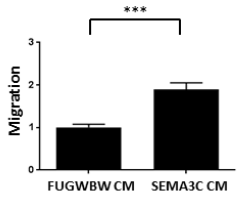
ZEB2-F: 5'-CGCTTGACATCACTGAAGGA-3’
ZEB2-R: 5'-CTTGCCACACTCTGTGCATT-3'

TGFβ1-F: 5’-CCAACTATTGCTTCAGCTCCA-3’
TGFβ1-R: 5'-TTATGCTGGTTGTACAGGG-3’

TGFβ2-F: 5'-CTGATCCTGCATCTGGTCACG-3’
TGFβ2-R: 5'-TGGGGGACTGGTGAGCTTC-3’

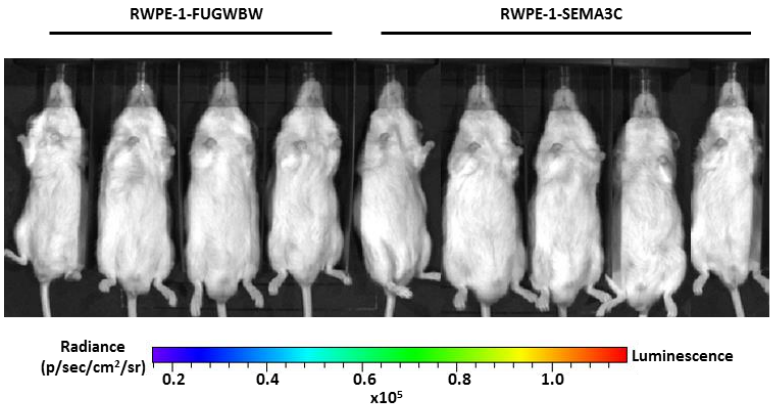
GAPDH-F: 5'-ATGACCCCTTCATTGACCTCA-3’
GAPDH-R: 5'-GAGATGATGACCCTTTTGGCT-3’

Appendix E. Directional migration of RWPE-1 cells to SEMA3C-containing conditioned media



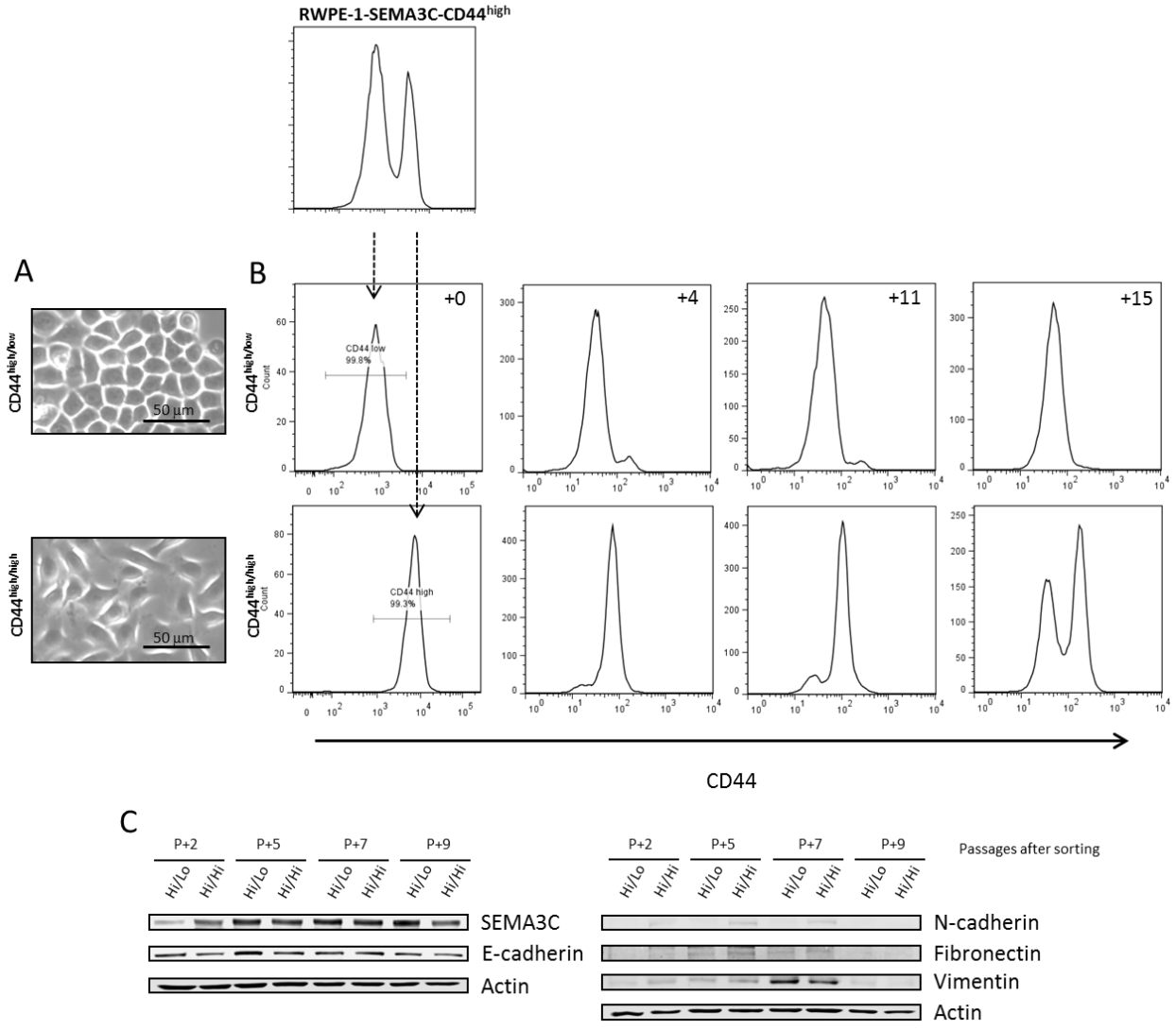
Appendix E. Directional migration of RWPE-1 cells to SEMA3C-containing conditioned media. RWPE-1-FUGWBW cells migrated more strongly toward conditioned media from SEMA3C-overexpressing RWPE-1-SEMA3C (SEMA3C CM) than to conditioned media from RWPE-1-FUGWBW (FUGWBW CM); y-axis is fold increase in migration over FUGWBW CM. Data represent mean, ± SD; *** $p < 0.001$.

Appendix F. Intracardiac injection of NOD scid gamma mice with RWPE-1 stable cells



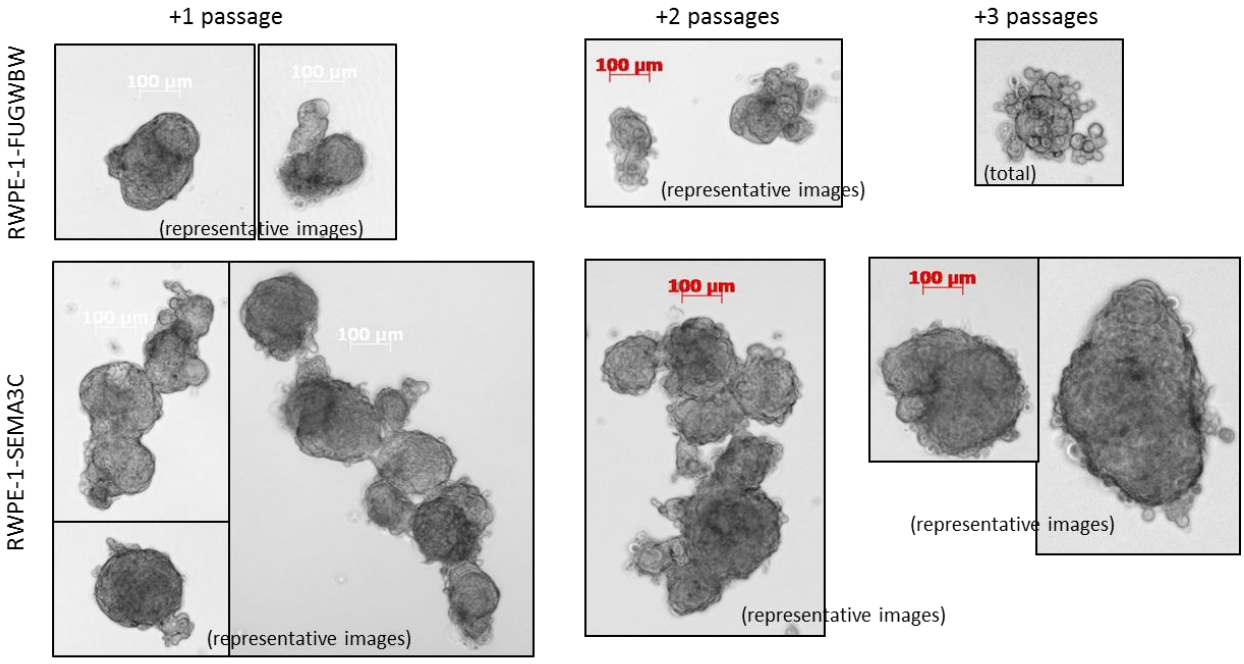
Appendix F. Intracardiac injection of NOD scid gamma mice with RWPE-1 stable cells. $5x10^5$ luciferase-expressing RWPE-1-FUGWBW and RWPE-1-SEMA3C were injected by ultrasound-guided intracardiac injection and monitored for tumor formation by IVIS.

Appendix G. RWPE-1-SEMA3C-CD44^{high} cells reconstitute the CD44^{low} population



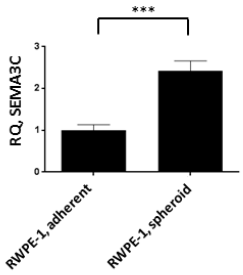
Appendix G. RWPE-1-SEMA3C-CD44^{high} cells reconstitute the CD44^{low} population. CD44^{high} cells were re-sorted on CD44 status into 'CD44$^{high/low}$' and 'CD44$^{high/high}$' populations; CD44$^{high/low}$ cells were cobble stone in morphology (A) and remained CD44-low (B). CD44$^{high/high}$ cells were spindle-shaped (A) and reconstituted the CD44^{low} population (B). Bar = 50 µm. CD44$^{high/high}$ cells were initially elevated in SEMA3C, N-cadherin, Fibronectin, and Vimentin expression and lower in E-cadherin expression compared to CD44$^{high/low}$ cells but expression of these proteins between the two populations became equal over successive passages (C).

Appendix H. Serial passaging of spheroids



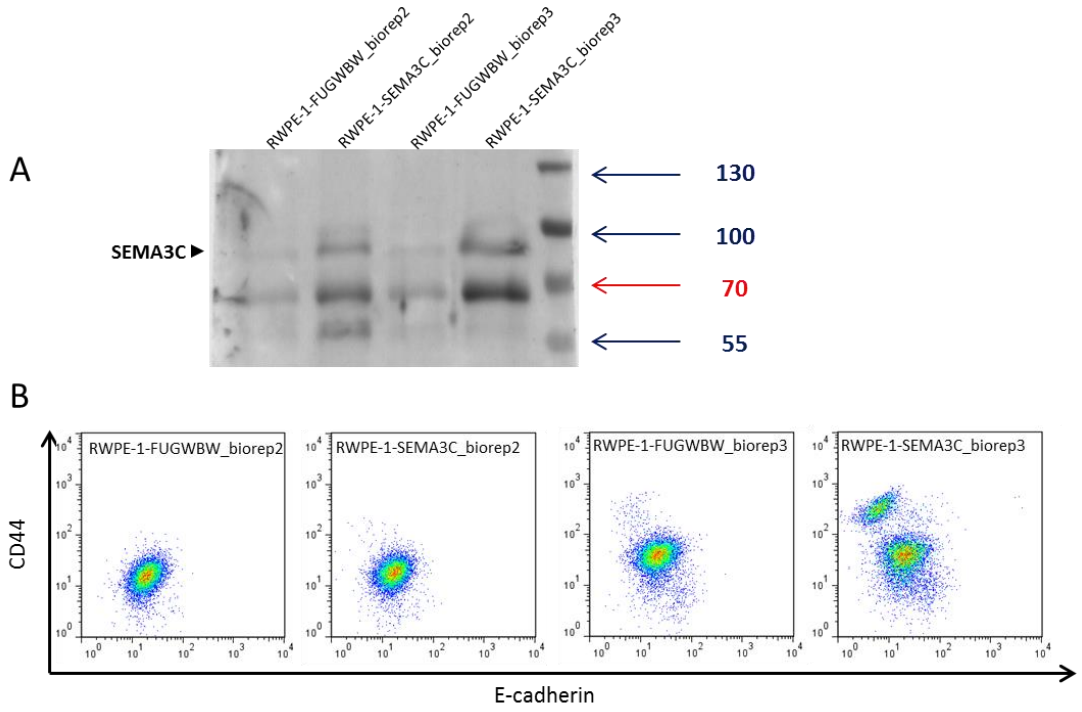
Appendix H. Serial passaging of spheroids. Spheroids were grown for approximately 7 days before being dissociated into constituent cells using trypsin. Cells were counted by trypan blue staining and hemocytometer at which time five-thousand cells were subcultured into fresh media. Spheroids were passaged like this a total of three times. Images are representative of the spheres present except in the case of the third passage of the RWPE-1-FUGWBW where the only sphere present was that which is shown.

Appendix I. SEMA3C is expressed at higher levels in RWPE-1 spheroids than in adherent RWPE-1



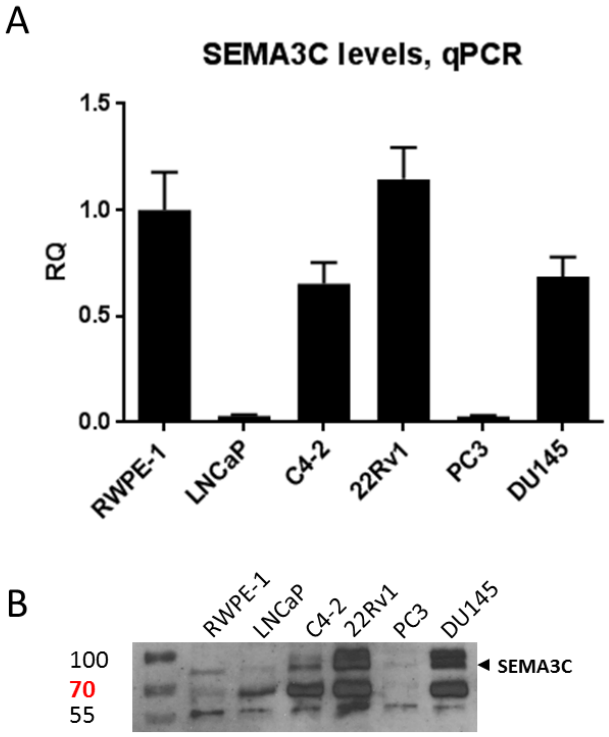
Appendix I. SEMA3C is expressed at higher levels in RWPE-1 spheroids than in adherent RWPE-1. SEMA3C levels were compared between RWPE-1 cells plated under adherent conditions versus anchorage-independent conditions (as spheroids) by qPCR. Data represent mean, ± SD; *** $p < 0.001$.

Appendix J. Repeat of RWPE-1 lentiviral transduction



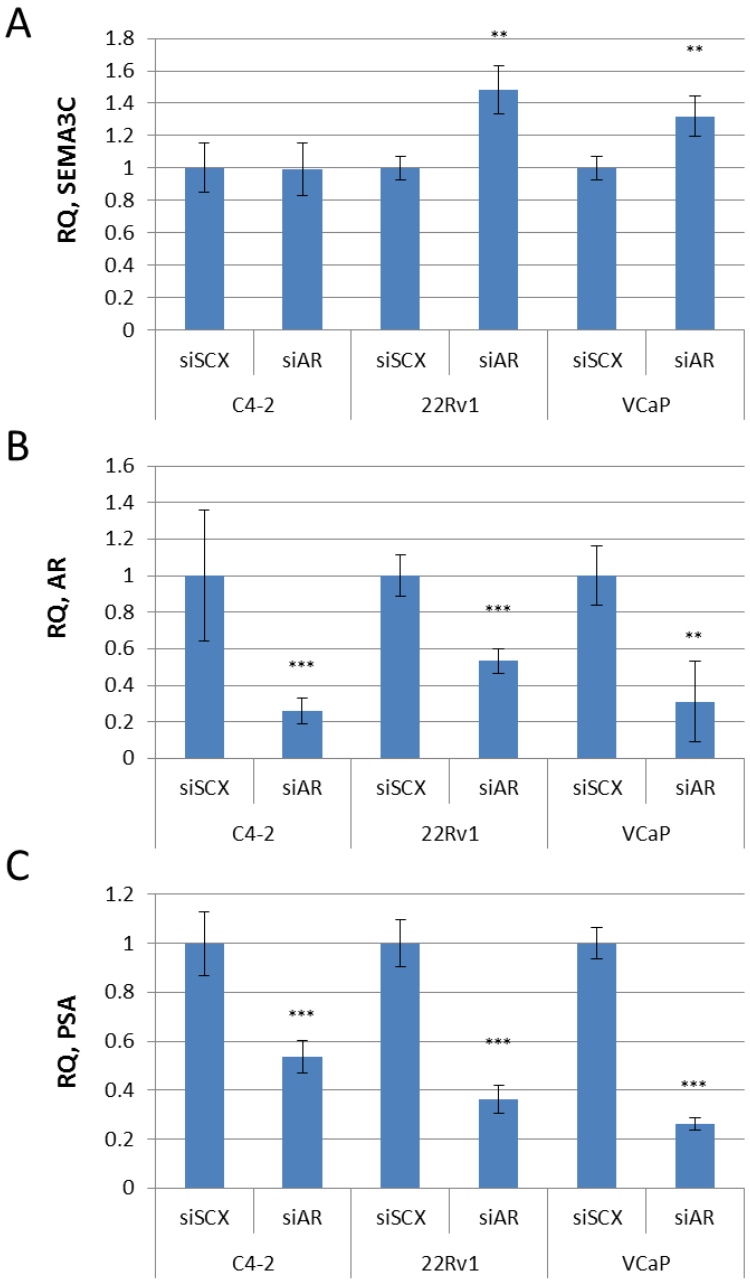
Appendix J. Repeat of RWPE-1 lentiviral transduction. Lentiviral transduction of RWPE-1 cells was repeated a second and third time. Overexpression of SEMA3C verified by Western blot of conditioned media (A). Upregulation of CD44 and coexpression of E-cadherin was determined by FACS (B).

Appendix K. SEMA3C expression in prostate cell lines



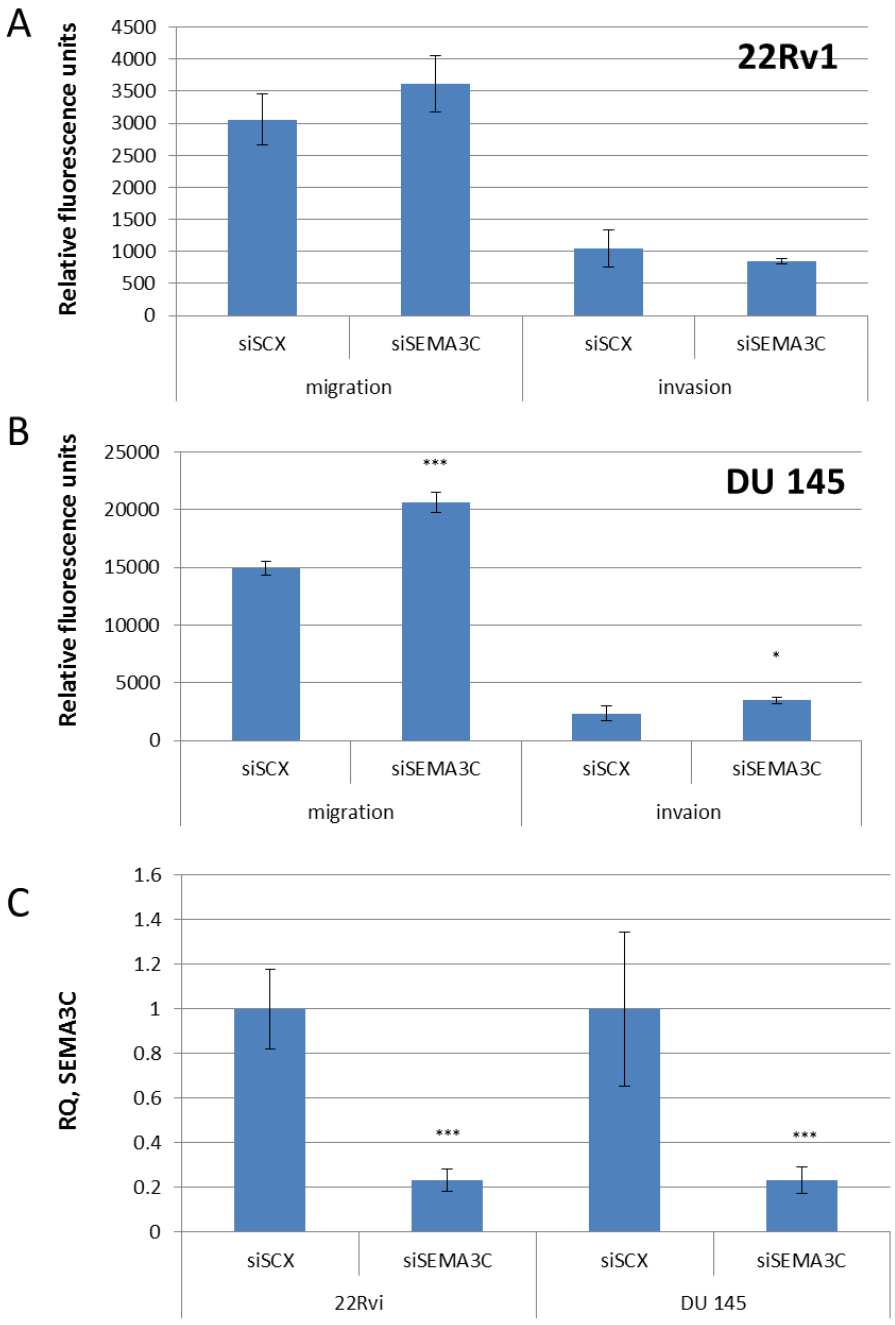
Appendix K. SEMA3C expression in prostate cell lines. SEMA3C mRNA (A) or protein (B) levels were compared across a panel of prostate lines: RWPE-1, LNCaP, C4-2, 22Rv1, PC3, and DU145. Cells were serum-starved for 24 hours in 0.2% charcoal-stripped serum in OptiMEM. Western blot detection of SEMA3C was done on conditioned media. Data represent mean, ± SD.

Appendix L. SEMA3C mRNA levels in AR knockdown of PCa cell lines



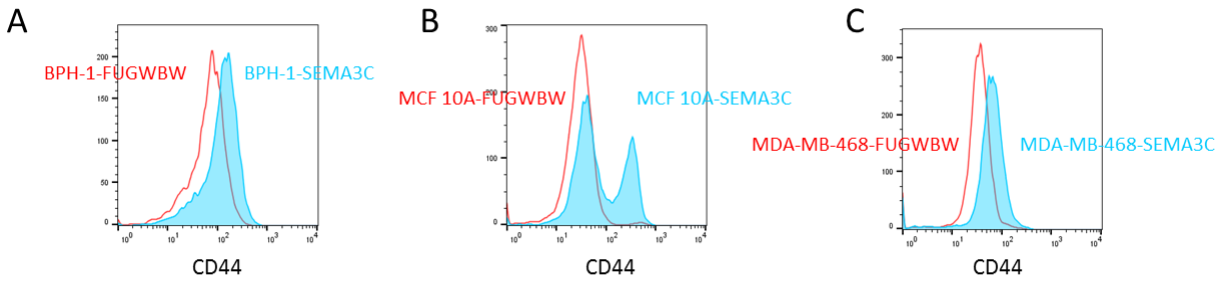
Appendix L. SEMA3C mRNA levels in AR knockdown of prostate cancer cell lines. The AR-positive prostate cancer cell lines: C4-2, 22Rv1, and VCaP were knocked down for AR using siRNA. Following this, qPCR was used to detect SEMA3C (A), AR (B), and PSA (C). Data represent mean, ± SD; ** $p < 0.01$, *** $p < 0.001$.

Appendix M. Migration and invasion in SEMA3C loss-of-function studies in 22Rv1 and DU 145 cells



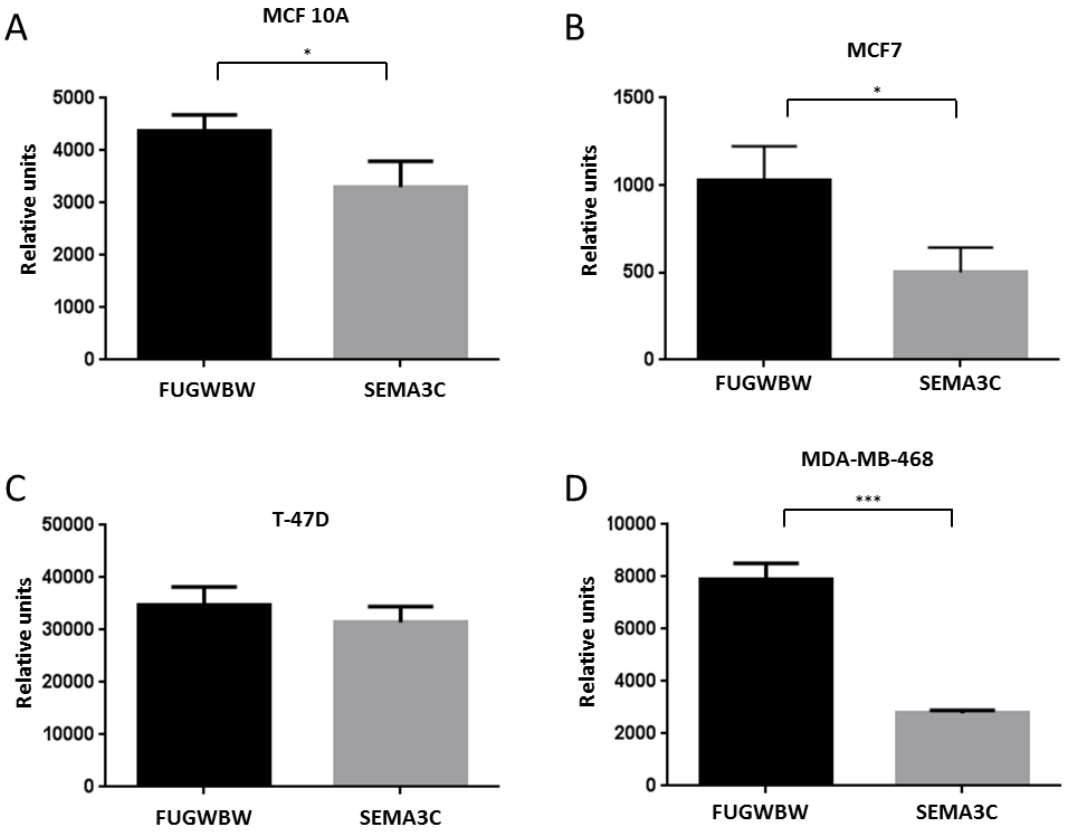
Appendix M. Migration and invasion in SEMA3C loss-of-function studies in 22Rv1 and DU 145 cells. SEMA3C-high prostate cancer cell lines, 22Rv1 (A) and DU 145 (B), were treated with siRNA directed against SEMA3C. 2 x 10^5 cells were then seeded for migration or invasion assays using a Boyden chamber approach. Data represent relative mean migration or invasion (C) Knockdown of SEMA3C was confirmed by qPCR. Data represent mean, ± SD; * $p < 0.05$, *** $p < 0.001$.

Appendix N. Upregulation of CD44 in additional prostate and breast cell lines overexpressing SEMA3C



Appendix N. Upregulation of CD44 in additional prostate and breast cell lines overexpressing SEMA3C. The CD44 status of an additional benign prostate line (BPH-1, A), a benign breast line (MCF 10A, B), and a breast cancer cell line (MDA-MB-468, C) stably overexpressing SEMA3C was documented by flow cytometry.

Appendix O. Migration of breast cell lines overexpressing SEMA3C



Appendix O. Migration of breast cell lines overexpressing SEMA3C. Breast and breast cancer cell lines MCF 10A (A), MCF7 (B), T-47D (C), and MDA-MB-468 (D) stably overexpressing SEMA3C were generated using a lentiviral approach. The migration of SEMA3C-overexpressing lines were compared to parental vector control cells using a Boyden chamber approach. Data represent mean, ± SD; * $p < 0.05$, *** $p < 0.001$.