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ABSTRACT

Title of Document: DEVELOPMENT OF ANIMAL MODELS AND MOLECULAR TOOLS TO INVESTIGATE THE FUNCTION OF PROSTATE SPECIFIC MEMBRANE ANTIGEN.

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Doctor of Philosophy
2023

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Prostate Specific Membrane Antigen (PSMA) is a transmembrane glycoprotein expressed in human prostate luminal epithelial cells. PSMA expression increases in most prostate cancer cases. PSMA overexpression is associated with an unfavorable prognosis, biochemical recurrence, and metastatic disease in patients treated for prostate cancer. PSMA has both folate hydrolase and glutamate carboxypeptidase enzymatic activity. Given its membrane localization and increased expression in prostate cancer, PSMA has received considerable attention as a diagnostic and therapeutic target. Remarkably, the physiological roles of PSMA in the prostate gland remain unknown, and no animal models expressing human PSMA exist. Neither mice nor rats express endogenous PSMA in the prostate, precluding testing of PSMA-targeting agents in wild type Muridae species. The principal goals of this work were to develop a genetically engineered animal model expressing

human PSMA in normal and malignant prostates. Multiple attempts to achieve this goal using three distinct molecular strategies in mice were unsuccessful. However, I succeeded in developing a transgenic rat model that conditionally expresses human PSMA in the prostate. By five weeks of age these rats display heterogenous PSMA expression in ventral and lateral prostate lobes. By twenty-five weeks, PSMA expression approaches homogeneity in luminal prostate epithelial cells without apparent pathological effect. Parallel efforts by others to develop a rat model of prostate cancer based on MYC oncogene expression and Pten tumor suppressor loss encountered technical roadblocks and were unsuccessful, which precluded analyses of PSMA function during malignant progression. Overcoming these technical roadblocks necessitated construction of large, complex, multifunctional transgenes that push the boundaries of extant cloning and recombineering technologies. To expedite this process, I optimized a DNA assembly technology and successfully deployed this approach to assemble up to twelve DNA fragments. I also discovered that non-specific DNA can significantly increase the likelihood of achieving success in the generation of complex assemblies. Taken together, these contributions provide a powerful framework to determine the roles of PSMA in normal and diseased prostate glands and provide new molecular tools to support development of next generation of PSMA-expressing animal models. Such models will be instrumental in advancing PSMA-directed diagnostics and therapeutics toward the clinic.

DEVELOPMENT OF ANIMAL MODELS AND MOLECULAR TOOLS TO INVESTIGATE THE FUNCTION OF PROSTATE SPECIFIC MEMBRANE ANTIGEN

By

Apurv M. Rege

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, Baltimore County, in partial fulfillment
of the requirements for the degree of
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Dedication

I dedicate this dissertation to my mother, without her unconditional love, encouragement, and support, throughout my life, this accomplishment would not have been possible.

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Abbreviations

ADC	Antibody drug conjugate
ADT	Androgen deprivation therapy
AJCC	American Joint Committee on Cancer
AP	Anterior prostate
AR	Androgen receptor
BPH	Benign Prostatic Hyperplasia
CDX	Cell-line derived xenografts
CK	Cytokeratin
CT	Computed tomography scan
CP/CPPS	Chronic prostatitis/ chronic pelvic pain syndrome
DHT	Dihydrotestosterone
DLP	Dorsolateral lobes
DP	Dorsal prostate
DRE	Digital rectal examination
ERSPC	European Randomized Study of Screening for Prostate Cancer
FOLH1	Folate hydrolase I
GDA	Gibson DNA Assembly
GGA	Golden Gate Assembly
GEM	Genetically engineered mouse models
GCPII	Glutamate carboxypeptidase II
GSTP1	Glutathione S-transferase Pi class
HGPIN	High-grade PIN

Hoxa10	Hox family of Antennapedia class homeobox gene a10
Hoxa13	Hox family of Antennapedia class homeobox gene a13
Hoxb13	Hox family of Antennapedia class homeobox gene b13
Hoxd13	Hox family of Antennapedia class homeobox gene d13
FOXA1	Forkhead box A1
KLK3	Kallikrein related peptidase 3
LGPIN	Low-grade PIN
LHRH	Luteinizing hormone -releasing hormone
LP	Lateral prostate
mCRPC	Metastatic castration-resistant prostate cancer
mCSPC	Metastatic castration-sensitive prostate cancer
MG1	Glycoprotein mucin 1
mGluR	Metabotropic glutamate receptor
MRI	Magnetic resonance imaging
NAA	N-acetyl aspartate
NAAG	N-acetyl-L-aspartyl-L-glutamate
NIH	National Institutes of Health
Nkx3.1	NKL family Antennapedia class homeobox gene 3.1
PCa	Prostate adenocarcinoma
PCR	Polymerase chain reaction
PDX	Patient derived xenografts
PET	Positron emission tomography scan
PhIP	2-Amino-1-methyl-6-phenylimidazo[4,5-b] pyridine

PIA	Proliferative inflammatory atrophy
PIN	Prostatic intraepithelial neoplasia
2-PMPA	2-(phosphonomethyl) pentanedioic acid
PIP2	PI 4,5-triphosphate
PIP3	PI 3,4,5-triphosphate
PTEN	Phosphatase and tensin homolog
PSA	Prostate specific antigen
PSMA	Prostate specific membrane antigen
scRNA-seq	Single cell RNA sequencing
SV40	Simian virus 40
TRAMP	Transgenic adenocarcinoma of the mouse prostate
TRUS	Transrectal ultrasound
UGE	Urogenital sinus epithelium
UGS	Urogenital sinus
USM	Urogenital mesenchyme
USPTF	United States Preventative task force
VP	Ventral prostate

Chapter 1: Introduction

1.1 The prostate gland

1.1.1 Prostate Anatomy

The prostate gland is an important sex organ that provides semen for the sperm providing the ideal environment for sperms survival and motility in the female reproductive tract. The prostate is a chestnut-sized accessory reproductive organ found in male mammals that varies in shape and form across species. The base of the prostate is located at the base of the bladder, surrounding the urethra and in front of the rectum with the apex ending before the urethra enters the penis (Figure 1) (Amin and Tickoo 2022).

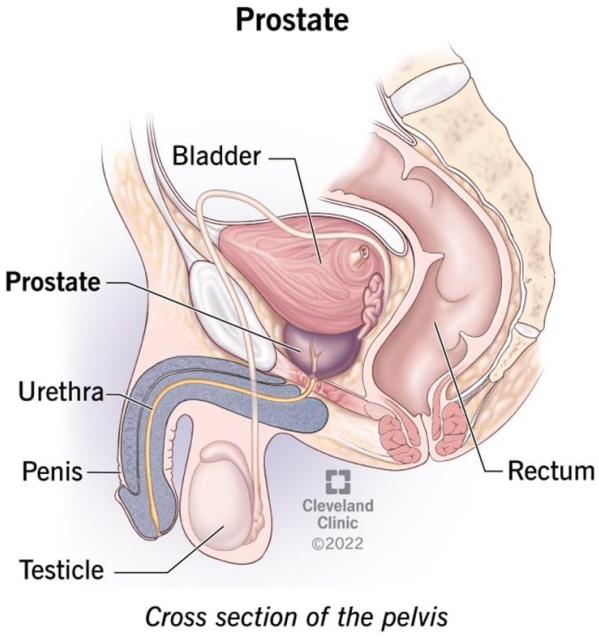

Figure 1. Anatomy of male reproductive system. Sagittal section of male pelvis showing different organs. The prostate is located below the bladder surrounding the urethra. Figure adapted from ("Prostate: Anatomy, Location, Function & Conditions").

The normal human prostate is a single glandular organ weighing approximately 15-20 grams and divided into three histological zones termed peripheral, transitional and central zones (Figure 2a) (Ittmann 2018; John E. McNeal 1981). The peripheral zone surrounds both the central and transition zones, the central zone surrounds the ejaculatory ducts, and the transition zone surrounds the urethra. The seminal vesicles in humans are located near the base of the prostate on either side (John E. McNeal 1981).

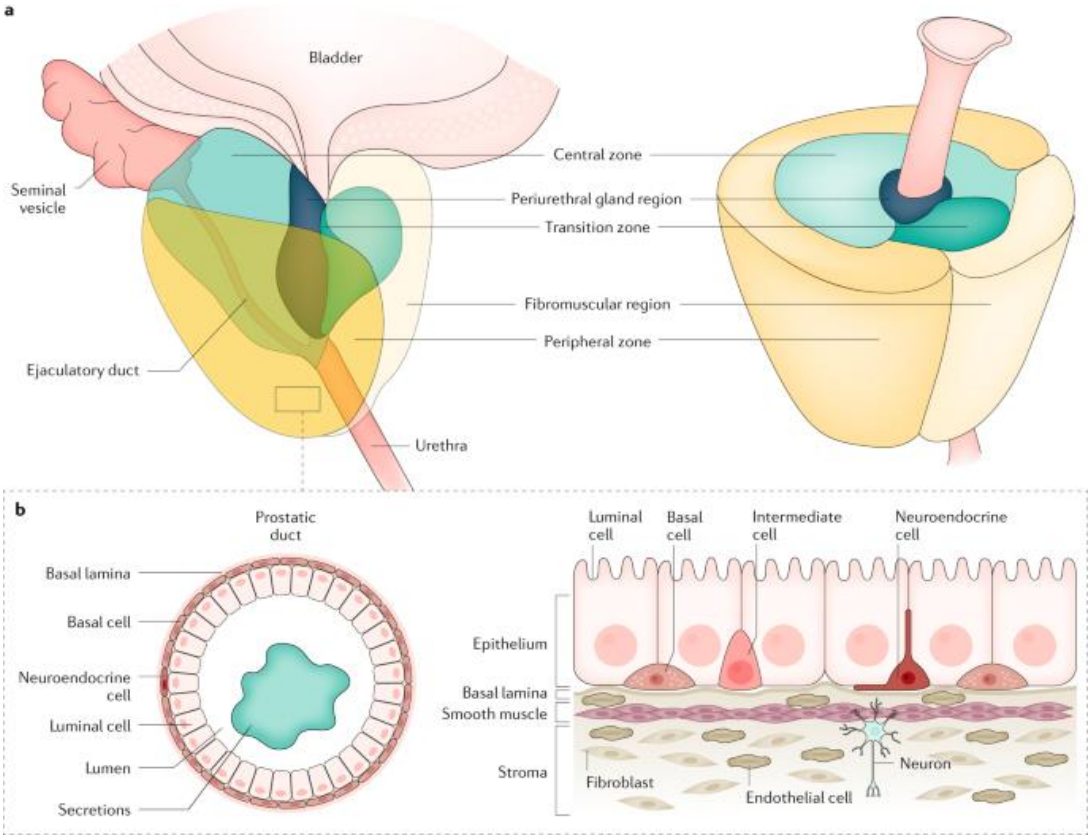

Figure 2. Schematic representation of human prostate (a) human prostate anatomy showing the physical location with regards to other organs and the different zones of the prostate. (b) histological structure of human prostate showing the different cell types and their arrangement in the architecture of the prostate duct. Adapted from (Verze, Cai, and Lorenzetti 2016b).

The prostate gland is comprised mainly of acinar gland and fibromuscular stroma as described by John E. McNeal and hence called McNeal prostate (John E.

McNeal 1981). According to McNeal's classification the peripheral zone is the largest making up ~70% of the prostate, followed by the central zone ~25% with the transition zone, the smallest of the three zones, making up the remainder (John E. McNeal 1981; Timms 2008; J. E. McNeal 1981). The majority of prostate cancers originate in the peripheral zone (~60-75%), followed by the transition zone (~10-20%), with originating in the central zone (~2-3%) (Haffner et al. 2009; J. E. McNeal et al. 1988). Benign Prostatic Hyperplasia (BPH), an inflammatory disease of the prostate, originates only in the transition zone.

The architecture of the prostate secretory ductal epithelium has been well described. In a normal prostate gland, the cells forming a continuous layer lining the basement membrane are termed basal cells, these cells express cytokeratin (CK) 5, CK 14 and p63, which are interspersed with neuroendocrine cells, these cells are marked by expression of chromogranin A and synaptophysin (Signoretti et al. 2000; Y. Wang et al. 2001; Di Sant'Agnese 1998). The columnar luminal epithelial cells line the interior of the prostatic lumen and secrete proteins into the lumen, these cells are marked by expression of cytokeratin (CK) 8 and 18 as well as prostate specific antigen (PSA) (Verhagen et al. 1988; Y. Wang et al. 2001). The basement membrane separates the epithelial compartment from the stroma, which is comprised principally by smooth muscle cells and fibroblasts (Figure 2b) (Cory Abate-Shen and Michael M Shen 2002; Shen and Abate-Shen 2010b). At the cellular level, early analysis suggested that three distinct types of epithelial cells were present in the prostatic ducts, namely luminal epithelial cells, basal epithelial cells, and neuroendocrine. These populations were distinguished based on their relative glandular position,

shape, cellular antigen expression, and later by their gene expression profiles (Shen and Abate-Shen 2010a; DeMarzo et al. 2003). Recent single cell RNA sequencing (scRNA-seq) studies performed by Henry et al. have identified two more previously unrecognized epithelial cell types expressing SCGB1A1 and KRT13 that are predominantly found in the prostatic urethra and proximal prostatic ducts respectively (Henry et al. 2018).

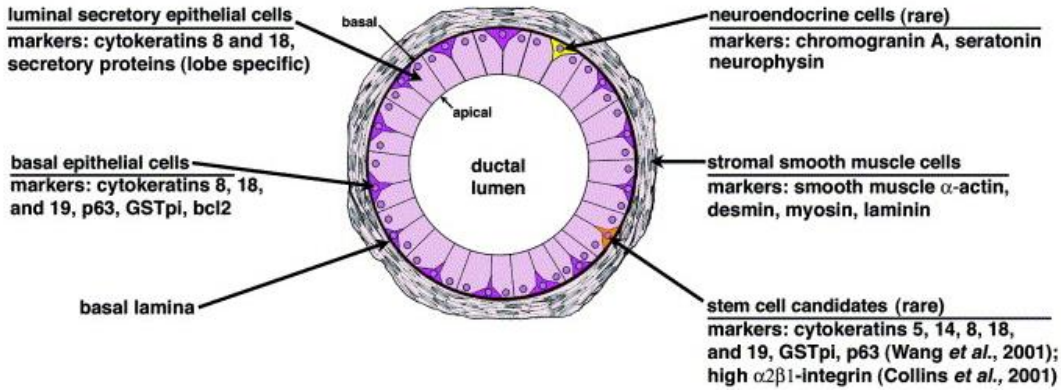

Figure 3 Shematic representation of a human prostate duct and lineage markers. Each type of prostate ductal cell expresses specific cell surface differentiation markers, as marked by the arrows below labels for each cell type. Adapted from (Marker et al. 2003a).

Several markers are commonly employed in immunohistochemical analyses to distinguish prostate epithelial cell populations. Luminal epithelial cells express cytokeratin 8, NKX3.1, and Kallikrein related peptidase 3 (KLK3) and are Androgen receptor (AR) positive. Basal epithelial cells are distinguished by expression of cytokeratin 5, cytokeratin 14, and transcription factor p63, and neuroendocrine cells express chromogranin A and synaptophysin (Figure 3) (Hudson et al. 2001; Yong Xue, Frank Smedts, Frans M.J. Debruyne, Jean J.M.C.H. de la Rosette 1998; Di Sant'Agnese 1998; Gurel et al. 2010).

1.1.2 Prostate development and function

The prostate development in all male mammals is highly similar across species despite the anatomical differences that are manifested in adult animals. Prostate development initiates in late embryogenesis and is completed during puberty under the influence of androgens. The prostate develops from the urogenital sinus (UGS). The UGS is develops from the ventral part of cloaca which also partitions into the hindgut. Colon and rectum arise from the hindgut whereas the bladder, prostate, bulbourethral glands, and urethra arise from the UGS. Prostate development initiates when the urogenital sinus epithelium (UGE) buds into the urogenital mesenchyme (USM). Our understanding of prostate development comes primarily from studies performed on mice and rats. Prostate development follows a chronological path and can be divided into six stages: (1) pre-bud stage, (2) initial budding, (3) bud elongation and branching, (4) ductal canalization, (5) differentiation of luminal and basal epithelial cells and, (6) secretory cytodifferentiation (Figure 4) (Cunha et al. 2018).

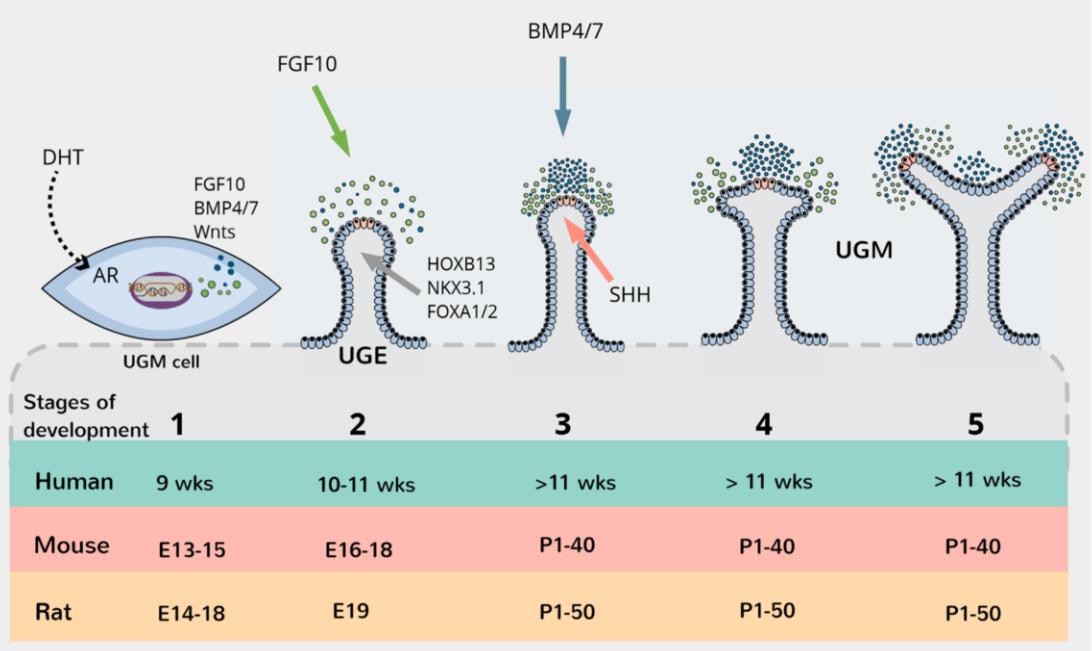

Figure 4. Timepoints and stages of prostate development in rodents and humans. (1) Pre-bud stage, (2) initial budding, (3) bud elongation, (4) branching, (5) ductal canalization. Solid arrows show localization of specific proteins, dotted arrows represent movement of DHT, adapted from (Buskin et al. 2021).

Androgens play a critical role in the prostate gland development. Fetal testosterone is important in the initiation and differentiation of the epithelial bud development from the UGS. Testosterone is converted by the enzyme 5α-reductase to dihydrotestosterone (DHT), which binds and activates the androgen receptor (AR) with higher affinity. Fetal testes produce testosterone around nine weeks in humans and around 15 weeks in rats and initiates prostate development from the UGS. The level of fetal testosterone is low to absent in female embryos, and in the absence of sufficient androgen signaling the UGS develops into the vagina, urethra, and distal colon. Epithelial/mesenchymal interactions are also vital in in budding, differentiation, and morphogenesis of the prostate (Marker et al. 2003b; Voigt, Feldman, and Dunning 1975; Cunha, Cooke, and Kurita 2004).

Male sperm viability is dependent on each organ of the urogenital system carrying out its function in a cooperative manner. The prostate gland's main function is to secrete an alkaline fluid that combines with seminal fluid from the seminal vesicles and sperm from the testes to form semen during ejaculation. Prostatic secretions and seminal fluid make up majority of the semen, ~20-30% and ~65-75% respectively (Kumar and Majumder 1995; Verze, Cai, and Lorenzetti 2016b). The prostatic secretions in human seminal plasma or semen are made up of proteins such as kallikreins (KLKs) and trace elements such as Zn^{2+}, metabolites such as citrate, and polyanionic spermine. Seminal vesicles principally secrete semenogelin proteins, testes contribute spermatozoa, as well as testosterone and insulin-like 3 protein. The bulbourethral glands primarily contributes glycoprotein mucin 1 (MG1) (Rebello et al. 2021; Gilany et al. 2015). By virtue of fibromuscular contraction the prostate also helps to push semen through the urethra. Prostatic secretions are not essential for male fertility, they are rather required for enhancing the sperm viability and motility. Lack of these secretions can thus result in reduced of male sperm viability and compromised fertility.

1.1.3 Genes in prostate development

The AR signaling pathway is the most extensively studied pathway in prostate organogenesis and is active throughout prostate development. AR is the target of steroid hormones produced by the testis, i.e. testosterone, which is converted to a more potent form DHT by the enzyme 5α-reductase. When DHT binds to the AR, it triggers a series of events, one of these events leading to the release of paracrine factors from the UGM that bind to receptors in UGE leading to morphologic

differentiation that drives prostate development (Baulieu, Lasnitzki, and Robel 1968; Brennen and Isaacs 2018). Cunha et. al. performed elaborate studies in mice to elucidate the role of AR in prostate organogenesis. There results show, the UGS can exhibit sexual dimorphism regardless of genetic sex due to the involvement of androgens (Lyon and Hawkes 1970; CUNHA 1975). For instance, male mouse embryos lacking functional AR undergo development of female genitalia and do not form prostate (Takeda, Lasnitzki, and Mizuno 1986). Conversely, female mouse embryos or male mouse embryos with impaired testis development can develop prostatic structures when exposed to adequate levels of androgens either during development in utero, or through grafting into male hosts (CUNHA 1975; Takeda, Lasnitzki, and Mizuno 1986).

Multiple families of homeobox genes which encode helix-turn-helix transcription factors, play an important role during development in anterior-posterior patterning in the body. The Hox family of Antennapedia class homeobox genes are divided into four genomically distinct clusters termed HOX A, HOX B, HOXC and, HOXD, and in mice Hoxa, Hoxb, Hoxc and Hoxd. Gene duplication over evolutionary time has generated 13 potential paralogous groups within each cluster. Several of the Hox genes have been demonstrated to play a role in prostate development. Loss of function of *Hoxa10, Hoxa13, Hoxb13* and *Hoxd13* in mice results in decreased prostate size, altered glandular shape and decreased branching in the lobes (Podlasek et al. 1999; Podlasek, Clemens, and Bushman 1999; Podlasek, Duboule, and Bushman 1997; Toivanen, R, Shen and 2017). During embryonic development Hoxb13 is expressed during the prostate bud initiation in the UGE and

is essential for normal budding and branching morphogenesis in the prostate (Cunha et al. 2018). In addition to its role in embryonic development, Hoxb13 continues to play a role in the adult prostate gland. It is expressed in the luminal cells of the prostate, where it regulates the expression of genes involved in prostate function and maintenance (Economides and Capecchi 2003; McMullin, Mutton, and Bieberich 2009a). Studies have associated Hoxb13 as a regulator of AR, and FOXA1 as a regulator of Hoxb13 (Norris et al. 2009; McMullin, Mutton, and Bieberich 2009a; McMullin et al. 2010). Mutations or alterations in the HOXB13 gene have been linked to increased susceptibility to prostate cancer. Certain germline mutations in HOXB13, such as G84E, have been identified in families with hereditary prostate cancer, indicating its potential involvement in the development and progression of the disease (Ewing et al. 2012).

NKX3.1/Nkx3.1 (human/mouse) is an NKL family Antennapedia class homeobox gene that plays an important role in prostate development. Nkx3.1 is one of the first genes to be expressed in response to AR signaling, is expressed throughout prostate development in the epithelium and later in adult luminal epithelial cells. Nkx3.1 expression in adult prostate continues to be regulated by androgens (Bieberich et al. 1996; Sciavolino et al. 1997; Marker et al. 2003b). The importance of the Nkx3.1 gene is demonstrated by experiments in which researchers manipulated its activity in mice. Mice with Nkx3.1 null mutations develop prostatic lobes with abnormal branching having epithelial hyperplasia with decreased secretory protein and abnormal cytodifferentiation (Bhatia-Gaur et al. 1999). In Nkx3.1 knockout mice, genes related to prostate development were downregulated, while genes related to the

seminal vesicles were upregulated. Conversely, when the Nkx3.1 gene was introduced into the cells of the seminal vesicles, those cells took on characteristics of prostate tissue instead (Dutta et al. 2016).

1.1.4 Rodent prostate

Mouse and rat are the most used animal research models for studying human diseases. Although the function of the prostate gland is conserved between the rodents and humans, anatomical architecture is divergent. The prostate is essentially anatomically identical in mouse and rats but varies in size with rats having bigger prostate than mice due to differences in overall body size. The rodent prostate is divided into four paired lobes anterior prostate (AP), dorsal prostate (DP), lateral prostate (LP), and) lobes according to their position relative to the urethra (Ferm 1987). The DP and LP are sometimes collectively called dorsolateral lobes (DLP). AP has also historically been referred to as the coagulating gland.

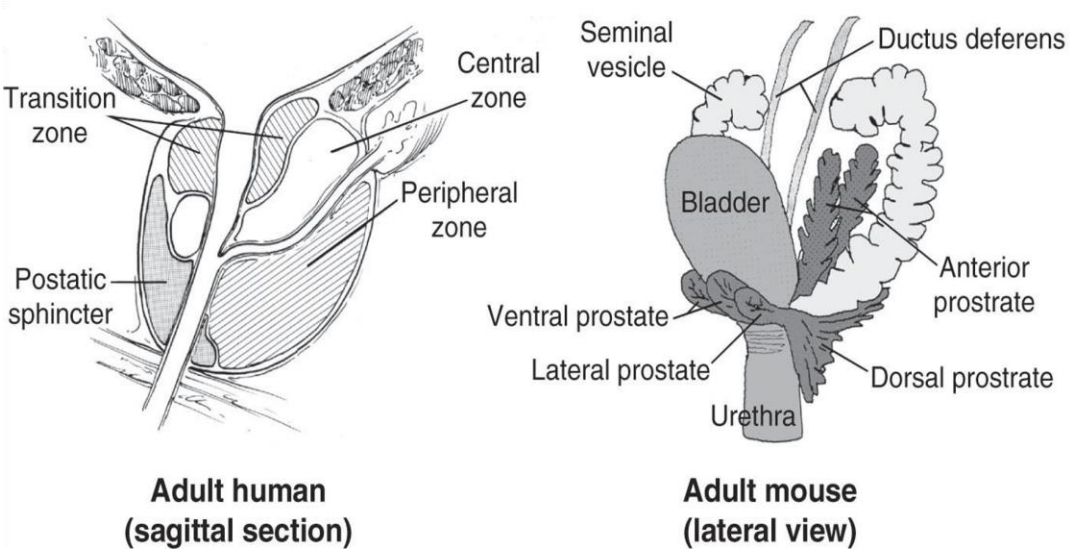

Figure 5. Schematic representation of human and mouse prostate glands. Human prostate gland is single lobe comprising of three distinct zones, mouse prostate gland is multi lobed and divided into four distinct lobes adapted from (Shen and Abate-Shen 2010b).

The VP is located at the base of the bladder in front of the urethra, the LP spans the region laterally between urethra and seminal vesicles, DP is located on the dorsal side of the urethra at the base of the seminal vesicles, and AP is attached to the seminal vesicles. There is no definitive direct homology between the lobes of the rodent prostate and human prostate zones. The rat DP has been suggested to be most functionally similar to the human peripheral zone and the rat AP is thought to be homologous to the human transition zone due to similarities in their gene signatures. In contrast, the rat VP does not seem to be similar to any of the human prostate zones (C. Lee and Holland 1987; Roy-Burman et al. 2004). Rodent models have contributed immensely to our understanding of various prostate diseases such as prostatitis, benign prostatic hyperplasia (BPH), and prostate cancer (Cory Abate-Shen and Michael M Shen 2002).

Prostatitis is a catch-all term for common benign and generally transient painful conditions of the prostate that can affect men of any age. Prostatitis is at times associated with prostate inflammation due to various factors including bacterial infection, viral infection, urine reflux, physical injury, hormonal changes, dietary habits, or a combination of two or more of these factors. Prostatitis symptoms may include painful urination, urine retention, increased frequency of urination, chronic pelvic pain, and sexual dysfunction. To facilitate accurate diagnosis of prostatitis the National Institutes of Health (NIH) have classified this condition into four categories; (I) Acute bacterial prostatitis, (II) Chronic bacterial prostatitis, (III) Chronic prostatitis/ chronic pelvic pain syndrome (CP/CPPS), and (IV) Asymptomatic prostatitis. Prostatitis is a non-life-threatening condition, that can become chronic and

affect the quality of life. Chronic inflammation of the prostate gland can lead to a pre-neoplastic lesion called proliferative inflammatory atrophy (PIA), is suspected to be a precursor to prostate cancer (De Marzo et al. 2007; Khan et al. 2017; Krieger, Leroy Nyberg, and Nickel 1999; DeMarzo AM, Nelson WG, Isaacs WB 2003).

BPH is a benign disease that results in prostate gland enlargement. BPH occurs in the transition zone of the human prostate due to hyperplastic growth in the fibromuscular, epithelial, and periurethral regions. As the prostate enlarges it can constrict the urethra shut and impinge upon the base of the bladder leading to urinary symptoms (i.e. urinary urgency) or, in extreme cases, to complete obstruction of lower urinary tract. BPH is an age-dependent disease that primarily affects men over the age of 50, but its etiology is still not well understood. Symptoms of BPH include poor urinary flow, urinary frequency, difficulty initiating urination, painful urination, post urination dribbling, and nocturia. BPH though generally not life-threatening, affects the quality of life of the patients and could lead to other co-morbidities (Kirby et al. 2004; Berry et al. 1984; Speakman et al. 2015).

1.2 Prostate Cancer

1.2.1 Epidemiology

Prostate cancer is the second most common cancer in American men after skin cancer, with the American cancer society estimating 288,300 new cases of prostate cancer in 2023. Of all new cancer cases in the United States of America (U.S.A), 14.7% is projected to be prostate cancer. One in eight American men will be diagnosed with prostate cancer during their lifetime. Prostate cancer risk increases

with age, making it the number one risk factor, with the majority of cases being diagnosed in men >65 years of age, while it is rare in men under 40. Millions of men worldwide are diagnosed with prostate cancer, with developed western countries having a higher incidence rate, which may be attributable to better screening and awareness. The most recent estimate for global prostate cancer incidence (2020) estimated that 1,414,259 men were diagnosed with prostate cancer, making it the fourth most diagnosed cancer worldwide (Figure 6). According to the American cancer society, 34,700 American men will die of prostate cancer in 2023 making it the second leading cause of cancer related deaths in men only behind lung cancer. Of all cancer related deaths in 2023, 5.7% are projected to be due to prostate cancer. One in 41 men diagnosed with prostate cancer will die of the disease. Globally, it was estimated that more than 350,000 men died of prostate cancer in 2020 (Figure 6). Although prostate cancer is a serious disease, most men diagnosed with it will not die from it. There are more than 3.1 million American men living with prostate cancer. The survival rate for prostate cancer depends on several factors such as stage upon diagnosis, age, health and, treatment options available for the patient. For American men, the five-year and ten-year relative survival rate for prostate cancer confined to the prostate or nearby organs is 97% and 98% respectively. For prostate cancer that has metastasized to distant organs the five year relative survival rate drops to 32% (Siegel Mph et al. 2023; Bray et al. 2018).

Prostate cancer incidence and mortality rates also show disparities among different ethnic groups living in U.S.A and displays the widest disparity of any cancer (Chowdhury-Paulino et al. 2021). In the U.S.A, African American men have a 64%

higher incidence rate of prostate cancer than Caucasian men and are twice as likely to die from the disease. Prostate cancer incidence is second highest in Caucasian American men followed by Hispanic and then Native American men. Native American men have the second highest mortality rate after African American men, followed by Caucasian and Hispanic American men. Asian American men have the lowest rates of incidence and mortality from prostate cancer in the U.S.A. The reasons underlying these disparities among different racial groups are not clear but have been attributed to social, economic, environmental, lifestyle and, genetic factors or a combination thereof (Siegel Mph et al. 2023; Chowdhury-Paulino et al. 2021; Hinata and Fujisawa 2022).

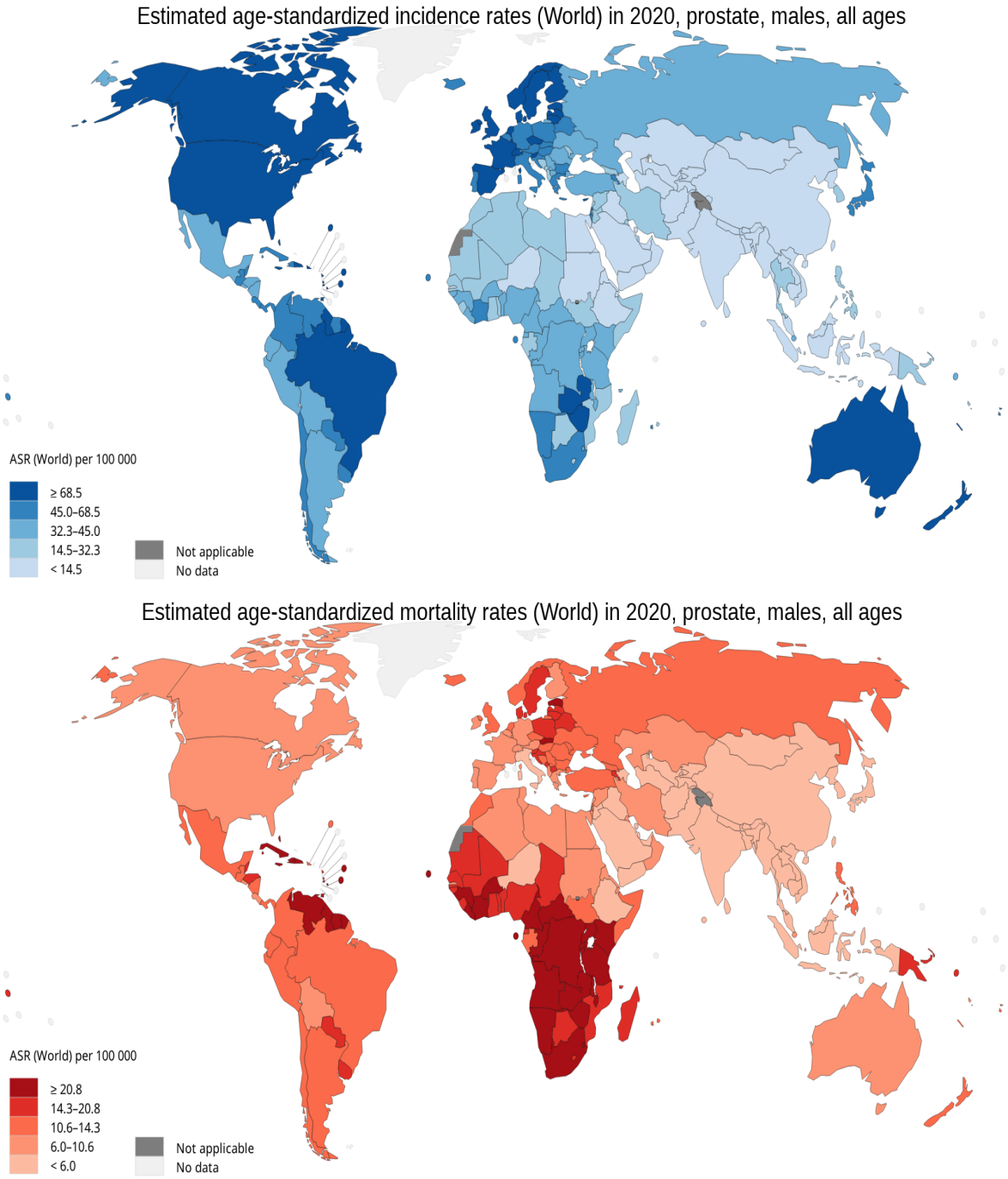

Figure 6. Prostate cancer incidence and mortality rate globally in 2020. Higher incidence of disease is observed in the developed parts of the world, but higher mortality rates are observed in the under-developed parts of the world. These disparities could be attributed to lack of accessible medical care and other socio-economic reasons. Data expressed as age-standardized rate (ASR), Adapted from Global Cancer Observatory 2020.

After age, the second most prominent risk factor for prostate cancer is family history of prostate cancer or cancer of any type. Men with first-degree relatives with a history of prostate cancer have more than two-fold increased risk of developing this disease. Genetic predisposition for prostate cancer is due to inheritance od specific germline mutations. Some of the important genes that are thought to play a role in genetic predisposition to prostate cancer include *BRCA1, BRCA2, HOXB13* and, mismatch repair related genes (Verze, Cai, and Lorenzetti 2016a). The highest risk for prostate cancer that's metastasizes has been associated with mutations in *BRCA2* and *HOXB13*, which increase the risk 7-8 fold and 3 fold, respectively (Mygatt and Osborn 2016; Karlsson et al. 2014; Bessede and Patard 2012; Pritchard et al. 2016). Other contributors to prostate cancer risk include heredity, diet, environmental factors, and lifestyle. A high animal fat diet or, excess consumption of red meat increases the risk of prostate cancer due to the presence of 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), present in charred meats, as shown in experiments in laboratory rats (Koh et al. 2010; W. G. Nelson, De Marzo, and Isaacs 2003).

1.2.2 Prostate Cancer development

Prostate cancer initiates with the development of preneoplastic lesions that emerge within the secretory epithelium. Prostatic intraepithelial neoplasia (PIN) characterized by the enlargement of the nucleus and nucleolus, crowding, irregular spacing, loss of basal cells and clumping of the luminal epithelial cells lining the prostatic ducts (Abate-Shen and Shen 2000; DeMarzo et al. 2003). PIN can be further

classified in low-grade PIN (LGPIN) or high-grade PIN (HGPIN). HGPIN is considered as a precursor to adenocarcinoma whereas LGPIN is considered benign. HGPIN and adenocarcinoma share molecular and morphological features such as tufting, micropapillary, flat and cribriform architectural patterns in epithelial appearance during pathological examination. At a molecular level loss of NKX3.1 gene is found in HGPIN in approximately 80% of the patients (D. Bostwick et al. 1996; Ming Zhou 2012; D. G. Bostwick and Qian 2004). Proliferative inflammatory atrophy (PIA) is suspected to be a precursor lesion that is observed in patients with long term chronic inflammation. PIA is identified by the atrophic columnar epithelial cells lining the prostatic duct, these lesions are focal and found mainly in the peripheral zone (De Marzo et al. 2007; Ming Zhou 2012; De Marzo et al. 1999).

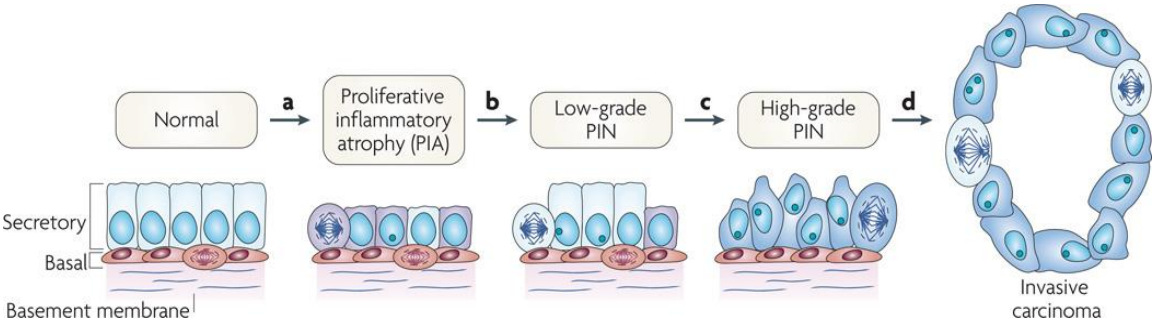

Figure 7. Schematic representation of prostate cancer initiation and progression from benign tissue to invasive carcinoma and eventually metastases. PIA is thought of as a pre-neoplastic lesion where the epithelial cells appear atrophic and is characteristically found in cases of chronic inflammation, PIN can be low-grade or high-grade and shares morphological features with adenocarcinoma such as enlarged nucleoli and cribiform ductal lining. adapted from (De Marzo et al. 2007).

Prostate adenocarcinoma (PCa) can be divided into localized disease, invasive carcinoma, and metastatic carcinoma. Metastatic PCa is an advanced stage of disease that is no longer organ confined and has spread to lymph node and/or bone (Figure 7). Metastatic PCa can be metastatic castration-sensitive prostate cancer (mCSPC) or

metastatic castration-resistant prostate cancer (mCRPC). PCa initiation and progression is believed to be strongly associated with the accumulation of somatic mutations in oncogenes and/or tumor suppressor genes over a patient's lifetime resulting in changes in gene expression leading to unregulated cell growth (Robinson et al. 2015; Abeshouse et al. 2015). PCa is characterized by several histological features like poorly differentiated tissue, prominent nucleoli, several mitotic figures, apoptotic cells, and loss of basal cell layer. On a genomic level several abnormalities that are observed in prostate cancer are somatic mutations, gene amplification, gene deletions, epigenetic changes, and gene translocations (W. G. Nelson, De Marzo, and Isaacs 2003; DeMarzo et al. 2003; Mygatt and Osborn 2016). One example of the aforementioned alterations that occur in PCa either just before or at initiation is the hypermethylation of glutathione S-transferase Pi class (*GSTP1*) gene promoter, an example of gene silencing through epigenetic changes. Other example examples include the activation of proto-oncogene MYC, somatic deletions resulting in decreased expression of the tumor suppressor genes NKX3.1 and PTEN (W. G. Nelson, De Marzo, and Isaacs 2003; Gurel et al. 2008; W. G. Nelson, De Marzo, and Yegnasubramanian 2009).

Human PCa is usually classified from stages I to IVB based on the staging system adopted by the American Joint Committee on Cancer (AJCC) TNM system, where T stands for tumor, N stands for lymph nodes, and M stands for metastases (Klein 2023; Humphrey 2004). There are several subcategories under this system which are based on the Gleason score and prostate specific antigen (PSA) test levels. A Gleason score is obtained by sum the of the two predominant grades of cancer that

are observed histologically in a specimen by a pathologist. A Gleason grade is on a scale of 1 to 5, whereas a Gleason score is on a scale of 2 to 10. Gleason grade 1 means that the tissue sample is more of less represents a normal prostate, meaning well differentiated. A Gleason grade of 5 means that the tissue sample is showing abnormal growth patterns, meaning poorly differentiated (Humphrey 2017). Generally, a higher Gleason score is indicative of worse prognosis due to the cancer being more aggressive.

1.2.3 Prostate Cancer Screening, Diagnosis and, Treatment

Some prostate cancer patients may not be symptomatic in the early stages, but symptoms may include difficulty urinating, nocturia, pelvic pain, erectile dysfunction, pain in the spinal cord, and weakness in the legs. Unfortunately, none of these symptoms are prostate cancer specific, and could also be due to BPH or prostatitis making accurate screening for prostate cancer challenging. Successful cancer screening should be able to detect aggressive and lethal cancer or their precursors versus indolent disease so appropriate therapy and be used to reduce mortality in patients. Screening for prostate cancer in an otherwise health individual is recommended if there is any familial history, as early detection can result in better outcomes especially in asymptomatic individuals. The European Randomized Study of Screening for Prostate Cancer (ERSPC), the largest single study that included 182,160 men documented a 20% reduction in prostate cancer related mortality associates with appropriate screening (Hugosson et al. 2019). The U.S. Preventative task force (USPTF) recommends >55 years to discuss with care providers regarding undergoing testing based on individual factors. There is no single standard test to

detect prostate cancer, however early detection and treatment results in better outcomes when treating localized prostate cancer. The most common screening tests performed for early detection are the digital rectal examination (DRE) and the serum prostate-specific antigen (PSA) test. A DRE involves insertion of a lubricated gloved finger into the rectum of the patient to palpate the prostate for any abnormalities in contour and/or texture. The PSA test is a blood test that quantifies the serum level of a specific glycoprotein that increases when prostate cancer is present. Although PSA screening is highly sensitive, detecting % of prostate cancer cases, it suffers from low specificity, since elevated PSA can occur for reasons unrelated to the presence of cancer. PSA screening alone is insufficient to distinguish between slowly progressing or indolent cancers, which are organ confined and may never be lethal, from more aggressive disease forms. Thus, a high PSA result may lead to unnecessary emotional stress and in some cases overtreatment. However, due to its high sensitivity, the PSA test is clearly useful for detecting early-stage prostate cancer in patient with risk factors. According to the American Urological Association and the American Cancer Society patients should receive counselling to make them aware of the risks/benefits PSA screening. Another form of PSA test is the Free PSA test in which the amount of non-bound or free PSA levels are tested as opposed to total PSA level. The ratio of free PSA to total PSA may be a better indicator of cancer. A PSA levels of 4 ng/mL is considered normal, 4-10 ng/mL is considered borderline and, >10 ng/mL is considered as a probability of having prostate cancer.

Depending on the results of PSA screening and the DRE, and considering the patient's medical history, a medical practitioner may perform additional tests such as

magnetic resonance imaging (MRI), transrectal ultrasound (TRUS) for imaging and, biopsy to confirm the presence or absence of cancer. Usually MRI, TRUS and biopsy are performed together to provide a complete picture of the stage of cancer. A Whole body scan, computed tomography scan (CT), or Positron emission tomography scan (PET) may also be performed to ascertain if the cancer is localized or has metastasized to other organs (Olleik et al. 2018; Hoffman 2011; Srivastava et al. 2019).

Treatments for prostate cancer depends on the stage of cancer determined by histopathological analysis of a biopsy, age, health, and medical history of the individual patient. A Gleason score is assigned following histopathological analysis by a pathologist, which is essentially a determination of the extent of differentiation of the prostate epithelium. For cancers with a Gleason score of 3+3, active surveillance is a common treatment option wherein patients are monitored closely. Many Gleason 3+3 cases grow slowly and may never become lethal and hence do not require treatment. Depending on the results of the DRE and PSA tests, performed either annually or semiannually, a urologist might suggest this as the best treatment option to preserve the patient's quality of life and to avoid over treatment (Chen et al. 2016).

For prostate cancers with higher Gleason scores that remain organ confined, surgery or radiation treatment maybe a viable option depending on the stage of cancer, as this might prevent the cancer from spreading to other areas while eliminating it altogether. Radical prostatectomy is the main type of surgery during which the doctor will remove the entire prostate as well as the seminal vesicles and

surrounding tissue. Due to advances in treatment options this surgery is not performed commonly due to the side effects on quality of life such as ED, urinary incontinence, or ever blood clots (Barnas et al. 2004; Mohler et al. 2019).

Hormone therapy or Androgen deprivation therapy (ADT) is usually used to treat prostate cancer patients when the cancer growth is being driven androgens themselves, such as Testosterone and DHT. Using this approach usually helps in shrinking or slowing the tumor growth as these androgens stimulate prostate cancer cell growth, as cancer cells are capable of producing it themselves. This form of therapy is usually used in conjunction with radiation or chemotherapy to treat cancers that may no longer be just organ confined or if they have recurred after initial surgery or radiation treatment as well. There are many forms of ADT that include drugs that either completely block androgen production or block AR such as Luteinizing hormone -releasing hormone (LHRH) agonists for example Leuprolide, Triptorelin, etc., LHRH antagonist for example Degarelix and Relugolix, anti-androgens like Flutamide, Abiraterone that blocks cells other than testis from making androgens and, newer anti-androgens such as Enzalutamide and Apalutamide which are also used to treat castration resistant prostate cancer. ADT is not a curative treatment for prostate cancer and also carries side effects such as ED, hot flashes, osteoporosis, anemia, depression etc. (Mohler et al. 2019; Lu-Yao et al. 2008; C. J. Nelson et al. 2008).

Chemotherapy for prostate cancer is usually used in conjunction with one of the other mentioned therapies or when other therapies have not been efficient in treating cancer that has metastasized to other organs. Drugs such as Docetaxel, Mitoxantrone, Cabazitaxel etc. are used to slow the cancer growth and improve

quality of life of the patient. General side effects of chemotherapy include hair loss, nausea, fatigue, diarrhea, increased chance of infections etc.

1.2.4 Genes involved in prostate cancer

MYC or *c-MYC* gene was characterized in early 1980s, is a human gene belonging to the MYC gene family and is a homolog of the avian myelocytomatosis oncogene (*v-MYC*), located on chromosome 8q24 and encodes a 64 KDa protein consisting of 439 amino acids (Bishop 1982; Fleming WH, Hamel A, MacDonald R, Ramsey E, Pettigrew NM, Johnston B, Dodd JG 1986). *c-MYC* has been implicated in in many cellular processes cell cycle progression, chromatin remodeling, cell differentiation, genomic instability and tumorigenesis (Altman, Stine, and Dang 2016; Gurel et al. 2008). Transcription of numerous genes is known to be controlled by MYC either directly or indirectly. Several studies have shown overexpression of MYC mRNA and protein overexpression in PCa lesions when compared to normal benign tissue and BPH, from human tissues, using northern blots, fluorescence in situ hybridization and immunohistochemistry respectively (Fleming WH, Hamel A, MacDonald R, Ramsey E, Pettigrew NM, Johnston B, Dodd JG 1986; Jenkins RB, Qian J, Lieber MM 1997; Yang et al. 2005; Fox et al. 1993). The mechanism driving overexpression of MYC remains unclear in PCa. The most widely believed explanation is the amplification of the genomic region in which MYC is located is amplified in PCa. However more studies are needed to understand the mechanisms for MYC overexpression in PCa (Jenkins RB, Qian J, Lieber MM 1997; Koh et al. 2010).

The Phosphatase and tensin homolog (*PTEN*) gene, a lipid and protein phosphatase belonging to the protein tyrosine phosphatase family of genes, is located on chromosome 10q23, acts as a tumor suppressor, and is found in nearly all human tissues. The PTEN gene encodes a 47 KDa protein containing 403 amino acids and is known to play a role in cell survival and is also known to have dual specificity phosphatase activity (Di Cristofano and Pandolfi 2000; Molinari and Frattini 2014). The primary function of PTEN is to maintain regulation of the PI-3-kinase (PI3K)/PTEN/Akt pathway by dephosphorylation of PI 3,4,5-triphosphate (PIP3) to PI 4,5-biphosphate (PIP2) preventing the phosphorylation of Protein kinase B (aka Akt) and activating a downstream cascade of signaling pathways involved in cell survival and apoptosis inhibition (Vazquez and Sellers 2000; Molinari and Frattini 2014). Loss of heterozygosity as well as homozygosity of the genomic region where PTEN is located are frequent in PCa cases resulting in unregulated activation of the PI3K/Akt signaling pathway. Loss of PTEN function can result in cell proliferation, apoptosis resistance and other aspects involved in cancer progression. Loss of PTEN function either by deletion or mutation has been identified in nearly 20% of primary prostate cancer cases and goes up to nearly 50% in castration resistant cancer cases (Molinari and Frattini 2014; Jamaspishvili et al. 2018).

Prostate cancer is a complex, heterogeneous disease with many genes playing a diversity of roles in its initiation and in addition to MYC and PTEN. Prominent examples of genes playing important roles in prostate carcinogenesis include AR, BRCA2 NKX3.1, and p53. To develop effective treatment paradigms, there is an urgent need to identify candidate genes to serve as diagnostic markers and/or

therapeutic targets. Prostate specific membrane antigen (PSMA) is one such candidate. Unlike the other prostate cancer-associated genes discussed above, PSMA has no known role in prostate development or function, and its basal expression level is low. Remarkably, its expression increases substantially upon the onset of carcinogenesis and increases during disease progression. A major goal of this body of work is to develop an animal model in which the functions of PSMA in prostate cancer can be explored.

1.3 Prostate specific membrane antigen (PSMA)

1.3.1 PSMA function

PSMA was discovered in 1986 as the antigen recognized by a monoclonal antibody developed using cell extract of the human prostate adenocarcinoma cell line LnCaP. PSMA is a type II transmembrane glycoprotein that has both folate hydrolase and glutamate carboxypeptidase activity and is encoded by the Folate hydrolase I (*FOLH1*) gene. *FOLH1,* consisting of 19 exons located on chromosome 11p11.2 (Israeli et al. 1993; Carter, Feldman, and Coyle 1996). Structurally, PSMA has a 707 amino acid extracellular domain , a 24 amino acid single helical transmembrane domain, and a 19 amino acid N-terminal domain encoding a 84 KDa protein (Figure 8) (HOROSZEWICZ JS, KAWINSKI E 1986; Israeli et al. 1993). The N-terminal domain interacts with membrane scaffold proteins including, clathrin, filamin A (FLNa), and calveolin-1 that govern endocytosis of PSMA-bound substrates (Anilkumar et al. 2006; S. A. Rajasekaran et al. 2003; A. Rajasekaran 2005). The extracellular domain makes up bulk of the protein and is the site of substrate/ligand

interactions. PSMA is presumed to act as a cell surface receptor, however, its natural ligands have not yet been determined. This extracellular domain is further subdivided into three domains: the protease/catalytic, apical and the C-terminal domain or dimerization domain (Figure 8) (Barinka et al. 2004; Davis et al. 2005).

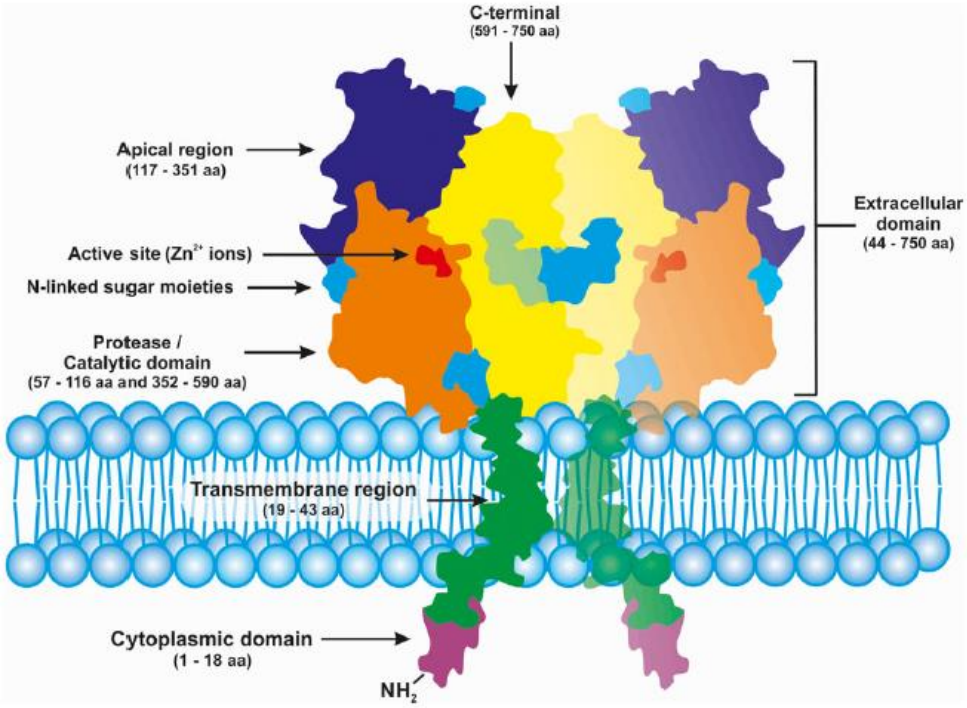

Figure 8. Schematic representation of PSMA structure. PSMA is a transmembrane protein with 707 extracellular domain, 24 amino acid transmembrane domain and 19 amino acid cytoplasmic domain, adapted from (Evans et al. 2016)

PSMA expression in a normal healthy human prostate is negligible. However, at the protein level PSMA expression increases 100-1000-fold in prostate cancer (Silver DA, Pellicer I, Fair WR, Heston WD 1997; D. G. Bostwick et al. 1998). Despite its designation, PSMA is not expressed uniquely in the prostate. Unfortunately, PSMA has been referred to in the literature using other designations, leading to considerable confusion. In the jejunal brush border cell literature, it is referred to as folate hydrolase I, whereas in the

nervous system literature it is described as N-acetyl-L-aspartyl-L-glutamate hydrolase (NAAG-Hydrolase). In literature describing its biochemical functions it is known as glutamate carboxypeptidase II (GCPII). (Israeli et al. 1993; Carter, Feldman, and Coyle 1996). In majority of the prostate cancer literature the product of FOLH1 gene is referred to as PSMA, and I will adhere to that convention in this thesis.

A major biochemical activity of PSMA first described in jejunal brush border cells is the hydrolysis of polyglutamated folates to monoglutamated folates and glutamates. The monoglutamated folates are then absorbed into the blood stream (Watt et al. 2001; Zhao, Matherly, and Goldman 2009). In the nervous system, the most abundant neuropeptide, N-acetyl-aspartyl-glutamate (NAAG) is hydrolyzed by PSMA to release glutamates and N-acetyl aspartate (NAA), which usually are recycled and are essential for normal brain function (Neale, Bzdega, and Wroblewska 2000). However, in cases when glutamate concentration exceeds a threshold, glutamate excitotoxicity can occur resulting in neurodegeneration (Ristau, O 'keefe, and Bacich 2014; Evans et al. 2016a). The biochemical function and physiological roles of PSMA in prostate cancer is yet not well understood, and a principal goal of the work presented in this dissertation is to develop an animal model in which this can be studied. The prevailing suggestion in the field to date is that in cancer cells, overexpression of PSMA leads to increased folate processing yielding glutamate that can be used in multiple catabolic pathways, thereby supporting malignant growth (Hong et al. 2022).

1.3.2 PSMA regulation

Regulation of PSMA expression in prostate gland is apparently complex and our currently partial understanding is derived from *in vitro* studies. Androgen receptor (AR) has been shown to negatively regulate PSMA, whereas several growth factors seem to positively regulate PSMA (Ghosh and Heston 2004; Israeli et al. 1993). From *in vitro* studies and known function of PSMA, Evans et al. have proposed a mechanism of PSMA regulation involving the PSMA enhancer (PSME) (Watt et al. 2001; O'Keefe et al. 2000); PSMA

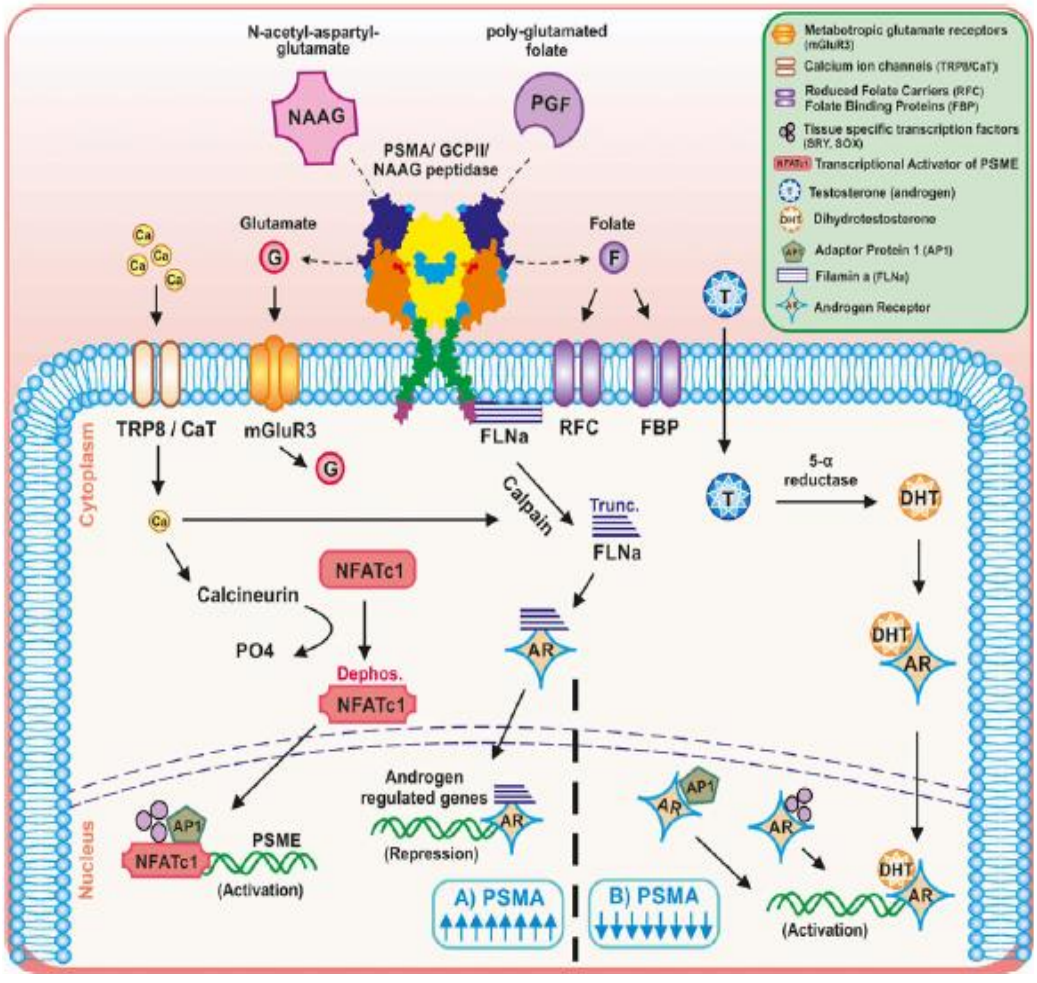

Figure 9. Schematic representation of PSMA regulation. Androgens and AR seems to negatively regulate PSMA, whereas other growth factors may be positively regulating PSMA adapted from (Evans et al. 2016)

hydrolyses polyglutamated folates into folates and glutamates. The folates and glutamates are internalized by their own specific receptors. Glutamates then bind to the metabotropic glutamate mGluR I receptor and activate the glutamatergic signaling system. Upon activation of the glutamatergic system Wissenbach et al. observed an influx of Ca^+ ions and propose Ca^+ ion dependent mTORC2 activation which in turn upregulates Akt (Wissenbach et al. 2001). Ca^+ ions influx can regulate PSMA in two ways. First, Ca^+ ions bind to calcineurin, which in turn dephosphorylates and activates NFATc1, which is a transcriptional activator of the PSME. NFATc1 then translocates to the nucleus and activates PSMA transcription. In the second pathway, Ca^+ ions activate calpain, which in turn cleaves FLNa, which is bound to the cytoplasmic domain of PSMA. The truncated form of FLNa binds to AR, which then translocates to the nucleus and suppresses AR regulated genes (Ghosh and Heston 2004). Androgens such as testosterone play an important role in regulating PSMA expression. Testosterone is converted to dihydrotestosterone (DHT) by the enzyme 5-α reductase in the prostate. DHT then binds to AR and activates it, thus localizing AR to the nucleus to bind its target genes and in concert with transcription factors which down regulate PSMA expression (Ghosh and Heston 2004). AR binding to PSME could explain the repression of PSMA under normal conditions but PSMA overexpression after androgen ablation (Figure 9) (Evans et al. 2016a).

Upon glutamate binding, activation of the mGluR I receptor also activates the downstream signaling of the PI3K/Akt/mTOR pathway via phosphorylation

the catalytic subunit p110β of PI3K which in turn leads to phosphorylation of Akt and activation of its downstream signaling independent of PTEN (Kaittanis et al. 2018b).

1.3.3 Potential roles of PSMA in prostate cancer metabolism.

Although the physiological role of PSMA in human prostate cancer has not been definitively established, the production of glutamate and monoglutamated folate from polyglutamated folates by PSMA provides ample opportunity for speculation. For example, recent studies in prostate cancer patients have shown positive correlations between increased blood serum glutamate levels and higher Gleason score as well as aggressive disease (Koochekpour et al. 2012). Importantly, serum glutamate levels also varied racially, with African American patients having higher glutamates levels that Caucasians (Koochekpour et al. 2012). Whether variation in serum glutamate plays a causal role in health disparities observed in the clinical presentation of prostate cancer among races is an interesting area for future investigation.

In addition to being the most abundant neurotransmitter, glutamate is a major source of energy for cells. As mentioned above glutamate binds a family of metabotropic glutamate receptors (mGluR's, i.e. mGluR1 and mGluR5) (Willard and Koochekpour 2013a). Interestingly, in human prostate cancer, mGlur1 has shown to be upregulated (Willard and Koochekpour 2013b; Pissimissis et al. 2009). However, free glutamates can also be internalized using the SLC7A11 glutamate/cysteine antiporter. Once inside the cell, glutamate can

enter the TCA cycle. Recently Nyugen et. al. showed in several cancer cell lines that NAAG acts as a reservoir for glutamate when supply is limited from other sources. They further showed in xenograft studies that inhibiting PSMA and glutaminase together results in decreased tumor size (T. Nguyen et al. 2019). These data are supportive of a model wherein PSMA has a pro-tumorigenic effect related to glutamate production. Another pro-tumorigenic function of PSMA has been suggested by Paschalis et. al. who observed in human prostate cancer biopsy samples that cells with aberrant DNA damage repair mechanism and loss of BRCA2 expressed higher PSMA on cancer cell membrane. As mentioned earlier PSMA's activation of PI3K-Akt signaling also suppresses homology dependent DNA repair and enhances non homologous recombination (Paschalis et al. 2019). PSMA's various properties combined with its expression profile in increasing grade of prostate cancer make it an important target to be exploited as a therapeutic and/or theranostic target. Although beyond the scope of this thesis, it is important to note that PSMA is also overexpressed in the neovasculature of many solid tumors and has also been implicated in various neurological diseases. These observations provide further impetus to develop animal models to dissect the physiological functions of this protein.

1.3.4 PSMA based diagnostic and therapeutic approaches.

The expression profile of PSMA in PCa has generated enormous interest in developing both diagnostics and targeted therapies that exploit its cell surface location. The identification of high affinity anti-PSMA antibodies as well as small molecule ligands has provided opportunities to generate drug-conjugated

delivery systems to direct therapeutic payloads preferentially to prostate cancer cells. The limited expression of PSMA in most other tissues provides a mechanism to mitigate toxicity due to off target effects. However, the relatively high expression of PSMA in the human salivary gland remains a concern (Bouchelouche and Choyke 2016). PSMA ligands conjugated with small molecule, chemotherapeutic agents, radioligands, immunotherapeutic molecules are currently being developed and one has recently been FDA approved (Szabo et al. 2015; Rowe et al. 2022).

Two distinct types of strategies have been undertaken to target PSMA in prostate cancer: those based on monoclonal antibodies, and those based on small high affinity ligands.

Immunotherapy, led by immune checkpoint inhibitors, has brought about a revolutionary transformation in the field of oncology treatments and is now considered the standard of care for numerous types of cancer (Giraudet et al. 2021). However, the effectiveness of immune checkpoint inhibitors in prostate cancer is unfortunately limited (Antonarakis et al. 2020). The lack of effectiveness is believed to stem from multiple factors such as the presence of immunosuppressive tumor microenvironment, low-levels of PD-L1 expression, a low tumor mutation burden, and other factors not yet fully understood (Rizzo et al. 2020). PSMA-targeted immunotherapy can be categorized into four major types: antibody-drug conjugates (ADC), chimeric antigen receptor T-cells, PSMA-directed vaccines, and bispecific T-cell re-directed therapy (Giraudet et al. 2021).

For example, Wang et al. developed an ADC with PSMA which was able to induce apoptosis 1000-fold higher in PSMA positive cells compared to PSMA negative cells in a mouse model of prostate cancer increasing the survival of treated mice. ADC based treatments have especially shown promise in patients that have undergone chemotherapy already (X. Wang et al. 2011). Ipilimuamab, an anti-cytotoxic T-lymphocyte antigen 4 antibody, is another example of PSMA-targeted immunotherapy that has shown an overall survival benefit when used as a single agent in phase III clinical trials (Cabel et al. 2017). PSMA's high expression in advanced and castration-resistant prostate cancer combined with its large extracellular domain makes it an ideal target for cancer immunotherapy.

Radiation therapy, since the discovery of x-rays, has been used to treat cancer. Nearly half of all cancer patients still receive radiation therapy alone or in combination with another treatment making it an important avenue for PSMA targeted radiotherapy (Delaney et al. 2005; Thariat et al. 2013). Monoclonal antibody J591 that binds the extracellular domain of PSMA and results in internalization upon binding has been conjugated to few different radionuclides. One of those ADC was the lutetium-177-J591 (177Lu-J591), which in a phase II clinical trial showed promising results with increasing survival rates and reducing PSA levels (D. P. Nguyen et al. 2016; Tagawa et al. 2013). There are many more molecules that are being explored to be used for PSMA based radiotherapy.

In addition to PSMA ligand-based treatments, efforts have also been made

to develop conventional drug inhibitors. One of the first potent inhibitors of PSMA was developed back in 1996 by Jackson et al. called 2-(phosphonomethyl) pentanedioic acid (2-PMPA). Although 2-PMPA was originally developed to treat neurological disorders, Nyugen et al. have shown the effectiveness of 2-PMPA from their *in vitro* and *in vivo* studies for treating PCa in animal models (T. Nguyen et al. 2019; Jackson et al. 1996). Given the effectiveness of PSMA ligand-based agents the future of small molecule PSMA inhibitor development remains unclear. However, it is conceivable that combinations of PSMA ligand-based and conventional small molecule inhibitors could be more clinically effective than single agent treatments.

PSMA based imaging is also another area that has been remarkably fruitful using PSMA-binding agents that are conjugated with imaging agents to provide high sensitivity prostate cancer detection. ProstaScint, the first FDA approved PSMA based imaging agent was comprised of antibody 7E11 conjugated with the radionuclide inidium-111 for whole body imaging to detect PCa lesions. The clinical success of this agent was limited due to high variability as it recognized an intracellular region of PSMA and so eventually fell out of favor (Kahn et al. 1994; Schuster et al. 2014). However, PSMA-binding small molecules ligands are now being used in conjunction with radionuclide tracers for imaging using positron emission tomography (PET) scans for detection. One of the most explored radionuclide tracers is gallium-68 (68Ga) that is conjugated with a small molecule termed PSMA-11. This 68Ga-PSMA11 PET has shown very promising results in detecting organ confined as well as metastatic disease even

in cases where PSA expression is low (A. Afshar-Oromieh et al. 2012). Another small molecule with high affinity for PSMA that has been developed recently is termed PSMA-617, which has the advantage of a rapid plasma clearance rate. PSMA-617 is currently being tested with various radionuclides for either imaging and therapy or as a theranostic agent wherein imaging and treatment are accomplished by the same agent (Ali Afshar-Oromieh et al. 2015; Sun et al. 2021). Recently, FDA granted approval for a PSMA-targeted small molecule PET-agent, Pyl, that is able to detect not just primary cancerous lesion but also metastatic lesions with high accuracy(Rowe et al. 2022).

Given the clear advantages of targeting PSMA for both diagnostic and therapeutic purposes, it is likely that this will remain an active area of investigation. However, the advancement of new agents toward the clinic would be greatly facilitated by the availability of a rodent animal model that expresses human PSMA in the prostate gland and in autochthonous prostate cancer. A majority of the work in this thesis is directed toward achieving the goal of developing such a model.

Section 1.4 Rodent models of prostate cancer

The lack of effective treatments for castration resistant prostate cancer is a pressing problem that needs to be addressed. One of the avenues of research to improve our understanding of the molecular mechanisms involved in prostate cancer initiation and progression and, to test new therapies has been to develop animal models which can recapitulate human prostate cancer. These models can be used to develop novel diagnostic as well as therapeutic agents for clinical use. The types of

animal models that have historically been used for this purpose can be broadly divided into two types: xenograft models using immunocompromised rodents, and genetically engineered immunocompetent transgenic rodent models.

1.4.1 Xenograft model

By a wide margin, the most extensively used models are based on xenografts generated by subcutaneous implantation of cell line-derived human prostate cancer cells. Less often, these cells are transplanted directly into the mouse prostate to generate orthotopic tumors. In either case such models are referred to as cell-line derived xenografts (CDX). CDX models have been the most widely used due mainly to the fact that they are not technically demanding and can be useful for rapid analysis of drug response. Depending on the number of cells implanted and the cell line used, tumors can develop within days to a few weeks. Another significant advantage of CDX's is that cells can be genetically manipulated prior to implantation, for example, to increase or dimmish expression of specific genes to explore their potential roles in cancer progression.

Another xenograft strategy involves the subcutaneous implantation of fragments of tumors derived directly from patients. These so-called patient derived xenografts (PDX) have proven to be quite difficult to establish in prostate cancer cases. As a result, and due to the difficulty in accessing patient material for many investigators, prostate PDX are not widely used. Because they are generated in immunocompromised mice, both CDX and PDX xenografts strategies cannot faithfully mimic the tumor immune microenvironment as it occurs in patients. As a result, these types of models are now considered secondary to immunocompetent

transgenic models (Figure 10) (Richmond and Su 2008; Samuel Aparicio, Manuel Hindalgo 2015; Grabowska et al. 2014).

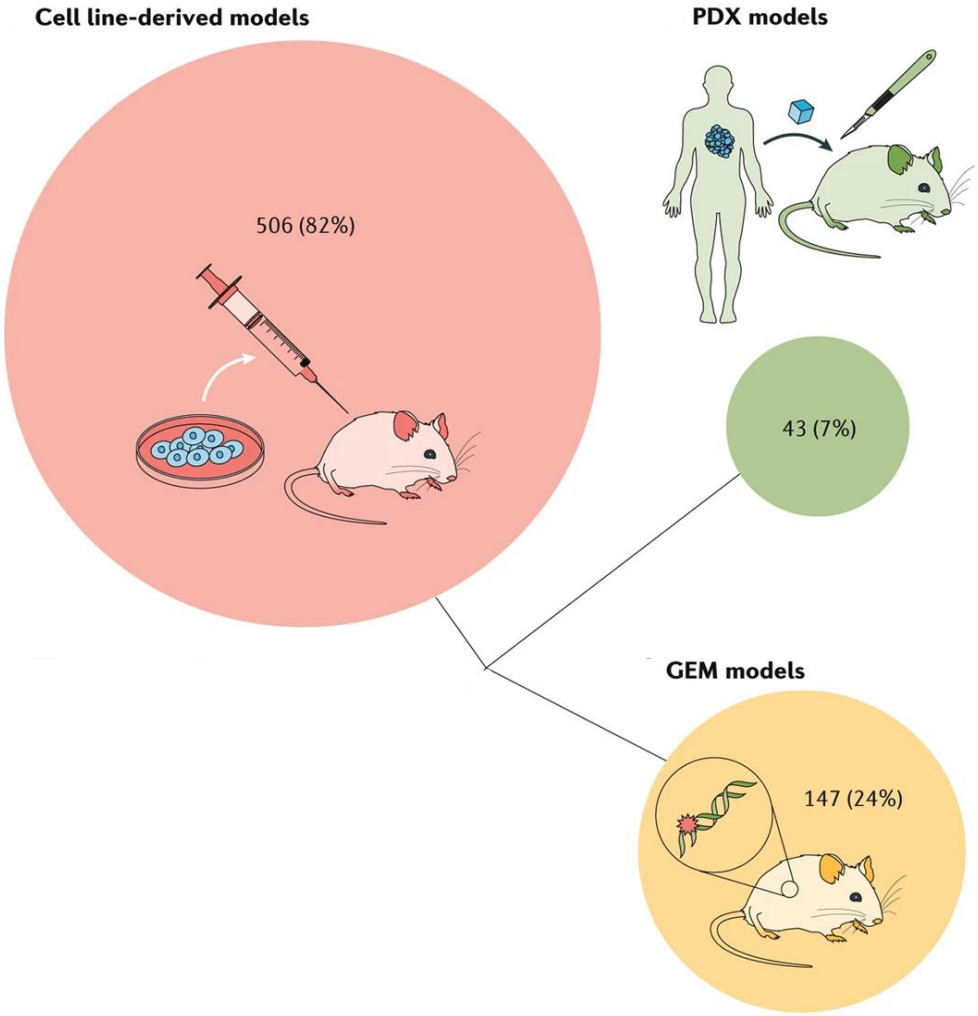

Figure 10. Schematic representation of different types of mouse models used in cancer research. CDX models are by far the most widely used in pre-clinical studies, due to the ease of generating them and providing rapid analysis, followed by GEM models and PDX respectively. GEM models are the only ones capable of recapitulating key molecular features in an immunocompetent animal, adapted from (Geaenbacher, Singhal, and Augustin 2017).

The mouse models of human cancer consortium (MMHCC) was formed by the National institutes of Health in 1999 in an effort to develop models of human cancers (Grabowska et al. 2014). Over the past two decades, progress has been made

in generating transgenic mouse models which closely recapitulate key features of human prostate cancer. Transgenic mouse models offer several clear advantages over xenograft models. (1) They can be designed to carry genomic changes that are thought to be drivers of human prostate cancer; (2) the tumor immune microenvironment is retained; (3) conditional gene expression can be achieved to study specific events at different ages; (4) pre-malignant and early cancer lesions be studied (Richmond and Su 2008).

1.4.2 Mouse models of prostate cancer: a brief overview

The primary objective of the first generation of genetically engineered mouse models (GEM) was to induce tumor formation in the mouse prostate gland by any means necessary. Transgenic adenocarcinoma of the mouse prostate (TRAMP) was the first model developed and used the prostate-specific rat probasin (PB) promoter to express both the large and small Simian virus 40 (SV40) tumor antigens. TRAMP mice had developed invasive carcinoma and lymph node metastases by 28 weeks. Although TRAMP contributed to our understanding of the molecular basis of PCa initiation and progression, a majority of the cancers that develop in this model have neuroendocrine features, and do not accur ly mimic human prostate adenocarcinoma (Greenberg et al. 1994, 1995; Grabowska et al. 2014).

The second generation of gain-of-function transgenic mouse prostate cancer models was ɒased on prostate-specific expression of the human MYC oncogene under the control of the PB promoter (termed Lo-MYC) and a modified PB promoter with additional AR binding sites (termed Hi-MYC) (Ellwood-Yen et al. 2003). Both Lo-MYC and Hi-MYC models more accurately phenocopied initiation and

progression of human adenocarcinoma, however, neither model developed highly aggressive disease with high penetrance. The first loss-of-function model used Cre-lox technology to disable the mouse Pten gene in a prostate-specific manner (Wu et al. 2001). Mice with homozygous loss of Pten function developed invasive cancers, albeit with slow kinetics, and metastasis was rare. These tumors also often had sarcomatoid features, which are rare in human prostate cancer cases. Thus, neither gain of human MYC nor loss of mouse Pten was sufficient to closely mimic highly aggressive human prostate cancer.

To develop a third-generation mouse prostate cancer model, our lab has developed a mouse model, termed BMPC, which combines overexpression of the human *MYC* oncogene in combination with genomic loss of tumor suppressor *Pten* in mouse prostate epithelial cells. In this model, the *Hoxb13* promoter drives *MYC* expression and directs *Pten* loss using the Cr-lox system, also under the control of the Hoxb13 promoter. BMPC mice faithfully mimic both early and advanced stages of aggressive human prostate cancer at the phenotypic and genotypic levels (Sreenath *et al.*, 1999; McMullin *et al.*, 2009; Hubbard *et al.*, 2016). In addition, the BMPC model has also shown that *Pten* loss combined with *MYC* overexpression leads to genomic instability, a hallmark of human prostate cancer, giving rise to adenocarcinomas that metastasize to several of the same distant organs as observed in human prostate cancer cases (Gretchen K Hubbard et al. 2016). Other third generation mouse models have been developed, however, none recapitulate the histopathologic and metastatic features of human prostate cancer as closely as the BMPC model (Aytes et al. 2013).

1.4.3 Rat models of prostate cancer.

Although currently, the most advanced rodent prostate cancer models are in mice, historically, rat models predate those in mice. Interestingly, only three species are known to develop spontaneous prostate cancer: humans, dogs, and rats. Why might it be advantageous to have rat models of prostate cancer as opposed to mouse? One reason is related to the fact in that the pharmaceutical industry, rats have historically been the species of choice for analyses of drug pharmacokinetics and toxicity. Thus, an abundant literature exists that describes rat drug metabolism. For some classes of drugs, rat metabolism more closely follows that in humans when compared to mouse drug metabolism. In addition, due to their larger body size, interventional studies (i.e. surgeries, implantation of cells, *etc*.) are less technically demanding. In addition, the higher blood volume of rats compared to mice provides more material for pharmacokinetic and metabolomic studies.

The first report of a spontaneous rat tumor was published by Dr. Dunning back in 1963, who observed a prostate tumor in an ~2-year-old Copenhagen strain rat (Tennant et al. 2000). In subsequent decades, other strains of rats have also been identified that develop spontaneous PCa, including Wistar rats, A X C rats, and ACI/SEG rats (M Pollard 1973; Shain, McCullough, and Segaloff 1975; T.Issacs 1984). The main drawback of these spontaneous rat models of autochthonous PCa is the long latency and low percentage of the cancer phenotype, which has prevented them form being widely used (Tennant et al. 2000).

To exploit his observation of spontaneous rat tumor formation and to overcome the challenges of long latency and low phenotypic penetrance, Dunning

developed an elegant allograft tumor system by establishing a cell line from one of the tumors (Marvin Rubenstein et al. 1995). After expanding the cell line in culture, he demonstrated its ability to generate flank allograft tumors, and later, orthotopic tumors in Copenhagen rats (D M Lubaroff, C W Reynolds 1978; Weber, Sinowatz, and Chandler 1982). Multiple sublines of the original Dunning cell line have also been derived (Tennant et al. 2000). The Dunning model is useful in that it does mimic important features of human prostate adenocarcinoma, including androgen dependence and metastasis to distant organs.

Rat xenograft models are not commonly used due to lack of immunodeficient strains and easily available mouse strains (Nascimento-Gonçalves et al. 2018; Tennant et al. 2000).

In some rat strains that do not spontaneously develop prostate cancers, it is possible to induce tumor formation by administration of chemicals and or hormones. For example, N-nitrosobis (2-oxopropyl) amine, N-methyl-N-nitrosourea, and 17β-estradiol (Pour 1981; Morris Pollard 1973; Özten et al. 2010).

In addition to spontaneous, allograft, and induced rat prostate cancer models, several transgenic rat models have been generated. To create the so-called transgenic rat with adenocarcinoma of the prostate (TRAP) model, the PB promoter was used to drive the SV40 large and small T antigens in the Sprague-Dawley strain. TRAP males develop androgen dependent tumors in the ventral and dorsolateral lobes, with onset at four weeks and are invasive by fifteen weeks (Asamoto; et al. 2001; Shirai et al. 2000). TRAP rats have also been crossed with the Lewis rat strain and the onset and

disease progression is similar to TRAP rats with 100% penetrance and were developed for cancer immunotherapy (Johnson et al. 2013).

Rodent prostate cancer models that accurately mimic histopathological and metabolic features of human prostate cancer provide critical reagents to probe the molecular basis of malignancy, and to test new therapeutic and surgical interventions. Given the inherent heterogeneity of human prostate cancer, it is unlikely that any one model will provide an optimal platform for all experimental lines of inquiry. Practically speaking, that suggests that models will continue to evolve and diversify as our understanding of the genetic, epigenetic, and environmental factors that influence human prostate cancer initiation and progression matures. Developing these models is time consuming, labor intensive, and, particularly in the case of transgenic models, technically demanding. Fortunately, the development of methods to modify the genome in precise ways using for example, CRISPR, customized zinc finger endonucleases, and TALENS has provided an incredibly powerful toolset to support transgenic model development. However, the increasing complexity of in vivo genomic manipulations has created a parallel need for in vitro technologies that generate evermore complex DNA assemblies that are subsequently used to accomplish the desired genetic manipulation. For example, the generation of multi-functional transgenes capable of directing expression of multiple open reading frames driven by a specific promoter/enhancer combination is often as time consuming and labor intensive as deriving the animal model itself. Furthermore, creating complex DNA assemblies is requires specific expertise that is not widely distributed in the research community. This may be due in part to the increasing commercialization of

general DNA cloning technologies. Whereas in past decades, conventional DNA cloning was a fundamental skill in which virtually all molecular biologists were trained, the advent of cost-effective commercial cloning services has fundamentally altered that training landscape. Now, many investigators simple purchase the DNA constructs they require. While this situation may be more efficient for simple cloning projects, there are few commercial vendors that offer services to create, *de novo*, the often-large complex DNA assemblies that are needed to create complex animal disease models. Thus, there is a need to both optimize existing DNA assembly approaches and to develop new methods to achieve a democratization of this important technological skill. In the course of this thesis work, the need became apparent for a complex DNA assembly to move the project forward. It was at that juncture that we paused our efforts to develop a PSMA-expressing rat model of human prostate cancer and pivoted toward overcoming the low efficiency of an existing DNA assembly technology.

1.5 DNA assembly techniques

The advent of DNA cloning technology revolutionized the field of biological science and our understanding of the complex mechanisms involved in gene function in normal physiology and disease (Ostrov et al. 2019). Developments in cloning technology over the last few decades have given us tools to write, or re-write, partial, or whole genomes in prokaryotic as well as eukaryotic organisms (Casini et al. 2015). Advancements in the assembly of DNA fragments into larger constructs are a crucial technology driving the vision behind synthetic biology (Ellis, Adie, and Baldwin 2011). Synthetic biology is an emerging field that aims to redesign and engineer

organisms with partial or entirely, new genomes using recombinant DNA technology for specific end goals (Ostrov et al. 2019). Synthetic biology is currently being applied in various disciplines to address their respective needs. Very early stages of synthetic biology trace back to the advent of molecular cloning using cohesive ends enabled by the discovery of restriction enzymes (Pingoud, Wilson, and Wende 2014; Loenen et al. 2014). The invention of PCR and its subsequent use in mainstream research brought about a paradigm shift in molecular biology and its applications, as custom double stranded DNA molecules could now be synthesized with user specified length and sequence (Zhu et al. 2020). The human genome sequencing project, that started in the late 1980's, also turbocharged the development of new technologies in molecular biology that made genetic engineering techniques readily available to mainstream researchers in terms of cost and accessibility (Olson 1993; Guyer and Collins 1993; Lanchbury 1998; Greenhalgh 2005; Moraes and Góes 2016). All these advancements in molecular cloning led us to a deeper understanding of gene functions and their roles in different molecular pathways involved in normal physiology and disease progression. However, to enable construction of multifunctional constructs it is important to have efficient and dependable DNA assembly techniques. An increasingly evident limiting factor constraining the progress of synthetic biology are the limitations of currently available DNA assembly techniques (Ellis, Adie, and Baldwin 2011; Casini et al. 2015).

1.5.1 BioBrick® Assembly

The goal of most DNA assemblies is to create an ordered arrangements of DNA fragments to create a larger DNA construct. For example, in

the case of creating a transgene to support the development of a new transgenic animal model, one might desire to assemble, in order, an enhancer, a 5' untranslated region (UTR), a promoter, one or more open reading frames, a 3', and a polyadenylation signal. In the early days of restriction enzyme-based DNA cloning this type of assembly was generally created using doubly digested DNA fragments to direct the order of assembly. Theoretically, only compatible 5' or 3' overhangs would be ligated, and many fragments could be joined. In practice, T4 DNA ligase would often tolerate mismatches in overhangs, resulting in unproductive ligation events. Experimentally, the rate of cloning success using this strategy diminishes exponentially as the number of fragments increases. As a result, the practical upper limit is 3-4 fragments, unless heroic measures to screen tens of thousands of resultant colonies are undertaken. In 2003, Tom Knight attempted to systematize and standardize this approach under the term BioBrick Assembly (Figure 12). In BioBrick, DNA "parts" has standardized 'prefix' and 'suffix' sequences that equate to restriction enzyme recognition sequences. The original enzymes in BioBrick assembly were EcoRI, XbaI, SpeI, and PstI. Care has to be taken that these same restriction sites are not present in the part being cloned. A drawback of this technique is the introduction of a scar sequence after ligation, which can potentially introduce unwanted features, for example, stop codons (Knight 2003; Ellis, Adie, and Baldwin 2011).

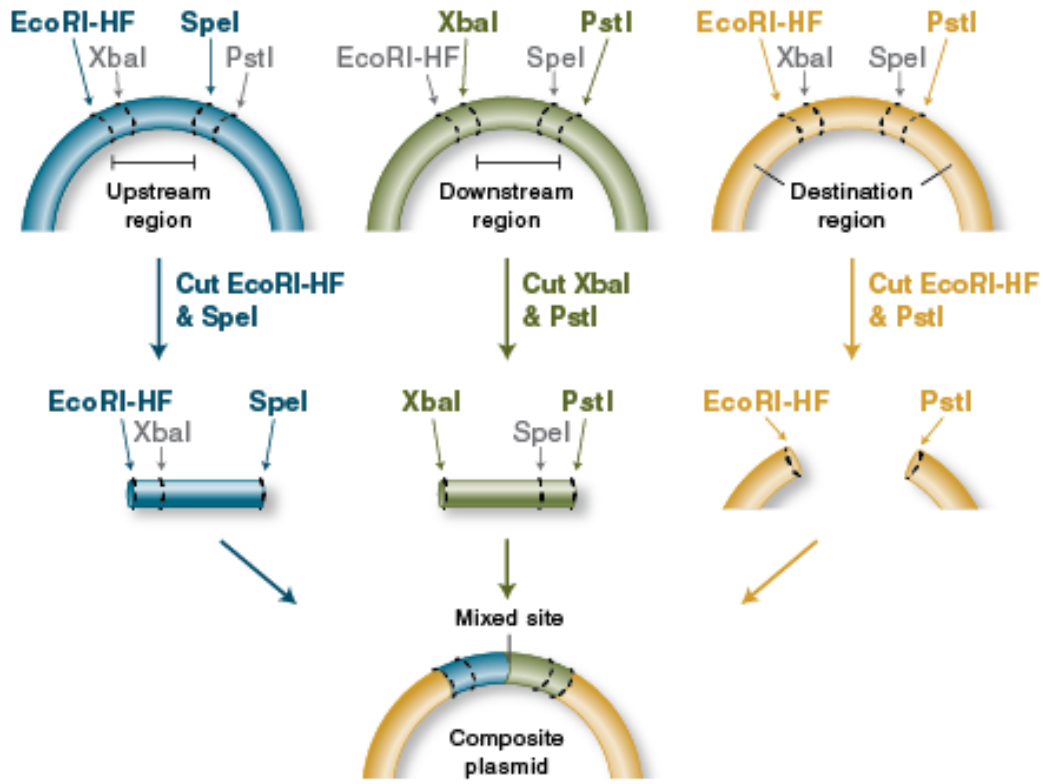

Figure 11. Schematic representation of BioBrick Assembly. BioBrick assembly has DNA fragments in a standardized manner in a registry that are flanked by identical restriction enzyme sites, that are not in the insert, thus allowing assembly of products, adapted from New England BioLabs.

1.5.2 Gibson Assembly

The Invention of Gibson DNA Assembly (GDA) cloning in 2009 was a significant milestone in the progress towards generating ordered joining of DNA fragments. In addition, GDA is the first truly scarless assembly technique since cohesive DNA ends are not generated by restriction enzyme digestion (Gibson et al. 2009b). GDA ushered in a new era of building precisely designed and scarless ordered DNA assemblies using homology-driven overlaps between adjacent DNA fragments (Figure 13). GDA is a single tube reaction using three enzymatic activities: exonuclease, polymerase, and ligase, to assemble the complete construct. A 5` DNA exonuclease ‘chews back’ the 5` end of each DNA fragment exposing single stranded

complimentary sequences for annealing to an adjacent fragment that has been designed to share end homology. The DNA polymerase activity then fills in the gaps in the annealed region, and lastly, DNA ligase seals the remaining nick and covalently links the DNA fragments together.

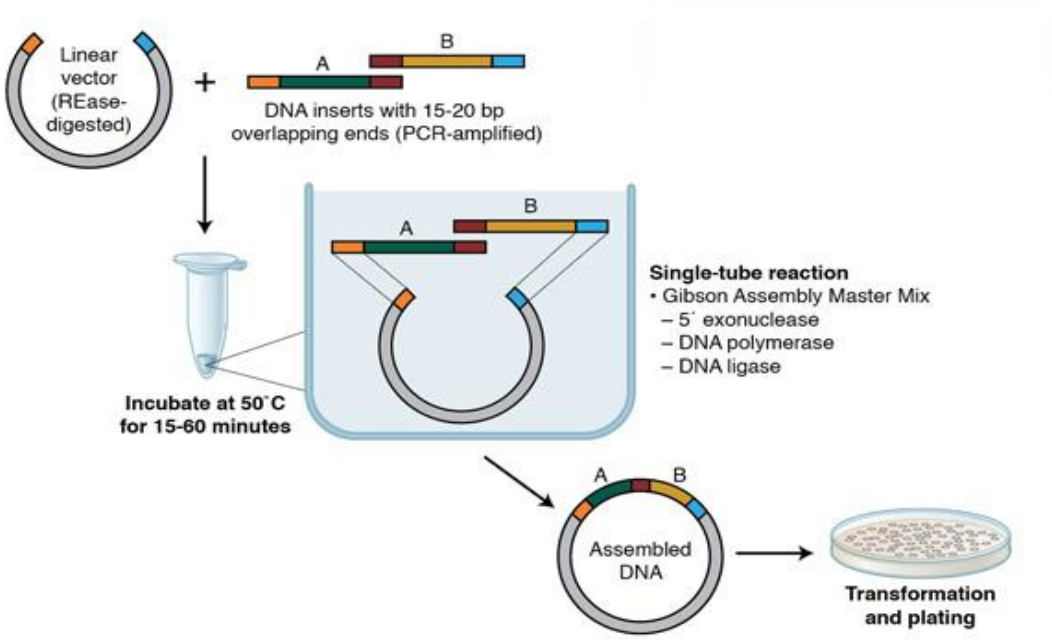

Figure 12. Schematic representation of the Gibson Assembly reaction. Gibson assembly uses three enzymes with complimentary functions to assemble double stranded DNA fragments in an ordered manner. 5' exonuclease chews back the ends of individual DNA fragment thus exposing the complimentary sequences that anneal to each other, DNA polymerase uses these annealed complimentary sequences and fills in the gaps and the DNA ligase seals the nicks together, adapted for New England BioLabs.

However, GDA has technical limitations including the size and number of DNA fragments that can be practically joined. The efficiency of GDA assembly drops significantly when joining together more than five DNA fragments and/or joining together DNA large fragments (Roth, Milenkovic, and Scott 2014; Gibson et al. 2009a). In addition, DNA sequence errors can be introduced due to the use of error-prone polymerases as well as the infidelity of DNA ligases. GDA is a labor intensive

and time-consuming process. GDA begins with linear DNA molecules, which necessitates linearization of circular vectors using restriction enzymes, typically followed by gel purification. The linearized vector is then combined with Gibson assembly mix to join all the DNA fragments and vector into the desired final circular DNA construct (Gibson et al. 2009b).

1.5.3 Golden Gate Assembly

Golden Gate Assembly (GGA) is one of the most widely used DNA assembly techniques to achieve cloning of multiple DNA fragments simultaneously and directionally using user defined homologous ends *in vitro* (Figure 14). GGA involves the use of Type IIS restriction enzymes, these enzymes recognize specific 5-7 nucleotide sequences, then cleave the DNA ~20 nucleotides distant from the recognition site. Thus, Type IIS restriction enzymes cut DNA at defined locations

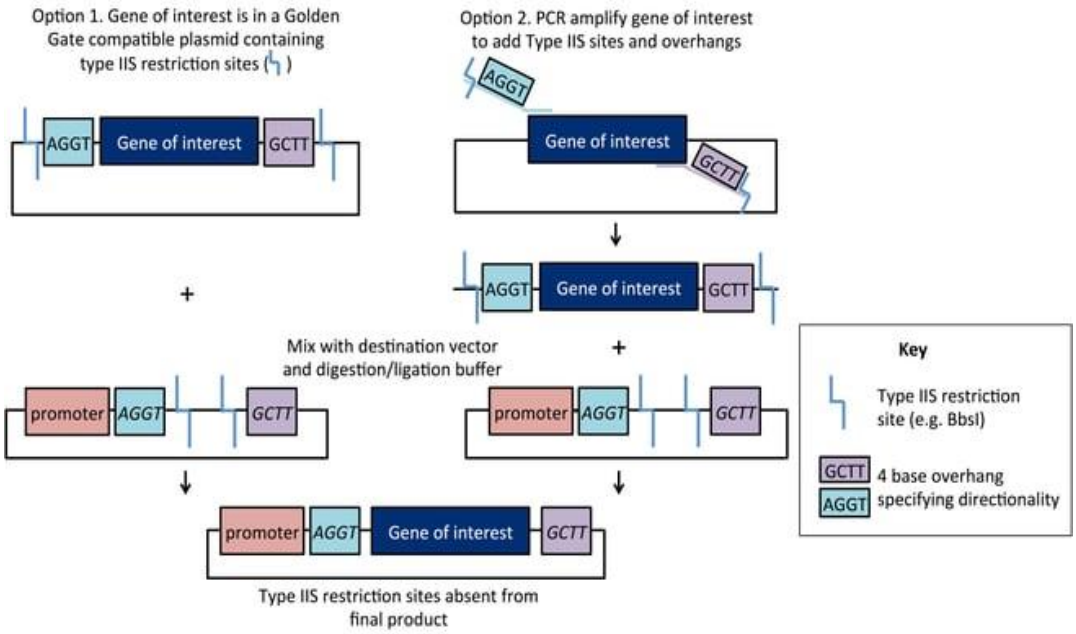

Figure 13. Schematic representation of the Golden Gate Assembly. GGA utilizes Type IIS restriction enzymes to insert the gene of interest in a compatible golden gate vector, both the insert and vector have complimentary 4-base overhang sequences that directs assembly, adapted from Addgene.

generating compatible overhangs within any DNA sequence. In GGA, the Type IIS recognition sites are generated by PCR and are eliminated from the final construct, allowing for scarless DNA assembly.

Advantages of GGA include the fact that it is a 30-minute, one tube reaction and can join up to 24 fragments in a user-defined order. However, it is not 100% sequence independent since care must be taken that the fragments to be inserted do not have the Type IIS recognition sequence.

Summary

The principal goal of this thesis project was to develop a rodent model of human prostate adenocarcinoma that recapitulates a key feature of the disease: expression of PSMA on the surface of prostate cancer cells. We began to address that goal by attempting to generate transgenic mice that express human PSMA in the prostate gland, with a view towards breeding that phenotype into the existing BMPC model described above. When those efforts stalled, we pivoted to attempt to develop a transgenic rat model with human PSMA expression in the prostate that could be bred to a rat model of prostate cancer that was being developed in parallel by another student. We achieved resounding success in developing a PSMA-expressing rat model, however development of the rat cancer model has proved to be elusive to date. In Chapter Two, we describe the results relating to development of the PSMA-expressing rodents. In the process of achieving these goals, we also made discoveries that allowed us to substantially improve upon existing homology-based DNA assembly workflows, increasing efficiency. Those data are described in Chapter

Three. In Chapter Four, we describe the implications of this body of work, and suggest future directions of investigations.

1.6 ***References***

Abate-Shen, C MM Shen - Genes & Development, and Undefined 2000. 2000. "Molecular Genetics of Prostate Cancer." *Genesdev.Cshlp.Org*. https://doi.org/10.1101/gad.819500.

Abeshouse, Adam, Jaeil Ahn, Rehan Akbani, Adrian Ally, Samirkumar Amin, Christopher D. Andry, Matti Annala, et al. 2015. "The Molecular Taxonomy of Primary Prostate Cancer." *Cell* 163 (4): 1011–25. https://doi.org/10.1016/J.CELL.2015.10.025.

Afshar-Oromieh, A., U. Haberkorn, M. Eder, M. Eisenhut, and C. M. Zechmann. 2012. "[68Ga]Gallium-Labelled PSMA Ligand as Superior PET Tracer for the Diagnosis of Prostate Cancer: Comparison with 18F-FECH." *European Journal of Nuclear Medicine and Molecular Imaging* 39 (6): 1085–86. https://doi.org/10.1007/S00259-012-2069-0.

Afshar-Oromieh, Ali, Henrik Hetzheim, Clemens Kratochwil, Martina Benesova, Matthias Eder, Oliver C Neels, Michael Eisenhut, et al. 2015. "The Theranostic PSMA Ligand PSMA-617 in the Diagnosis of Prostate Cancer by PET/CT: Biodistribution in Humans, Radiation Dosimetry, and First Evaluation of Tumor Lesions." *Journal of Nuclear Medicine : Official Publication, Society of Nuclear Medicine* 56 (11): 1697–1705. https://doi.org/10.2967/jnumed.115.161299.

Altman, Brian J., Zachary E. Stine, and Chi V. Dang. 2016. "From Krebs to Clinic:

Glutamine Metabolism to Cancer Therapy." *Nature Reviews Cancer* 16 (10): 619–34. https://doi.org/10.1038/nrc.2016.71.

Amin, MB, and SK Tickoo. 2022. *Diagnostic Pathology: Genitourinary E-Book*. https://books.google.com/books?hl=en&lr=&id=ISl3EAAAQBAJ&oi=fnd&pg=PP1&ots=U1LiDG4jvL&sig=sn7egP1AzZvjWqS7IBsM82xGHBg.

Anilkumar, Gopalakrishnapillai, Sonali P Barwe, Jason J Christiansen, Sigrid A Rajasekaran, Donald B Kohn, and Ayyappan K Rajasekaran. 2006. "Association of Prostate-Specific Membrane Antigen with Caveolin-1 and Its Caveolae-Dependent Internalization in Microvascular Endothelial Cells: Implications for Targeting to Tumor Vasculature." https://doi.org/10.1016/j.mvr.2006.03.004.

Asamoto;, Makoto, Naomi Hokaiwado;, Young-Man Cho;, Satoru Takahashi;, Yoshihisa Ikeda;, Katsumi Imaida;, and Tomoyuki Shirai. 2001. "Prostate Carcinomas Developing in Transgenic Rats with SV40 T Antigen Expression under Probasin Promoter Control Are Strictly Androgen Dependent." *Cancer Research* 61 (12): 4693–4700. https://aacrjournals.org/cancerres/article/61/12/4693/507602/Prostate-Carcinomas-Developing-in-Transgenic-Rats.

Barinka, Cyril, Pavel Sácha, Jan Sklenár, Petr Man, Karel Bezouska, Barbara S Slusher, and Jan Konvalinka. 2004. "Identification of the N-Glycosylation Sites on Glutamate Carboxypeptidase II Necessary for Proteolytic Activity." *Protein Science : A Publication of the Protein Society* 13 (6): 1627–35. https://doi.org/10.1110/ps.04622104.

Barnas, Jennifer L., Steven Pierpaoli, Patricia Ladd, Rolando Valenzuela, Nadid

Aviv, Marilyn Parker, W. Bedford Waters, Robert C. Flanigan, and John P. Mulhall. 2004. “The Prevalence and Nature of Orgasmic Dysfunction after Radical Prostatectomy.” *BJU International* 94 (4): 603–5. https://doi.org/10.1111/J.1464-410X.2004.05009.X.

Baulieu, E. E., Ilse Lasnitzki, and P. Robel. 1968. “Metabolism of Testosterone and Action of Metabolites on Prostate Glands Grown in Organ Culture.” *Nature* 219 (5159): 1155–56. https://doi.org/10.1038/2191155A0.

Berry, S. J., D. S. Coffey, P. C. Walsh, and L. L. Ewing. 1984. “The Development of Human Benign Prostatic Hyperplasia with Age.” *The Journal of Urology* 132 (3): 474–79. https://doi.org/10.1016/S0022-5347(17)49698-4.

Bessede, Thomas, and Jean-Jacques Patard. 2012. “Words of Wisdom. Re: Germline Mutations in HOXB13 and Prostate-Cancer Risk.” *European Urology* 61 (5): 1062. https://doi.org/10.1016/j.eururo.2012.02.010.

Bhatia-Gaur, Rajula, Annemarie A. Donjacour, Peter J. Sciavolino, Minjung Kim, Nishita Desai, Peter Young, Christine R. Norton, et al. 1999. “Roles for Nkx3.1 in Prostate Development and Cancer.” *Genes and Development* 13 (8): 966–77. https://doi.org/10.1101/GAD.13.8.966.

Bieberich, Charles J., Kazuyuki Fujita, Wei Wu He, and Gilbert Jaj. 1996. “Prostate-Specific and Androgen-Dependent Expression of a Novel Homeobox Gene.” *The Journal of Biological Chemistry* 271 (50): 31779–82. https://doi.org/10.1074/JBC.271.50.31779.

Bishop, J. Michael. 1982. “Retroviruses and Cancer Genes.” *Advances in Cancer Research* 37 (C): 1–32. https://doi.org/10.1016/S0065-230X(08)60880-5.

Bostwick, David G., Anna Pacelli, Michael Blute, Patrick Roche, and Gerald P. Murphy. 1998. “Prostate Specific Membrane Antigen Expression in Prostatic Intraepithelial Neoplasia and Adenocarcinoma: A Study of 184 Cases.” *Cancer* 82 (11): 2256–61. https://doi.org/10.1002/(SICI)1097-0142(19980601)82:11<2256::AID-CNCR22>3.0.CO;2-S.

Bostwick, David G., and Junqi Qian. 2004. “High-Grade Prostatic Intraepithelial Neoplasia.” *Modern Pathology : An Official Journal of the United States and Canadian Academy of Pathology, Inc* 17 (3): 360–79. https://doi.org/10.1038/MODPATHOL.3800053.

Bostwick, DG, A Pacelli, A Lopez-Beltran - The Prostate, and Undefined 1996. 1996. “Molecular Biology of Prostatic Intraepithelial Neoplasia.” *Prostate*, 117–34. https://onlinelibrary.wiley.com/doi/abs/10.1002/(SICI)1097-0045(199608)29:2%3C117::AID-PROS7%3E3.0.CO;2-C.

Bouchelouche, Kirsten, and Peter L. Choyke. 2016. “Prostate-Specific Membrane Antigen Positron Emission Tomography in Prostate Cancer: A Step toward Personalized Medicine.” *Current Opinion in Oncology*. Lippincott Williams and Wilkins. https://doi.org/10.1097/CCO.0000000000000277.

Bray, Freddie, Jacques Ferlay, Isabelle Soerjomataram, Rebecca L. Siegel, Lindsey A. Torre, and Ahmedin Jemal. 2018. “Global Cancer Statistics 2018: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries.” *CA: A Cancer Journal for Clinicians* 68 (6): 394–424. https://doi.org/10.3322/caac.21492.

Brennen, W. Nathaniel, and John T. Isaacs. 2018. “Mesenchymal Stem Cells and the

Embryonic Reawakening Theory of BPH." *Nature Reviews Urology* 15 (11): 703–15. https://doi.org/10.1038/S41585-018-0087-9.

Buskin, Adriana, Parmveer Singh, Oliver Lorenz, Craig Robson, Douglas W. Strand, and Rakesh Heer. 2021. "A Review of Prostate Organogenesis and a Role for IPSC-Derived Prostate Organoids to Study Prostate Development and Disease." *International Journal of Molecular Sciences 2021, Vol. 22, Page 13097* 22 (23): 13097. https://doi.org/10.3390/IJMS222313097.

Carter, Ruth E, Alexis R Feldman, and Joseph T Coyle. 1996. "Prostate-Specific Membrane Antigen Is a Hydrolase with Substrate and Pharmacologic Characteristics of a Neuropeptidase." *Neurobiology* 93: 749–53. http://www.pnas.org/content/93/2/749.long.

Casini, Arturo, Marko Storch, Geoffrey S. Baldwin, and Tom Ellis. 2015. "Bricks and Blueprints: Methods and Standards for DNA Assembly." *Nature Reviews Molecular Cell Biology 2015 16:9* 16 (9): 568–76. https://doi.org/10.1038/nrm4014.

Chen, Ronald C., R. Bryan Rumble, D. Andrew Loblaw, Antonio Finelli, Behfar Ehdaie, Matthew R. Cooperberg, Scott C. Morgan, et al. 2016. "Active Surveillance for the Management of Localized Prostate Cancer (Cancer Care Ontario Guideline): American Society of Clinical Oncology Clinical Practice Guideline Endorsement." *Journal of Clinical Oncology : Official Journal of the American Society of Clinical Oncology* 34 (18): 2182–90. https://doi.org/10.1200/JCO.2015.65.7759.

Chowdhury-Paulino, Ilkania M., Caroline Ericsson, Randy Vince, Daniel E. Spratt,

Daniel J. George, and Lorelei A. Mucci. 2021. “Racial Disparities in Prostate Cancer among Black Men: Epidemiology and Outcomes.” *Prostate Cancer and Prostatic Diseases 2021 25:3* 25 (3): 397–402. https://doi.org/10.1038/s41391-021-00451-z.

Cory Abate-Shen, and Michael M Shen. 2002. “Mouse Models of Prostate Carcinogenesis.” *Trends in Genetics* 18 (5): S1–5. https://doi.org/10.1016/S0168-9525(02)02683-5.

Cristofano, Antonio Di, and Pier Paolo Pandolfi. 2000. “The Multiple Roles of PTEN in Tumor Suppression.” *Cell* 100 (4): 387–90. https://doi.org/10.1016/S0092-8674(00)80674-1.

Cunha, Gerald R., Mei Cao, Omar Franco, and Laurence S. Baskin. 2020. “A Comparison of Prostatic Development in Xenografts of Human Fetal Prostate and Human Female Fetal Proximal Urethra Grown in Dihydrotestosterone-Treated Hosts.” *Differentiation; Research in Biological Diversity* 115 (September): 37. https://doi.org/10.1016/J.DIFF.2020.06.001.

Cunha, Gerald R., Paul S. Cooke, and Takeshi Kurita. 2004. “Role of Stromal-Epithelial Interactions in Hormonal Responses.” *Archives of Histology and Cytology* 67 (5): 417–34. https://doi.org/10.1679/AOHC.67.417.

Cunha, Gerald R., Chad M. Vezina, Dylan Isaacson, William A. Ricke, Barry G. Timms, Mei Cao, Omar Franco, and Laurence S. Baskin. 2018. “Development of the Human Prostate.” *Differentiation; Research in Biological Diversity* 103 (September): 24. https://doi.org/10.1016/J.DIFF.2018.08.005.

Davis, Mindy I, Melanie J Bennett, Leonard M Thomas, and Pamela J Bjorkman.

2005. “Crystal Structure of Prostate-Specific Membrane Antigen, a Tumor Marker and Peptidase.” *British Journal of Pharmacology* 102 (17): 5981–86. https://doi.org/doi: 10.1073/pnas.0502101102.

Delaney, Geoff, Susannah Jacob, Carolyn Featherstone, and Michael Barton. 2005. “The Role of Radiotherapy in Cancer Treatment: Estimating Optimal Utilization from a Review of Evidence-Based Clinical Guidelines.” *Cancer* 104 (6): 1129–37. https://doi.org/10.1002/CNCR.21324.

DeMarzo AM, Nelson WG, Isaacs WB, Epstein JI. 2003. “Pathological and Molecular Aspects of Prostate Cancer.” *The Lancet* 361 (9361): 955–64. https://doi.org/10.1016/S0140-6736(03)12779-1.

DeMarzo, Angelo M., William G. Nelson, William B. Isaacs, and Jonathan I. Epstein. 2003. “Pathological and Molecular Aspects of Prostate Cancer.” *Lancet (London, England)* 361 (9361): 955–64. https://doi.org/10.1016/S0140-6736(03)12779-1.

Dutta, Aditya, Clémentine Le Magnen, Antonina Mitrofanova, Xuesong Ouyang, Andrea Califano, and Cory Abate-Shen. 2016. “Identification of an NKX3.1-G9a-UTY Transcriptional Regulatory Network That Controls Prostate Differentiation.” *Science* 352 (6293): 1576–80. https://doi.org/10.1126/SCIENCE.AAD9512.

Ellis, Tom, Tom Adie, and Geoff S. Baldwin. 2011. “DNA Assembly for Synthetic Biology: From Parts to Pathways and Beyond.” *Integrative Biology* 3 (2): 109–18. https://doi.org/10.1039/C0IB00070A.

Evans, James C., Meenakshi Malhotra, John F. Cryan, and Caitriona M. O’Driscoll.

2016. “The Therapeutic and Diagnostic Potential of the Prostate Specific Membrane Antigen/Glutamate Carboxypeptidase II (PSMA/GCPII) in Cancer and Neurological Disease.” *British Journal of Pharmacology,* 3041–79. https://doi.org/10.1111/bph.13576.

Ewing, Charles M, Anna M Ray, Ethan M Lange, Kimberly A Zuhlke, Christiane M Robbins, Waibhav D Tembe, Kathleen E Wiley, et al. 2012. “Germline Mutations in HOXB13 and Prostate-Cancer Risk.” *Https://Doi.Org/10.1056/NEJMoa1110000* 366 (2): 141–49. https://doi.org/10.1056/NEJMOA1110000.

Ferm, Vergil H. 1987. “Embryology and Comparative Anatomy, Rodent Reproductive Tract,” 3–7. https://doi.org/10.1007/978-3-642-72550-0_1.

Fleming WH, Hamel A, MacDonald R, Ramsey E, Pettigrew NM, Johnston B, Dodd JG, Matusik RJ. 1986. “Expression of the C-Myc Protooncogene in Human Prostatic Carcinoma and Benign Prostatic Hyperplasia.” *Cancer Res.* March (46(3)): 1535–38.

Fox, S. B., R. A. Persad, J. Royds, R. N. Kore, P. B. Silcocks, and C. C. Collins. 1993. “P53 and C-Myc Expression in Stage A1 Prostatic Adenocarcinoma: Useful Prognostic Determinants?” *The Journal of Urology* 150 (2 Pt 1): 490–94. https://doi.org/10.1016/S0022-5347(17)35533-7.

Ghosh, Arundhati, and Warren D.W. Heston. 2004. “Tumor Target Prostate Specific Membrane Antigen (PSMA) and Its Regulation in Prostate Cancer.” *Journal of Cellular Biochemistry* 91 (3): 528–39. https://doi.org/10.1002/jcb.10661.

Gibson, Daniel G., Lei Young, Ray Yuan Chuang, J. Craig Venter, Clyde A.

Hutchison, and Hamilton O. Smith. 2009a. “Enzymatic Assembly of DNA Molecules up to Several Hundred Kilobases.” *Nature Methods* 6 (5): 343–45. https://doi.org/10.1038/nmeth.1318.

———. 2009b. “Enzymatic Assembly of DNA Molecules up to Several Hundred Kilobases.” *Nature Methods 2009 6:5* 6 (5): 343–45. https://doi.org/10.1038/nmeth.1318.

Gilany, Kambiz, Arash Minai-Tehrani, Elham Savadi-Shiraz, Hassan Rezadoost, and Niknam Lakpour. 2015. “Exploring the Human Seminal Plasma Proteome: An Unexplored Gold Mine of Biomarker for Male Infertility and Male Reproduction Disorder.” *Journal of Reproduction & Infertility* 16 (2): 61–71. https://pubmed.ncbi.nlm.nih.gov/25927022/.

Grabowska, Magdalena M., David J. Degraff, Xiuping Yu, Ren Jie Jin, Zhenbang Chen, Alexander D. Borowsky, and Robert J. Matusik. 2014. “Mouse Models of Prostate Cancer: Picking the Best Model for the Question.” *Cancer and Metastasis Reviews* 33 (2–3): 377–97. https://doi.org/10.1007/S10555-013-9487-8/TABLES/2.

Greenberg, N. M., F. Demayo, M. J. Finegold, D. Medina, W. D. Tilley, J. O. Aspinall, G. R. Cunha, A. A. Donjacour, R. J. Matusik, and J. M. Rosen. 1995. “Prostate Cancer in a Transgenic Mouse.” *Proceedings of the National Academy of Sciences of the United States of America* 92 (8): 3439–43. https://doi.org/10.1073/PNAS.92.8.3439.

Greenberg, N M, F J DeMayo, P C Sheppard, R Barrios, R Lebovitz, M Finegold, R Angelopoulou, et al. 1994. “The Rat Probasin Gene Promoter Directs

Hormonally and Developmentally Regulated Expression of a Heterologous Gene Specifically to the Prostate in Transgenic Mice." *Molecular Endocrinology (Baltimore, Md.)* 8 (2): 230–39. https://doi.org/10.1210/MEND.8.2.8170479.

Greenhalgh, Trisha. 2005. "The Human Genome Project." *Journal of the Royal Society of Medicine* 98 (12): 545–545. https://doi.org/10.1177/014107680509801212.

Gurel, Bora, Tehmina Z. Ali, Elizabeth A. Montgomery, Shahnaz Begum, Jessica Hicks, Michael Goggins, Charles G. Eberhart, et al. 2010. "NKX3.1 as a Marker of Prostatic Origin in Metastatic Tumors." *The American Journal of Surgical Pathology* 34 (8): 1097. https://doi.org/10.1097/PAS.0B013E3181E6CBF3.

Gurel, Bora, Tsuyoshi Iwata, Cheryl M. Koh, Robert B. Jenkins, Fusheng Lan, Chi Van Dang, Jessica L. Hicks, et al. 2008. "Nuclear MYC Protein Overexpression Is an Early Alteration in Human Prostate Carcinogenesis." *Modern Pathology : An Official Journal of the United States and Canadian Academy of Pathology, Inc* 21 (9): 1156–67. https://doi.org/10.1038/MODPATHOL.2008.111.

Guyer, Mark S., and Francis S. Collins. 1993. "The Human Genome Project and the Future of Medicine." *American Journal of Diseases of Children (1960)* 147 (11): 1145–52. https://doi.org/10.1001/ARCHPEDI.1993.02160350019003.

Haffner, Jérémie, Eric Potiron, Sébastien Bouyé, Philippe Puech, Xavier Leroy, Laurent Lemaitre, and Arnauld Villers. 2009. "Peripheral Zone Prostate Cancers: Location and Intraprostatic Patterns of Spread at Histopathology." *The Prostate* 69 (3): 276–82. https://doi.org/10.1002/PROS.20881.

Henry, Gervaise H., Alicia Malewska, Diya B. Joseph, Venkat S. Malladi, Jeon Lee,

Jose Torrealba, Ryan J. Mauck, et al. 2018. “A Cellular Anatomy of the Normal Adult Human Prostate and Prostatic Urethra.” *Cell Reports* 25 (12): 3530-3542.e5. https://doi.org/10.1016/j.celrep.2018.11.086.

Hinata, Nobuyuki, and Masato Fujisawa. 2022. “Racial Differences in Prostate Cancer Characteristics and Cancer-Specific Mortality: An Overview.” *The World Journal of Men’s Health* 40 (2): 217. https://doi.org/10.5534/WJMH.210070.

Hoffman, Richard M. 2011. “Clinical Practice. Screening for Prostate Cancer.” *The New England Journal of Medicine* 365 (21): 2013–19. https://doi.org/10.1056/NEJMCP1103642.

Hong, Xi, Liang Mao, Luwei Xu, Qiang Hu, and Ruipeng Jia. 2022. “Prostate-Specific Membrane Antigen Modulates the Progression of Prostate Cancer by Regulating the Synthesis of Arginine and Proline and the Expression of Androgen Receptors and Fos Proto-Oncogenes.” *Bioengineered* 13 (1): 995. https://doi.org/10.1080/21655979.2021.2016086.

HOROSZEWICZ JS, KAWINSKI E, MURPHY GP. 1986. “MONOCLONAL-ANTIBODIES TO A NEW ANTIGENIC MARKER IN EPITHELIAL PROSTATIC CELLS AND SERUM OF PROSTATIC-CANCER PATIENTS-Web of Science Core Collection.” *Anticancer Research* 7 (5): 927–36. https://www.webofscience.com/wos/woscc/full-record/WOS:A1987L903900001?SID=USW2EC0E37bh8aZY2oBa1l5xazrNW.

Hubbard, Gretchen K, Laura N Mutton, May Khalili, Ryan P McMullin, Jessica L Hicks, Daniella Bianchi-Frias, Lucas A Horn, et al. 2016. “Combined MYC

Activation and Pten Loss Are Sufficient to Create Genomic Instability and Lethal Metastatic Prostate Cancer." *Cancer Research* 76 (2): 283–92. https://doi.org/10.1158/0008-5472.CAN-14-3280.

Hudson, D. L., A. T. Guy, P. Fry, M. J. O'Hare, F. M. Watt, and J. R.W. Masters. 2001. "Epithelial Cell Differentiation Pathways in the Human Prostate: Identification of Intermediate Phenotypes by Keratin Expression." *The Journal of Histochemistry and Cytochemistry : Official Journal of the Histochemistry Society* 49 (2): 271–78. https://doi.org/10.1177/002215540104900214.

Hugosson, Jonas, Monique J. Roobol, Marianne Månsson, Teuvo L.J. Tammela, Marco Zappa, Vera Nelen, Maciej Kwiatkowski, et al. 2019. "A 16-Yr Follow-up of the European Randomized Study of Screening for Prostate Cancer." *European Urology* 76 (1): 43–51. https://doi.org/10.1016/J.EURURO.2019.02.009.

Humphrey, Peter A. 2004. "Gleason Grading and Prognostic Factors in Carcinoma of the Prostate." *Modern Pathology : An Official Journal of the United States and Canadian Academy of Pathology, Inc* 17 (3): 292–306. https://doi.org/10.1038/MODPATHOL.3800054.

———. 2017. "Histopathology of Prostate Cancer." *Cold Spring Harbor Perspectives in Medicine* 7 (10). https://doi.org/10.1101/CSHPERSPECT.A030411.

Israeli, R S, C T Powell, W R Fair, and W D Heston. 1993. "Molecular Cloning of a Complementary DNA Encoding a Prostate-Specific Membrane Antigen." *Cancer Research* 53 (2): 227–30.

http://www.ncbi.nlm.nih.gov/pubmed/8417812.

Ittmann, Michael. 2018. “Anatomy and Histology of the Human and Murine Prostate.” *Cold Spring Harbor Perspectives in Medicine* 8 (5). https://doi.org/10.1101/CSHPERSPECT.A030346.

Jackson, Paul F., Derek C. Cole, Barbara S. Slusher, Susan L. Stetz, Laurie E. Ross, Bruce A. Donzanti, and Diane Amy Trainor. 1996. “Design, Synthesis, and Biological Activity of a Potent Inhibitor of the Neuropeptidase N-Acetylated Alpha-Linked Acidic Dipeptidase.” *Journal of Medicinal Chemistry* 39 (2): 619–22. https://doi.org/10.1021/JM950801Q.

Jamaspishvili, Tamara, David M. Berman, Ashley E. Ross, Howard I. Scher, Angelo M. De Marzo, Jeremy A. Squire, and Tamara L. Lotan. 2018. “Clinical Implications of PTEN Loss in Prostate Cancer.” *Nature Reviews. Urology* 15 (4): 222–34. https://doi.org/10.1038/NRUROL.2018.9.

Jenkins RB, Qian J, Lieber MM, Bostwick DG. 1997. “Detection of C-Myc Oncogene Amplification and Chromosomal Anomalies in Metastatic Prostatic Carcinoma by Fluorescence in Situ Hybridization.” *Cancer Res.* Feb (57(3)): 524–31.

Johnson, Laura E., Jordan T. Becker, Jason A. Dubovsky, Brian M. Olson, and Douglas G. McNeel. 2013. “Prostate Carcinoma in Transgenic Lewis Rats - a Tumor Model for Evaluation of Immunological Treatments.” *Chinese Clinical Oncology* 2 (1). https://doi.org/10.3978/J.ISSN.2304-3865.2012.11.06.

Kahn, D., R. D. Williams, D. W. Seldin, J. A. Libertino, M. Hirschhorn, R. Dreicer, G. J. Weiner, D. Bushnell, and J. Gulfo. 1994. “Radioimmunoscintigraphy with

111indium Labeled CYT-356 for the Detection of Occult Prostate Cancer Recurrence." *The Journal of Urology* 152 (5 Pt 1): 1490–95. https://doi.org/10.1016/S0022-5347(17)32453-9.

Kaittanis, Charalambos, Chrysafis Andreou, Haley Hieronymus, Ninghui Mao, Catherine A Foss, Matthias Eiber, Gregor Weirich, et al. 2018. "Prostate-Specific Membrane Antigen Cleavage of Vitamin B9 Stimulates Oncogenic Signaling through Metabotropic Glutamate Receptors." *The Journal of Experimental Medicine* 215 (1): 159–75. https://doi.org/10.1084/jem.20171052.

Karlsson, Robert, Markus Aly, Mark Clements, Lilly Zheng, Jan Adolfsson, Jianfeng Xu, Henrik Grönberg, and Fredrik Wiklund. 2014. "A Population-Based Assessment of Germline HOXB13 G84E Mutation and Prostate Cancer Risk." *European Urology* 65 (1): 169–76. https://doi.org/10.1016/J.EURURO.2012.07.027.

Khan, Farhan Ullah, Awais Ullah Ihsan, Hidayat Ullah Khan, Ruby Jana, Junaid Wazir, Puregmaa Khongorzul, Muhammad Waqar, and Xiaohui Zhou. 2017. "Comprehensive Overview of Prostatitis." *Biomedicine & Pharmacotherapy* 94 (October): 1064–76. https://doi.org/10.1016/J.BIOPHA.2017.08.016.

Kim, J., M. Roh, I. Doubinskaia, G. N. Algarroba, I. E.A. Eltoum, and S. A. Abdulkadir. 2012. "A Mouse Model of Heterogeneous, c-MYC-Initiated Prostate Cancer with Loss of Pten and P53." *Oncogene* 31 (3): 322–32. https://doi.org/10.1038/ONC.2011.236.

Kirby, RS, JD McConnell, JM Fitzpatrick, and CG Roehrborn. 2004. *Textbook of Benign Prostatic Hyperplasia*.

https://books.google.com/books?hl=en&lr=&id=Ex3NvHbC8yEC&oi=fnd&pg=PR8&ots=rNWpZLY6ov&sig=fKusVyVhvKsSBLfIOQd5y-mTlAk.

Klein, Eric A. 2023. “Localized Prostate Cancer: Risk Stratification and Choice of Initial Treatment.” UpToDate. 2023. https://medilib.ir/uptodate/show/6943.

Knight, Thomas. 2003. “Idempotent Vector Design for Standard Assembly of Biobricks.” https://dspace.mit.edu/handle/1721.1/21168.

Koh, Cheryl M, Charles J Bieberich, Chi V Dang, William G Nelson, Srinivasan Yegnasubramanian, and Angelo M De Marzo. 2010. “MYC and Prostate Cancer.” *Genes & Cancer* 1 (6): 617–28. https://doi.org/10.1177/1947601910379132.

Koochekpour, Shahriar, Sunipa Majumdar, Gissou Azabdaftari, Kristopher Attwood, Ray Scioneaux, Dhatchayini Subramani, Charles Manhardt, et al. 2012. “Serum Glutamate Levels Correlate with Gleason Score and Glutamate Blockade Decreases Proliferation, Migration, and Invasion and Induces Apoptosis in Prostate Cancer Cells.” *Clinical Cancer Research* 18 (21): 5888–5901. https://doi.org/10.1158/1078-0432.CCR-12-1308.

Krieger, John N., Jr Leroy Nyberg, and J. Curtis Nickel. 1999. “NIH Consensus Definition and Classification of Prostatitis.” *JAMA* 282 (3): 236–37. https://doi.org/10-1001/pubs.jama-issn-0098-7484-282-3-jac90006.

Kumar, V. L., and P. K. Majumder. 1995. “Prostate Gland: Structure, Functions and Regulation.” *International Urology and Nephrology* 27 (3): 231–43. https://doi.org/10.1007/BF02564756.

Lanchbury, J. S. 1998. “The Human Genome Project.” *British Journal of*

Rheumatology 37 (2): 119–21. https://doi.org/10.1093/RHEUMATOLOGY/37.2.119.

Lee, Chung, and James M. Holland. 1987. "Anatomy, Histology, and Ultrastructure (Correlation with Function), Prostate, Rat," 239–51. https://doi.org/10.1007/978-3-642-72550-0_37.

Loenen, Wil A.M., David T.F. Dryden, Elisabeth A. Raleigh, and Geoffrey G. Wilson. 2014. "Type I Restriction Enzymes and Their Relatives." *Nucleic Acids Research* 42 (1): 20–44. https://doi.org/10.1093/NAR/GKT847.

Lu-Yao, Grace L., Peter C. Albertsen, Dirk F. Moore, Weichung Shih, Yong Lin, Robert S. DiPaola, and Siu Long Yao. 2008. "Survival Following Primary Androgen Deprivation Therapy among Men with Localized Prostate Cancer." *JAMA* 300 (2): 173–81. https://doi.org/10.1001/JAMA.300.2.173.

Lyon, Mary F., and Susan G. Hawkes. 1970. "X-Linked Gene for Testicular Feminization in the Mouse." *Nature* 227 (5264): 1217–19. https://doi.org/10.1038/2271217A0.

Marker, Paul C., Annemarie A. Donjacour, Rajvir Dahiya, and Gerald R. Cunha. 2003. "Hormonal, Cellular, and Molecular Control of Prostatic Development." *Developmental Biology* 253 (2): 165–74. https://doi.org/10.1016/S0012-1606(02)00031-3.

Marzo, Angelo M. De, Valerie L. Marchi, Jonathan I. Epstein, and William G. Nelson. 1999. "Proliferative Inflammatory Atrophy of the Prostate : Implications for Prostatic Carcinogenesis." *The American Journal of Pathology* 155 (6): 1985. https://doi.org/10.1016/S0002-9440(10)65517-4.

Marzo, Angelo M. De, Elizabeth A. Platz, Siobhan Sutcliffe, Jianfeng Xu, Henrik Grönberg, Charles G. Drake, Yasutomo Nakai, William B. Isaacs, and William G. Nelson. 2007. “Inflammation in Prostate Carcinogenesis.” *Nature Reviews. Cancer* 7 (4): 256–69. https://doi.org/10.1038/NRC2090.

McMullin, Ryan P., Albert Dobi, Laura N. Mutton, András Orosz, Shilpi Maheshwari, Cooduvalli S. Shashikant, and Charles J. Bieberich. 2010. “A FOXA1-Binding Enhancer Regulates Hoxb13 Expression in the Prostate Gland.” *Proceedings of the National Academy of Sciences of the United States of America* 107 (1): 98–103. https://doi.org/10.1073/PNAS.0902001107.

McMullin, Ryan P., Laura N. Mutton, and Charles J. Bieberich. 2009a. “Hoxb13 Regulatory Elements Mediate Transgene Expression during Prostate Organogenesis and Carcinogenesis.” *Developmental Dynamics* 238 (3): 664–72. https://doi.org/10.1002/dvdy.21870.

———. 2009b. “Hoxb13 Regulatory Elements Mediate Transgene Expression during Prostate Organogenesis and Carcinogenesis.” *Developmental Dynamics* 238 (3): 664–72. https://doi.org/10.1002/dvdy.21870.

McNeal, J. E. 1981. “Normal and Pathologic Anatomy of Prostate.” *Urology* 17 (Suppl 3): 11–16. https://pubmed.ncbi.nlm.nih.gov/6163241/.

McNeal, J. E., E. A. Redwine, F. S. Freiha, and T. A. Stamey. 1988. “Zonal Distribution of Prostatic Adenocarcinoma. Correlation with Histologic Pattern and Direction of Spread.” *The American Journal of Surgical Pathology* 12 (12): 897–906. https://doi.org/10.1097/00000478-198812000-00001.

McNeal, John E. 1981. “The Zonal Anatomy of the Prostate.” *The Prostate* 2 (1): 35–

49. https://doi.org/10.1002/PROS.2990020105.

Ming Zhou, George J. Netto and Jonathan I. Epstein. 2012. “Prostatic Intraepithelial Neoplasia.” *Uropathology,* January, 52–54. https://doi.org/10.1016/B978-1-4377-2523-0.00026-7.

Mohler, James L., Emmanuel S. Antonarakis, Andrew J. Armstrong, Anthony V. D’Amico, Brian J. Davis, Tanya Dorff, James A. Eastham, et al. 2019. “Prostate Cancer, Version 2.2019, NCCN Clinical Practice Guidelines in Oncology.” *Journal of the National Comprehensive Cancer Network* 17 (5): 479–505. https://doi.org/10.6004/JNCCN.2019.0023.

Molinari, Francesca, and Milo Frattini. 2014. “Functions and Regulation of the PTEN Gene in Colorectal Cancer.” *Frontiers in Oncology* 4 JAN (January): 326. https://doi.org/10.3389/FONC.2013.00326/BIBTEX.

Moraes, Fernanda, and Andréa Góes. 2016. “A Decade of Human Genome Project Conclusion: Scientific Diffusion about Our Genome Knowledge.” *Biochemistry and Molecular Biology Education : A Bimonthly Publication of the International Union of Biochemistry and Molecular Biology* 44 (3): 215–23. https://doi.org/10.1002/BMB.20952.

Mygatt, Justin G, and David J Osborn. 2016. “DNA-Repair Gene Mutations in Metastatic Prostate Cancer.” *The New England Journal of Medicine* 375 (18): 1803–4. https://doi.org/10.1056/NEJMc1611137.

Nandana, Srinivas, Katharine Ellwood-Yen, Charles Sawyers, Marcia Wills, Brandy Weidow, Thomas Case, Valeri Vasioukhin, and Robert Matusik. 2010. “Hepsin Cooperates with MYC in the Progression of Adenocarcinoma in a Prostate

Cancer Mouse Model." *The Prostate* 70 (6): 591–600. https://doi.org/10.1002/PROS.21093.

Nascimento-Gonçalves, Elisabete, Ana I. Faustino-Rocha, Fernanda Seixas, Mário Ginja, Bruno Colaço, Rita Ferreira, Margarida Fardilha, and Paula A. Oliveira. 2018. "Modelling Human Prostate Cancer: Rat Models." *Life Sciences* 203 (June): 210–24. https://doi.org/10.1016/J.LFS.2018.04.014.

Neale, Joseph H, Tomasz Bzdega, and Barbara Wroblewska. 2000. "N-Acetylaspartylglutamate: The Most Abundant Peptide Neurotransmitter in the Mammalian Central Nervous System." *Journal of Neurochemistry* 75 (2): 443–52. https://doi.org/10.1046/j.1471-4159.2000.0750443.x.

Nelson, Christian J., Jennifer S. Lee, Maria C. Gamboa, and Andrew J. Roth. 2008. "Cognitive Effects of Hormone Therapy in Men With Prostate Cancer." *Cancer* 113 (5): 1097. https://doi.org/10.1002/CNCR.23658.

Nelson, William G., Angelo M. De Marzo, and William B. Isaacs. 2003. "Prostate Cancer." *New England Journal of Medicine* 349 (4): 366–81. https://doi.org/10.1056/NEJMra021562.

Nelson, William G., Angelo M. De Marzo, and Srinivasan Yegnasubramanian. 2009. "Epigenetic Alterations in Human Prostate Cancers." *Endocrinology* 150 (9): 3991–4002. https://doi.org/10.1210/EN.2009-0573.

Nguyen, D. P., P. L. Xiong, H. Liu, S. Pan, W. Leconet, V. Navarro, M. Guo, et al. 2016. "Induction of PSMA and Internalization of an Anti-PSMA MAb in the Vascular Compartment." *Molecular Cancer Research*. https://doi.org/10.1158/1541-7786.MCR-16-0193.

Nguyen, Tu, Brian James Kirsch, Ryoichi Asaka, Karim Nabi, Addison Quinones, Jessica Tan, Marjorie Justine Antonio, et al. 2019. “Uncovering the Role of N-Acetyl-Aspartyl-Glutamate as a Glutamate Reservoir in Cancer.” *Cell Reports* 27 (2): 491-501.e6. https://doi.org/10.1016/j.celrep.2019.03.036.

Norris, John D., Ching Yi Chang, Bryan M. Wittmann, Rebecca S. Kunder, Huaxia Cui, Daju Fan, James D. Joseph, and Donald P. McDonnell. 2009. “The Homeodomain Protein HOXB13 Regulates the Cellular Response to Androgens.” *Molecular Cell* 36 (3): 405–16. https://doi.org/10.1016/J.MOLCEL.2009.10.020.

O’Keefe, Denise S., Atsushi Uchida, Dean J. Bacich, Fujiko B. Watt, Anna Martorana, Peter L. Molloy, and Warren D.W. Heston. 2000. “Prostate-Specific Suicide Gene Therapy Using the Prostate-Specific Membrane Antigen Promoter and Enhancer.” *The Prostate* 45 (2): 149–57. https://doi.org/10.1002/1097-0045(20001001)45:2<149::AID-PROS9>3.0.CO;2-O.

Olleik, Ghadeer, Wassim Kassouf, Armen Aprikian, Jason Hu, Marie Vanhuyse, Fabio Cury, Stuart Peacock, Elin Bonnevier, Ebba Palenius, and Alice Dragomir. 2018. “Evaluation of New Tests and Interventions for Prostate Cancer Management: A Systematic Review.” *Journal of the National Comprehensive Cancer Network* 16 (11): 1340–51. https://doi.org/10.6004/JNCCN.2018.7055.

Olson, M. V. 1993. “The Human Genome Project.” *Proceedings of the National Academy of Sciences of the United States of America* 90 (10): 4338–44. https://doi.org/10.1073/PNAS.90.10.4338.

Ostrov, Nili, Jacob Beal, Tom Ellis, D. Benjamin Gordon, Bogumil J. Karas, Henry

H. Lee, Scott C. Lenaghan, et al. 2019. “Technological Challenges and Milestones for Writing Genomes.” *Science* 366 (6463): 310–12. https://doi.org/10.1126/SCIENCE.AAY0339.

Paschalis, Alec, Beshara Sheehan, Ruth Riisnaes, Daniel Nava Rodrigues, Bora Gurel, Claudia Bertan, Ana Ferreira, et al. 2019. “Prostate-Specific Membrane Antigen Heterogeneity and DNA Repair Defects in Prostate Cancer.” *European Urology* 76 (4): 469–78. https://doi.org/10.1016/j.eururo.2019.06.030.

Pingoud, Alfred, Geoffrey G. Wilson, and Wolfgang Wende. 2014. “Type II Restriction Endonucleases--a Historical Perspective and More.” *Nucleic Acids Research* 42 (12): 7489–7527. https://doi.org/10.1093/NAR/GKU447.

Pissimissis, Nikolaos, Efstathia Papageorgiou, Peter Lembessis, Athanasios Armakolas, and Michael Koutsilieris. 2009. “The Glutamatergic System Expression in Human PC-3 and LNCaP Prostate Cancer Cells.” *Anticancer Research* 29 (1): 371–77. http://www.ncbi.nlm.nih.gov/pubmed/19331175.

Podlasek, Carol A., J. Quentin Clemens, and Wade Bushman. 1999. “HOXA-13 Gene Mutation Results in Abnormal Seminal Vesicle and Prostate Development.” *Journal of Urology* 161 (5): 1655–61. https://doi.org/10.1016/S0022-5347(05)68999-9.

Podlasek, Carol A, Denis Duboule, and Wade Bushman. 1997. “Male Accessory Sex Organ Morphogenesis Is Altered by Loss of Function of Hoxd-13.” *Dev. Dyn* 208: 454–65. https://doi.org/10.1002/(SICI)1097-0177(199704)208:4.

Podlasek, Carol A, Robert M Seo, J Quentin Clemens, Liang Ma, Richard L Maas, and Wade Bushman. 1999. “Hoxa-10 Deficient Male Mice Exhibit Abnormal

Development of the Accessory Sex Organs." *Wiley Online Library* 214: 1–12. https://doi.org/10.1002/(SICI)1097-0177(199901)214:1<1::AID-DVDY1>3.0.CO;2-2.

Pollard, M. 1973. "Spontaneous Prostate Adenocarcinomas in Aged Germfree Wistar Rats." - *Journal of the National Cancer* 51 (4): 1235–41. https://academic.oup.com/jnci/article-abstract/51/4/1235/1079968.

Pritchard, Colin C., Joaquin Mateo, Michael F. Walsh, Navonil De Sarkar, Wassim Abida, Himisha Beltran, Andrea Garofalo, et al. 2016. "Inherited DNA-Repair Gene Mutations in Men with Metastatic Prostate Cancer." *The New England Journal of Medicine* 375 (5): 443–53. https://doi.org/10.1056/NEJMOA1603144.

"Prostate: Anatomy, Location, Function & Conditions." n.d. Accessed May 3, 2023. https://my.clevelandclinic.org/health/body/23965-prostate.

Rajasekaran, Ak. 2005. "Is Prostate-Specific Membrane Antigen a Multifunctional Protein?" *Am J Physiol Cell Physiol* 288 (67): 975–81. https://doi.org/10.1152/ajpcell.00506.2004.

Rajasekaran, Sigrid A, Gopalakrishnapillai Anilkumar, Eri Oshima, James U Bowie, He Liu, Warren Heston, Neil H Bander, and Ayyappan K Rajasekaran. 2003. "A Novel Cytoplasmic Tail MXXXL Motif Mediates the Internalization of Prostate-Specific Membrane Antigen." *Molecular Biology of the Cell* 14: 4835–45. https://doi.org/10.1091/mbc.E02–11.

Rebello, Richard J., Christoph Oing, Karen E. Knudsen, Stacy Loeb, David C. Johnson, Robert E. Reiter, Silke Gillessen, Theodorus Van der Kwast, and

Robert G. Bristow. 2021. “Prostate Cancer.” *Nature Reviews Disease Primers* 7 (1). https://doi.org/10.1038/s41572-020-00243-0.

Richmond, Ann, and Yingjun Su. 2008. “Mouse Xenograft Models vs GEM Models for Human Cancer Therapeutics.” *Disease Models & Mechanisms* 1: 78–82. https://doi.org/10.1242/dmm.000976.

Ristau, Benjamin T, Denise S O ’keefe, and Dean J Bacich. 2014. “The Prostate-Specific Membrane Antigen: Lessons and Current Clinical Implications from 20 Years of Research.” *Urologic Oncology: Seminars and Original Investigations* 32: 272–79. https://doi.org/10.1016/j.urolonc.2013.09.003.

Robinson, Dan, Eliezer M. Van Allen, Yi Mi Wu, Nikolaus Schultz, Robert J. Lonigro, Juan Miguel Mosquera, Bruce Montgomery, et al. 2015. “Integrative Clinical Genomics of Advanced Prostate Cancer.” *Cell* 161 (5): 1215–28. https://doi.org/10.1016/J.CELL.2015.05.001.

Roth, Theodore L., Ljiljana Milenkovic, and Matthew P. Scott. 2014. “A Rapid and Simple Method for DNA Engineering Using Cycled Ligation Assembly.” *PLoS ONE* 9 (9): 107329. https://doi.org/10.1371/JOURNAL.PONE.0107329.

Rowe, Steven P., Andreas Buck, Ralph A. Bundschuh, Constantin Lapa, Sebastian E. Serfling, Thorsten Derlin, Takahiro Higuchi, Michael A. Gorin, Martin G. Pomper, and Rudolf A. Werner. 2022. “[18F]DCFPyL PET/CT for Imaging of Prostate Cancer.” *Nuklearmedizin. Nuclear Medicine* 61 (3): 240–46. https://doi.org/10.1055/A-1659-0010.

Roy-Burman, P., H. Wu, W. C. Powell, J. Hagenkord, and M. B. Cohen. 2004. “Genetically Defined Mouse Models That Mimic Natural Aspects of Human

Prostate Cancer Development." *Endocrine-Related Cancer* 11 (2): 225–54. https://doi.org/10.1677/ERC.0.0110225.

Samuel Aparicio, Manuel Hindalgo, Andrew L. Kung. 2015. "Examining the Utility of Patient-Derived Xenograft Mouse Models." *Nat Rev Cancer* 15: 311–16.

Sant'Agnese, P. Anthony Di. 1998. "Neuroendocrine Cells of the Prostate and Neuroendocrine Differentiation in Prostatic Carcinoma: A Review of Morphologic Aspects." *Urology* 51 (5A Suppl): 121–24. https://doi.org/10.1016/S0090-4295(98)00064-8.

Schuster, David M., Peter T. Nieh, Ashesh B. Jani, Rianot Amzat, F. Dubois Bowman, Raghuveer K. Halkar, Viraj A. Master, et al. 2014. "Anti-3-[(18)F]FACBC Positron Emission Tomography-Computerized Tomography and (111)In-Capromab Pendetide Single Photon Emission Computerized Tomography-Computerized Tomography for Recurrent Prostate Carcinoma: Results of a Prospective Clinical Trial." *The Journal of Urology* 191 (5): 1446–53. https://doi.org/10.1016/J.JURO.2013.10.065.

Sciavolino, Peter J, Elliott W Abrams, L U Yang, Leif P Austenberg, Michael M Shen, and Cory Abate-Shen. 1997. "Tissue-Specific Expression of Murine Nkx3.1 in the Male Urogenital System." *Dev. Dyn* 209: 127–38. https://doi.org/10.1002/(SICI)1097-0177(199705)209:1.

Shain, S. A., B. McCullough, and A. Segaloff. 1975. "Spontaneous Adenocarcinomas of the Ventral Prostate of Aged A X C Rats." *JNCI: Journal of the National Cancer Institute* 55 (1): 177–80. https://doi.org/10.1093/JNCI/55.1.177.

Shen, Michael M., and Cory Abate-Shen. 2010a. "Molecular Genetics of Prostate

Cancer: New Prospects for Old Challenges." *Genes & Development* 24 (18): 1967. https://doi.org/10.1101/GAD.1965810.

Shen, Michael M, and Cory Abate-Shen. 2010b. "Molecular Genetics of Prostate Cancer: New Prospects for Old Challenges." *Genes & Development* 24 (18): 1967–2000. https://doi.org/10.1101/gad.1965810.

Shirai, Tomoyuki, Satoru Takahashi, Lin Cui, Mitsuru Futakuchi, Koji Kato, Seiko Tamano, and Katsumi Imaida. 2000. "Experimental Prostate Carcinogenesis — Rodent Models." *Mutation Research/Reviews in Mutation Research* 462 (2–3): 219–26. https://doi.org/10.1016/S1383-5742(00)00039-9.

Siegel Mph, Rebecca L, Kimberly D Miller, Nikita Sandeep, Wagle Mbbs, | Ahmedin, Jemal Dvm, and Rebecca L Siegel. 2023. "Cancer Statistics, 2023." *CA: A Cancer Journal for Clinicians* 73 (1): 17–48. https://doi.org/10.3322/CAAC.21763.

Signoretti, Sabina, David Waltregny, James Dilks, Beth Isaac, Douglas Lin, Levi Garraway, Annie Yang, Rodolfo Montironi, Frank McKeon, and Massimo Loda. 2000. "P63 Is a Prostate Basal Cell Marker and Is Required for Prostate Development." *The American Journal of Pathology* 157 (6): 1769–75. https://doi.org/10.1016/S0002-9440(10)64814-6.

Silver DA, Pellicer I, Fair WR, Heston WD, Cordon-Cardo C. 1997. "Prostate-Specific Membrane Antigen Expression in Normal and Malignant Human Tissues." *Clin Cancer Res.* 81 (5).

Speakman, Mark, Roger Kirby, Scott Doyle, and Chris Ioannou. 2015. "Burden of Male Lower Urinary Tract Symptoms (LUTS) Suggestive of Benign Prostatic

Hyperplasia (BPH) - Focus on the UK." *BJU International* 115 (4): 508–19. https://doi.org/10.1111/BJU.12745.

Sreenath, Taduru, Andras Orosz, Kazuyuki Fujita, and Charles J. Bieberich. 1999. "Androgen-Independent Expression of Hoxb-13 in the Mouse Prostate." *The Prostate* 41 (3): 203–7. https://doi.org/10.1002/(SICI)1097-0045(19991101)41:3<203::AID-PROS8>3.0.CO;2-J.

Srivastava, Sudhir, Eugene J. Koay, Alexander D. Borowsky, Angelo M. De Marzo, Sharmistha Ghosh, Paul D. Wagner, and Barnett S. Kramer. 2019. "Cancer Overdiagnosis: A Biological Challenge and Clinical Dilemma." *Nature Reviews. Cancer* 19 (6): 349–58. https://doi.org/10.1038/S41568-019-0142-8.

Sun, Michael, Muhammad Junaid Niaz, Muhammad Obaid Niaz, and Scott T. Tagawa. 2021. "Prostate-Specific Membrane Antigen (PSMA)-Targeted Radionuclide Therapies for Prostate Cancer." *Current Oncology Reports* 23 (5). https://doi.org/10.1007/S11912-021-01042-W.

Szabo, Zsolt, Esther Mena, Steven P Rowe, Donika Plyku, Rosa Nidal, Mario A Eisenberger, Emmanuel S Antonarakis, et al. 2015. "Initial Evaluation of [18 F]DCFPyL for Prostate-Specific Membrane Antigen (PSMA)-Targeted PET Imaging of Prostate Cancer HHS Public Access." *Mol Imaging Biol* 17 (4): 565–74. https://doi.org/10.1007/s11307-015-0850-8.

Tagawa, Scott T., Matthew I. Milowsky, Michael Morris, Shankar Vallabhajosula, Paul Christos, Naveed H. Akhtar, Joseph Osborne, et al. 2013. "Phase II Study of Lutetium-177-Labeled Anti-Prostate-Specific Membrane Antigen Monoclonal Antibody J591 for Metastatic Castration-Resistant Prostate Cancer." *Clinical*

Cancer Research : An Official Journal of the American Association for Cancer Research 19 (18): 5182–91. https://doi.org/10.1158/1078-0432.CCR-13-0231.

Takeda, H., I. Lasnitzki, and T. Mizuno. 1986. "Analysis of Prostatic Bud Induction by Brief Androgen Treatment in the Fetal Rat Urogenital Sinus." *Journal of Endocrinology* 110 (3): 467–70. https://doi.org/10.1677/JOE.0.1100467.

Tennant, TR, H Kim, … M Sokoloff - The, and Undefined 2000. 2000. "The Dunning Model." *The Prostate* 43: 295–302. https://onlinelibrary.wiley.com/doi/abs/10.1002/1097-0045(20000601)43:4%3C295::AID-PROS9%3E3.0.CO;2-W.

Thariat, Juliette, Jean Michel Hannoun-Levi, Arthur Sun Myint, Te Vuong, and Jean Pierre Gérard. 2013. "Past, Present, and Future of Radiotherapy for the Benefit of Patients." *Nature Reviews. Clinical Oncology* 10 (1): 52–60. https://doi.org/10.1038/NRCLINONC.2012.203.

Thomson, Axel A., and Paul C. Marker. 2006. "Branching Morphogenesis in the Prostate Gland and Seminal Vesicles." *Differentiation* 74 (7): 382–92. https://doi.org/10.1111/j.1432-0436.2006.00101.x.

Timms, Barry G. 2008. "Prostate Development: A Historical Perspective." *Differentiation; Research in Biological Diversity* 76 (6): 565–77. https://doi.org/10.1111/J.1432-0436.2008.00278.X.

Toivanen, R, Shen, MM -, and Undefined 2017. 2017. "Prostate Organogenesis: Tissue Induction, Hormonal Regulation and Cell Type Specification." *Journals.Biologists.Com* 144 (8): 1382–98. https://doi.org/10.1242/dev.148270.

Vazquez, Francisca, and William R. Sellers. 2000. "The PTEN Tumor Suppressor

Protein: An Antagonist of Phosphoinositide 3- Kinase Signaling." *Biochimica et Biophysica Acta - Reviews on Cancer* 1470 (1). https://doi.org/10.1016/S0304-419X(99)00032-3.

Verhagen, Anja P.M., Tilly W. Aalders, Frans C.S. Ramaekers, Frans M.J. Debruyne, and Jack A. Schalken. 1988. "Differential Expression of Keratins in the Basal and Luminal Compartments of Rat Prostatic Epithelium during Degeneration and Regeneration." *The Prostate* 13 (1): 25–38. https://doi.org/10.1002/PROS.2990130104.

Verze, Paolo, Tommaso Cai, and Stefano Lorenzetti. 2016a. "The Role of the Prostate in Male Fertility, Health and Disease." *Nature Reviews Urology* 13 (7): 379–86. https://doi.org/10.1038/nrurol.2016.89.

———. 2016b. "The Role of the Prostate in Male Fertility, Health and Disease." *Nature Reviews. Urology* 13 (7): 379–86. https://doi.org/10.1038/NRUROL.2016.89.

Voigt, Walter, Mark Feldman, and W. F. Dunning. 1975. "5alpha-Dihydrotestosterone-Binding Proteins and Androgen Sensitivity in Prostatic Cancers of Copenhangen Rats." *Cancer Research* 35 (7): 1840–46. https://pubmed.ncbi.nlm.nih.gov/165887/.

Wang, Guocan, Di Zhao, Denise J. Spring, and Ronald A. Depinho. 2018. "Genetics and Biology of Prostate Cancer." *Genes & Development* 32 (17–18): 1105. https://doi.org/10.1101/GAD.315739.118.

Wang, Shunyou, Jing Gao, Qunying Lei, Nora Rozengurt, Colin Pritchard, Jing Jiao, George V. Thomas, et al. 2003. "Prostate-Specific Deletion of the Murine Pten

Tumor Suppressor Gene Leads to Metastatic Prostate Cancer." *Cancer Cell* 4 (3): 209–21. https://doi.org/10.1016/S1535-6108(03)00215-0.

Wang, Xinning, Dangshe Ma, William C. Olson, and Warren D.W. Heston. 2011. "In Vitro and in Vivo Responses of Advanced Prostate Tumors to PSMA ADC, an Auristatin-Conjugated Antibody to Prostate-Specific Membrane Antigen." *Molecular Cancer Therapeutics* 10 (9): 1728–39. https://doi.org/10.1158/1535-7163.MCT-11-0191.

Wang, Yuzhuo, Simon W. Hayward, Mei Cao, Kristina A. Thayer, and Gerald R. Cunha. 2001. "Cell Differentiation Lineage in the Prostate." *Differentiation* 68 (4–5): 270–79. https://doi.org/10.1046/J.1432-0436.2001.680414.X.

Watt, Fujiko, Anna Martorana, Diana E Brookes, Thu Ho, Elizabeth Kingsley, Denise S O 'keefe, Pamela J Russell, Warren D W Heston, and Peter L Molloy. 2001. "A Tissue-Specific Enhancer of the Prostate-Specific Membrane Antigen Gene, FOLH1." https://doi.org/10.1006/geno.2000.6446.

Willard, Stacey S, and Shahriar Koochekpour. 2013a. "Glutamate, Glutamate Receptors, and Downstream Signaling Pathways." *International Journal of Biological Sciences* 9 (9): 948–59. https://doi.org/10.7150/ijbs.6426.

———. 2013b. "Glutamate Signaling in Benign and Malignant Disorders: Current Status, Future Perspectives, and Therapeutic Implications." *International Journal of Biological Sciences* 9 (7): 728–42. https://doi.org/10.7150/ijbs.6475.

Wissenbach, U, B A Niemeyer, T Fixemer, A Schneidewind, C Trost, A Cavalie, K Reus, E Meese, H Bonkhoff, and V Flockerzi. 2001. "Expression of CaT-like, a Novel Calcium-Selective Channel, Correlates with the Malignancy of Prostate

Cancer." *The Journal of Biological Chemistry* 276 (22): 19461–68. https://doi.org/10.1074/jbc.M009895200.

Wu, Xiantuo, Jian Wu, Jiapeng Huang, William C. Powell, Jian Feng Zhang, Robert J. Matusik, Frank O. Sangiorgi, Robert E. Maxson, Henry M. Sucov, and Pradip Roy-Burman. 2001. "Generation of a Prostate Epithelial Cell-Specific Cre Transgenic Mouse Model for Tissue-Specific Gene Ablation." *Mechanisms of Development* 101 (1–2): 61–69. https://doi.org/10.1016/S0925-4773(00)00551-7.

Yang, Guang, Terry L. Timme, Anna Frolov, Thomas M. Wheeler, and Timothy C. Thompson. 2005. "Combined C-Myc and Caveolin-1 Expression in Human Prostate Carcinoma Predicts Prostate Carcinoma Progression." *Cancer* 103 (6): 1186–94. https://doi.org/10.1002/CNCR.20905.

Yong Xue, Frank Smedts, Frans M.J. Debruyne, Jean J.M.C.H. de la Rosette, Jack A. Schalken. 1998. "Identification of Intermediate Cell Types by Keratin Expression in the Developing Human Prostate." The Prostate. 1998. https://onlinelibrary.wiley.com/doi/abs/10.1002/%28SICI%291097-0045%2819980301%2934%3A4%3C292%3A%3AAID-PROS7%3E3.0.CO%3B2-J?sid=nlm%3Apubmed.

Zhao, Rongbao, Larry H Matherly, and I David Goldman. 2009. "Membrane Transporters and Folate Homeostasis: Intestinal Absorption and Transport into Systemic Compartments and Tissues." *Expert Reviews in Molecular Medicine* 11 (January): e4. https://doi.org/10.1017/S1462399409000969.

Zhu, Hanliang, Haoqing Zhang, Ying Xu, Soňa Laššáková, Marie Korabečná, and

Pavel Neužil. 2020. “PCR Past, Present and Future.” *BioTechniques* 69 (4): 317–25. https://doi.org/10.2144/BTN-2020-0057.

Chapter 2: Developing and characterizing novel rodent models of prostate cancer expressing human Prostate Specific Membrane Antigen (PSMA).

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Introduction

Prostate cancer is the second most prevalent cause of cancer related deaths among American men, with approximately 288,300 new cases of prostate cancer being diagnosed in 2023. Approximately 34,700 cases of men diagnosed with prostate cancer would be fatal in the U.S (Siegel Mph et al. 2023). Risk for prostate cancer increases with age. Other contributors to prostate cancer risk include heredity, diet, environmental factors and, lifestyle (Koh et al. 2010; W. G. Nelson, De Marzo, and Isaacs 2003). Prostate cancer shows variations in incidence and mortality rates across different ethnic groups in the United States (Chowdhury-Paulino et al. 2021). These racial disparities are particularly prominent in prostate cancer compared to other cancer types. The reasons behind these differences among racial groups are not fully understood, but they are believed to stem from a mix of social, economic, environmental, lifestyle, and genetic factors (Siegel Mph et al. 2023; Chowdhury-Paulino et al. 2021; Hinata and Fujisawa 2022).

Prostate cancer proceeds through a morphological progression beginning with the pre-malignant lesion termed prostatic intraepithelial neoplasia (PIN) which may progress to adenocarcinoma (W. G. Nelson, De Marzo, and Isaacs 2003). Adenocarcinomas are initially intra-ductal but can progress to become invasive cancers, left untreated these cancers can metastasize and have a predilection for lymph nodes (LN), bone, liver, and brain (Bubendorf et al. 2000). Androgens regulate the growth of prostate cancer by maintaining the ratio of proliferating cells to apoptotic cells by stimulating growth and survival and inhibiting apoptosis (Feldman and Feldman 2001). In nearly all cases upon androgen depravation therapy the cancer

initially regresses but later relapses in an androgen independent form. This type of disease, termed castration resistant prostate cancer (CRPC) is often refractory to treatment and prone to metastasis. Currently, there are no effective treatments available to treat the androgen independent metastatic prostate cancer. There is an urgent need to identify biomarkers and develop therapeutic approaches to treat metastatic castration resistant prostate cancer (mCRPC). Prostate specific membrane antigen (PSMA) is a molecule expressed on the surface of prostate epithelial cells that may be useful in both regards (Israeli et al. 1993; Sweat et al. 1998).

PSMA is a type II transmembrane glycoprotein that has both folate hydrolase and glutamate carboxypeptidase activity. PSMA is primarily expressed in the epithelial cells of the prostate gland once they become neoplastic (Israeli et al. 1993; Carter, Feldman, and Coyle 1996). PSMA expression in a normal healthy human prostate is negligible, but in prostate cancer tissue it is significantly higher (100-1000-fold) (Silver DA, Pellicer I, Fair WR, Heston WD 1997; D. G. Bostwick et al. 1998). PSMA expression in prostate cancer tissue increases with severity of disease as it found to be highest in aggressive metastatic disease (Perner et al. 2007; Queisser et al. 2015). High PSMA expression at time of initial diagnosis is also considered to be predictive of disease recurrence after initial treatment (Hupe et al. 2018). Also, data from recent clinical studies in human prostate cancer patients have observed disparities in PSMA expression along racial lines that correlate to the incidence and mortality rates observed in prostate cancer patients (Koochekpour et al. 2012). In addition to its expression in the prostate, PSMA is also overexpressed in the neovasculature of many solid tumors and has been implicated in various neurological

diseases (Lauri et al. 2022). Although the exact role of PSMA in prostate cancer is not fully understood, it is widely believed that cancer cells overexpressing PSMA exhibit an increased ability in folate and glutamate processing suggesting a potential role in cancer cell metabolism (Koochekpour 2013; Hong et al. 2022). The various properties of PSMA, combined with its expression profile in increasing grades of prostate cancer, make it an important target for the development of diagnostics, therapeutics, and theranostics.

Rat and mouse models are both excellent organisms for investigating the impact of PSMA expression in presence of prostate cancer. Unfortunately, even though numerous transgenic rodent models of prostate cancer exist none of them express PSMA. In this thesis we propose to develop both rat and mouse models of prostate cancer expressing PSMA selectively only in the prostate gland. By using these models, we can potentially gain a better understanding of PSMA's role in prostate cancer and provide a critical tool for the evaluation of PSMA-directed agents.

In our efforts to develop next generation rodent models of prostate cancer, we decided to simultaneously attempt to develop both mouse and rat models expressing PSMA selectively in the prostate gland. Although out attempts to develop transgenic mouse strains expressing PSMA were not successful, we did succeed in developing a rat model with conditional, prostate-restricted expression of human PSMA. The results of those studies, and their implications of this advancement will be discussed.

Methods and materials

Generation of PSMA BAC

A BAC containing the human PSMA genomic locus cloned in the pBACGK1.1 vector was obtained from a collaborator, Dr. Shawn Lupold at the Johns Hopkins University School of Medicine. In addition to the *FOLH1/PSMA* gene, the BAC contained genomic regions 40 kb upstream and 120 kb downstream of the exons encoding the PSMA protein. These regions were included to maximize the likelihood that the cis-acting regulatory elements of the human PSMA gene could be incorporated, thus providing the BAC transgene with the enhancer or other elements required for robust expression in the prostate gland. The PSMA BAC in E. coli DH10B was grown on chloramphenicol selective media and extracted using commercial large construct kit (Qiagen, Cat# 12462). The BAC purification process was optimized until a yield of 70-100 µg of pure intact BAC was achieved. The yield was determined using UV Spectrophotometry and the intactness was confirmed by gel electrophoresis. The purified PSMA BAC was then linearized using PI-SceI (New England Biolabs, Cat# R0696S). Following linearization, PSMA BAC was further purified using sucrose fraction on a 10-40% gradient by centrifugation. Sucrose gradient fractions were then analyzed on an agarose gel to identify fractions containing the BAC. Fractions with the highest BAC concentration were then dialyzed against microinjection buffer (10 mM TRIS, 0.25mM EDTA, pH 7.5) to prepare the BAC ready for pronuclear microinjection. The linearized BAC was further purified by filtering through a 0.22-micron filter (Cat# GVHP00010). The ultra purified PSMA BAC was injected into the nucleus of fertilized embryos via

pronuclear injection and the injected embryos were implanted in foster CD-1 IGS female mice (Charles River, Strain# 022).

Preparation of the Hoxb13Myc-2A-Hoxb13Cre-2A-PSMA-β-globin (MCP) BAC

A complex BAC transgene designed to express human MYC, Cre, and human PSMA under the control of mouse Hoxb13 regulatory elements was designed and generated by a previous student using recombineering as described (Briceno 2015). A key feature of this transgene was the use of picornavirus 2A peptide technology so that all three open reading frames (MYC, Cre, and PSMA) would be translated from a single transgene mRNA. The BAC was termed MCP. DH10B cells containing MCP BAC were grown on kanamycin selective media and extracted using a midi-prep kit (Macherey-Nagel Nucleobond Xtra EF midi kit, Cat# 740420.5). The purified MCP construct was then linearized using PI-SceI (New England Biolabs, Cat# R0696S). Following linearization, it was further purified using sucrose gradient centrifugation and microinjected as described above for the human PSMA BAC, generate founder generation transgeneic mice.

Preparation of the Hoxb13Myc-2A-Hoxb13Cre-2A-rtTA-β-globin 3' (MCR) BAC

A complex BAC transgene designed to express human MYC, Cre, and the reverse tetracycline transactivator (rtTA) under the control of mouse Hoxb13 regulatory elements was designed and generated by a previous student using recombineering as described (M. Rubenstein 2022). As with the MCP BAC, a key

feature of this transgene was the use of picornavirus 2A peptide technology so that all three open reading frames (MYC, Cre, and rtTA) would be translated from a single transgene mRNA. The MCP BAC in DH10B cells was purified and prepared for microinjection into single cell FVB embryos as described above for the MCP BAC.

Generation of a conditional human PSMA transgene

To generate a condition transgene capable of expressing human PSMA, designed a construct the employed a 'flox-stop-flox' approach. In this approach, a cytomegalovirus (CMV) promoter/enhancer is cloned upstream of two separate gene expression cassettes. The first cassette downstream of the CMV promoter is comprised of a neomycin resistance gene with a polyadenylation signal and is flanked by 37-bp loxP sites. The second cassette contains the open reading frame of human PSMA and a chicken ß-globin intron and polyadenylation signal. In the absence of Cre recombinase, the neomycin resistance gene is constitutively transcribed. In the presence of Cre, the neomycin resistance cassette is removed leaving a single loxP site upstream of the PSMA expression cassette. To generate this construct, we obtained plasmid pCALNL-GFP from (Addgene, plasmid # 13770), in which the second cassette downstream encodes green fluorescent protein (GFP). To replace GFP with the human PSMA cDNA we used standard Gibson Assembly. The vector and neomycin resistance cassette were PCR amplified to contain homology to the PSMA expression cassette and assembled in a two-part Gibson reaction. Correct insertion of the PSMA cassette was determined by restriction enzyme digestion, and sequence fidelity was confirmed by Sanger DNA sequencing of miniprep DNA. The construct will hence forth be referred to as 'flox-stop PSMA' (FSP). The FSP plasmid

was linearized using a double digest by SalI (New England BioLabs, Cat# R3138S) and BamHI (New England BioLabs, Cat#R3136S) ,prepared for microinjection as described above, and microinjected into single-cell FVB embryos as described (G. K. Hubbard 2013; M. Rubenstein 2022).

Animal husbandry

CD-1 IGS mice and Sprague-Dawley rats were obtained from Charles River (Kingston, NY). All mice were housed 2-5 per cage, whereas rats were housed 2-3 per cage in temperature (22°C ± 2°C) and humidity (55% ± 15%) controlled rooms on a 12-hour light/dark cycle. Water and food were available to animals ad libitum. PSMA BAC mice, MCP mice, MCR rats, and FSP rats were generated using pronuclear injection of their respective transgene into single cell mouse or rat embryos as described previously (G. K. Hubbard 2013; M. Rubenstein 2022). All experiments were reviewed and approved by the UMBC Institutional Animal Care and Use Committee.

Mating scheme and PCR genotyping

To obtain F_1 generation offspring, MCP founder mice were mated to homozygous floxed Pten mice ($Pten^{fl/fl}$) that were originally obtained from Jackson laboratory (stock number 004597), to generate mice with the genotype $MCP^{+}/Pten^{fl/fl}$. The DNA for genotyping the mice was obtained using a standard laboratory protocol as described previously (M. Rubenstein 2022; G. K. Hubbard 2013). Integration of the MCP transgene was identified by PCR using the following primers that yielded a 700 bp product: Forward primer: 5`-CAAGGCCTGGGGAGAGTGAAGAGA-3`;

Reverse primer: 5`- GCAGCCTGCACCTGAGGAGTGAATT-3`. *Pten*$^{fl/fl}$ mice were genotyped as described previously using primers: Forward primer: 5`-GTCTCTGGTCCTTACTTCC-3`; Reverse primer: 5`-ACGAGACTAGTGAGAAGTGC-3` (G. K. Hubbard 2013).

The flox-stop PSMA rats (FSP) were mated to MCR rats to generate rats with the double transgenic with genotypes MCR^{+}/flox-stop PSMA^{+}. The MCR transgene was identified by PCR using following primers: Forward primer: 5`-CGTTCGAGCTGGGAGCGATTTAAAACGCT-3`; Reverse primer: 5`-CCGAAGGGAGAAGGGTGTGACCGCAACGTAGG-3`. The flox-stop PSMA transgene was identified by using the following primers: Forward primer: 5`-CAAGGCCTGGGGAGAGTGAAGAGA-3`; Reverse primer: 5`-GCAGCCTGCACCTGAGGAGTGAATT-3`.

BMPC (Hoxb13-MYC^{+}/Hoxb13Cre^{+}/Pten$^{fl/fl}$) triple transgenic mice were bred and maintained as described previously (Gretchen K Hubbard et al. 2016).

Southern Blot analysis of MCR and MCP transgenic animals

Ten micrograms of purified genomic DNA was digested overnight with BamHI, buffer and separated on a 0.8% agarose at 30V for 12 hours. The gel was processed for Southern blotting as described previously (G. K. Hubbard 2013). A ^{32}P labeled DNA probe that recognizes a region of the Hoxb13 locus was generated by linear PCR and used to probe Southern blot membranes. Using this probe, in DNA from animals in which the MCR BAC had integrated. BamHI digestion produce a

transgenic band at 2.9 kb and an endogenous band from the mouse Hoxb13 locus at 4.6 kb.

Prostate dissection and tissue processing

Mice and rats were dissected immediately following euthanasia by CO2 asphyxiation followed by cervical dislocation. All prostate lobes were dissected individually from the urogenital tract and fixed in 10% neutral buffered formalin for 48 hours at room temperature. After fixation tissues were transferred to sterile 1X phosphate buffered saline and stored at 4°C. Samples were then transferred to the Johns Hopkins histology core where they were embedded into paraffin blocks using standard protocol.

Histopathological analysis

Hematoxylin and eosin (H&E) staining was performed at the Johns Hopkins histology core according using standard protocols. Immunohistochemistry (IHC) was performed by our collaborator Dr. Angelo DeMarzo using the Ventana Discovery ULTRA (Roche) automated IHC platform using antibodies for MYC (32072, Abcam), Pten (9188, Cell Signaling technology), and PSMA (58779, Abcam).

Glutamate-Glo™ assay

To determine the blood serum glutamate levels rats we performed the Glutamate-Glo™ assay (Promega, Cat# J7021) as per the manufacture instructions. All samples used in the assay were prepared in technical triplicates.

Results

Generation of transgenic mice expressing human PSMA in the prostate gland

The linearized human BAC containing the *FolH1/PSMA* locus was injected into the pronucleus of single-cell FVB embryos which were transferred to CD-1 foster mothers. Eighty-two F_0 generation pups were born, and upon weaning, were screened by genomic PCR and Southern blot for the presence of the human BAC (Table 1). Of the 82 mice screened, one, designated PSMA-79, was positive by both PCR and Southern blot for integration of the human PSMA transgene (Figure 1). PSMA-79 was mated to two FVB females to determine whether the transgene could be transmitted through the germline. The first two F_1 litters yielded 19 pups, 10 of which were male. Surprisingly, Southern blot analysis revealed that all 10 males were positive for presence of PSMA, whereas all 9 females were negative.

Table 2-1. Summary table of screened Folh1/PSMA transgenic mice. Only one founder FOLH1/PSMA transgene positive mice was born out of eighty-two.

Total No. of founders screened	82
Total No. of positive founders	1
Total No. of F1 screened	19
Total No. of positive F1	10

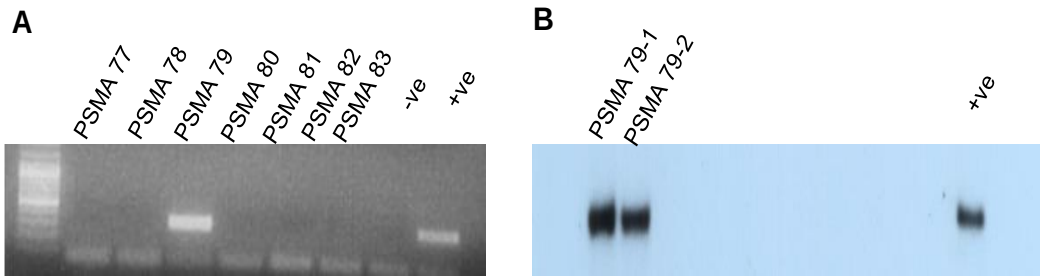

Figure 1. Genotyping using PCR and Southern blot. PCR results showing positive identification of PSMA 79 (A) and positive identification of PSMA-79-1/2 by Southern blot (B). PSMA DNA microinjection fragment was used as positive control.

To determine if human PSMA was expressed at the mRNA level in the prostate gland in strain PSMA-79, reverse transcriptase PCR (RT-PCR) was performed on total RNA from the prostate gland of transgene-positive PSMA-79 male. RNA extracted from the prostate gland of an age matched FVB male served as a negative control. RNA from the human prostate cancer cell line LNCaP which constitutively expresses PSMA was used as a positive control. RT-PCR using LNCaP RNA yielded a strong signal from a PCR product of the expected size (Figure 2), and RT-PCR using FVB RNA yielded no detectable specific signal. RT-PCR from the PSMA-79 prostate RNA yielded a very faint signal of the expected size (~300 bp). These data indicated that the human PSMA gene in the context of the BAC transgene in strain PSMA-79 was being transcribed, but at a substantially lower level relative to LNCaP cells (Figure 2).

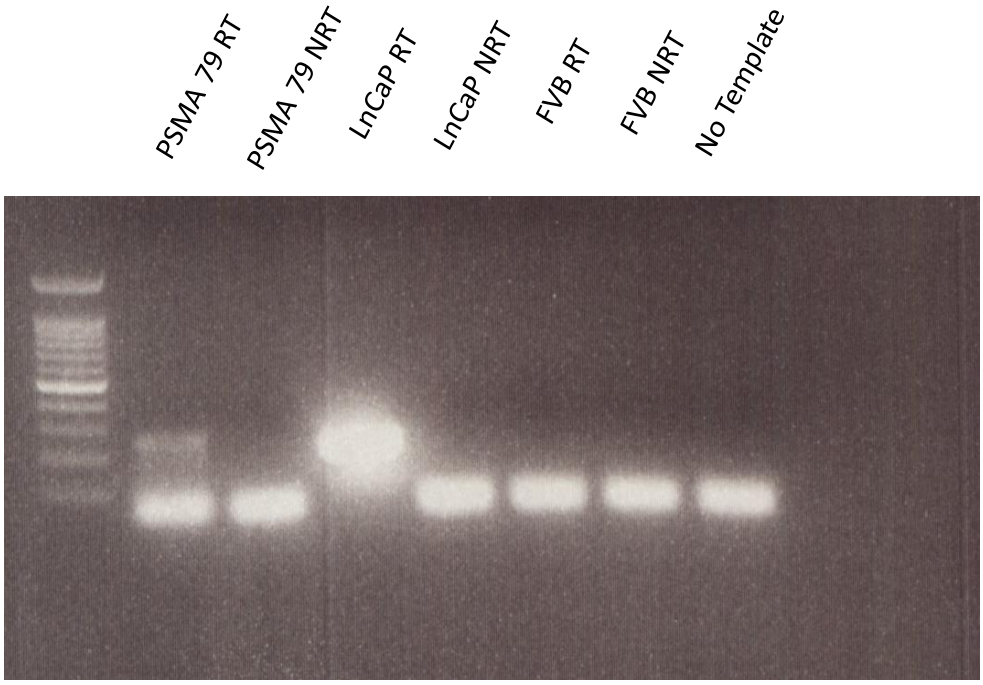

Figure 2. RT-PCR on mRNA from mouse prostate. Results show very low PSMA mRNA expression in PSMA 79 prostate RT sample, FVB prostate sample was used as negative control as it does not express PSMA in the prostate. RT and NRT refers to presence or absence of enzyme reverse transcriptase respectively. NTC refers to no template control.

To determine if human PSMA protein was expressed in the prostate gland of PSMA-79 transgenic offspring at a detectable level, Southern blot-positive males were euthanized, and prostate glands were dissected and prepared for immunohistochemical analysis of formalin-fixed paraffin embedded tissue sections along with prostate glands from FVB controls. Sections from anterior, dorsal, ventral, and lateral were stained with anti-human PSMA specific antibodies and counterstained with hematoxylin by our collaborator Dr. Angelo DeMarzo. Microscopic analysis of the resulting slides showed no difference IHC signal between PSMA-79 offspring and FVB controls. These data indicate that the low level of transcription of the human PSMA transgene observed by RT-PCR in

strainPSMA-79 prostates was insufficient to result in a detectable level of PSMA protein.

Generation and analysis of transgenic mice carrying the MCP BAC

The linearized Hoxb13 BAC containing the MCP transgene was injected into the pro-nuclei of single-cell FVB embryos which were then transferred to CD-1 foster mothers. 135 F_0 generation pups were born, and upon weaning, were screened by genomic PCR for the presence of the MCP transgene. Historically, in the same laboratory, ~10% of mice arising from embryos injected with BAC transgenes were transgenic. These data suggest that expressing PSMA under the control of *Hoxb13* regulatory elements is incompatible with life.

Table 2-2. List of MCP mice, PCR screening of F_0 mice arising from embryos injected with the MCR BAC.

Total founders screened	135
Total transgene positive founders	0

Generation and analysis of transgenic mice carrying a conditional PSMA transgene (FSP).

The linearized FSP transgene comprised of the human PSMA cDNA preceded by a floxed neomycin resistance cassette was injected into the pronucleus of single-cell FVB mouse embryos which were then transferred to mouse CD-1 foster mothers. 76 F_0 generation pups were born. Upon weaning, the founder generation mice were screened by genomic PCR for the presence of the FSP transgene.

Of the 76 mice screened, four, designated FSP-12, FSP-47, FSP-49, and FSP-73 were transgene positive. To determine whether the transgene could be transmitted through the germline, all four FSP+ founders were mated to animals carrying the Hoxb13-Cre transgene. A combination of PCR and Southern blot analyses revealed that all four FSP F_0 mice transmitted the transgene to offspring.

To determine if the flox-stop PSMA transgene was expressed in the prostate gland of FSP+ transgenic offspring, males PCR positive for both the Hoxb13-Cre and FSP transgene (hereafter, Hoxb13-Cre^+/FSP^+) were euthanized, and prostate glands were dissected, and RNA prepared. RT-PCR was performed to detect the PSMA mRNA, and LNCaP mRNA served as a positive control. All four Hoxb13-Cre^+/FSP^+ strains were negative for PSMA mRNA expression (data not shown).

To determine if PSMA could be detected in Hoxb13-Cre^+/FSP^+ mice, Hoxb13-Cre^+/FSP^-12 prostate glands were prepared for immunohistochemical analysis of formalin-fixed paraffin embedded tissue. Sections from singly transgenic FSP^+ prostate glands served as controls. Sections from anterior, dorsal, ventral, and lateral prostate lobes were stained with anti-human PSMA-specific antibodies and counterstained with hematoxylin by our collaborator Dr. Angelo DeMarzo. Microscopic analysis of the resulting slides showed no difference in specific staining between double transgenic Hoxb13-Cre^+/FSP^+ and singly transgenic FSP^+ controls. These data indicate that human PSMA transgene is not expressed at a detectable level in offspring of Hoxb13-Cre^+/FSP-12 mice (Figure 3).

To determine if the flox-stop PSMA transgene was expressed in the prostate gland of FSP-12 transgenic offspring, PCR positive males for Hoxb13-

Cre and flox-stop PSMA (Hoxb13-Cre^{+}/FSP^{+}) were euthanized, and prostate glands were dissected and prepared for immunohistochemical analysis of formalin-fixed paraffin embedded tissue sections along with prostate glands from flox-stop PSMA controls (FSP^{+}). Sections from anterior, dorsal, ventral, and lateral were stained with anti-human PSMA specific antibody and counterstained with hematoxylin by our collaborator Dr. Angelo DeMarzo. Microscopic analysis of the resulting slides showed no difference in specific staining between Hoxb13-Cre^{+}/FSP^{+} offspring and flox-stop FSP^{+} controls. These data indicate that human PSMA transgene is not expressed at a detectable level in offspring of FSP-12 mice (Figure 3).

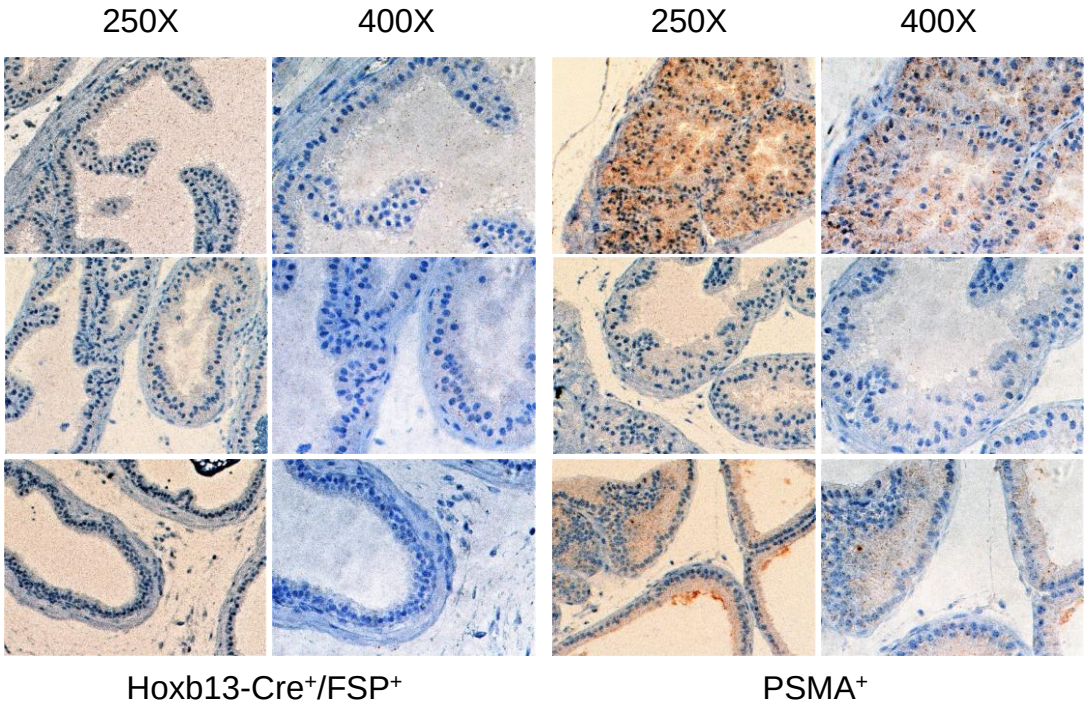

Figure 3. IHC for PSMA in Flox-stop PSMA mice. Immunohistochemistry results show no staining for PSMA in the ventral prostate of 4-week-old double transgenic Hoxb13-Cre+/FSP+, and control FSP+ mice, shown in two different magnifications of 250X and 400X.

Generation and analysis of transgenic rats carrying a conditional PSMA transgene (FSP)

Of the four F_0 generation rats, one rat pup designated CRP-1, was positive for the FSP transgene. CRP-1 was mated to MCR^+ female rats to determine the transmittance of the transgene through the germline. The mating yielded 16 F_1 generation pups who were positive for both MCR and MCR^+/FSP^+.

To determine if the flox-stop PSMA gene was expressed in the prostate gland in MCR^+/FSP^+, both at the RNA and protein level, we euthanized two 5-week-old MCR^+/FSP^+ rats. FSP^+ rats served as control. The prostate glands were dissected and split for immunohistochemical analysis and RT-PCR by formalin-fixing tissue and individually frozen on dry ice respectively.

To determine if PSMA cDNA was expressed at the mRNA level in MCR^+/FSP^+ prostate glands, RT-PCR was performed by extracting total RNA from the ventral lobe. RNA from an FSP^+ ventral lobe was prepared in parallel as a negative control. An ~300 bp RT-PCR product was observed exclusively in the MCR^+/FSP^+ sample demonstrating that the Cre protein expressed from the MCR transgene had excised the flox-stop-flox cassette from the FSP transgene, allowing transcription of an mRNA encoding human PSMA (Figure 4).

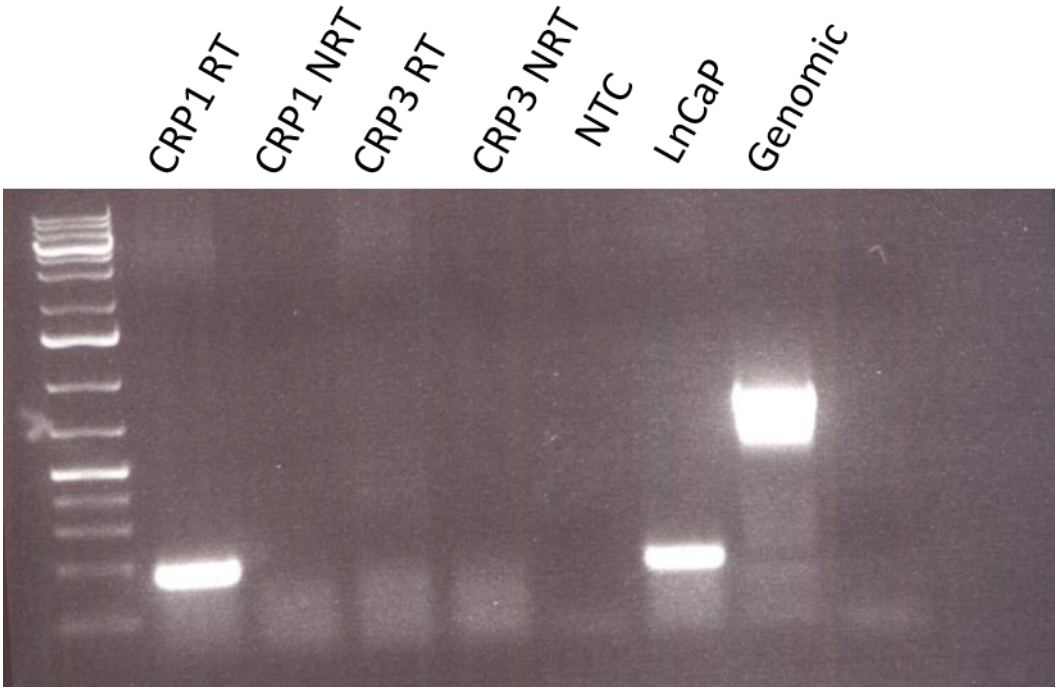

Figure 4. RT-PCR showing robust PSMA mRNA expression in CRP-1 prostate sample, CRP-3 prostate sample was used as negative control and does not express PSMA in the prostate. RT and NRT refers to presence or absence of enzyme reverse transcriptase respectively. NTC refers to no template control.

To determine if human PSMA transcribed from the FSP transgene was expressed at the protein level in the prostate gland sections of 5-week-old MCR^+/FSP^+ rats, sections of all four prostate lobes were stained with an anti-human PSMA-specific antibody and counterstained with hematoxylin. Microscopic analysis of the resulting slides showed positive staining for PSMA in the MCR^+/FSP^+ ventral, lateral, and dorsal lobes, but not in the anterior lobe (Figure 5 and data not shown). In contrast, and consistent with the RT-PCR data described above, no staining was observed in the FSP^+ negative control. These data demonstrate that FSP transgene in MCR^+/FSP^+ prostate glands properly encodes human PSMA. Within the three MCR^+/FSP^+ PSMA-expressing lobes, transgene expression was heterogeneous and non-uniform (Figure 5).

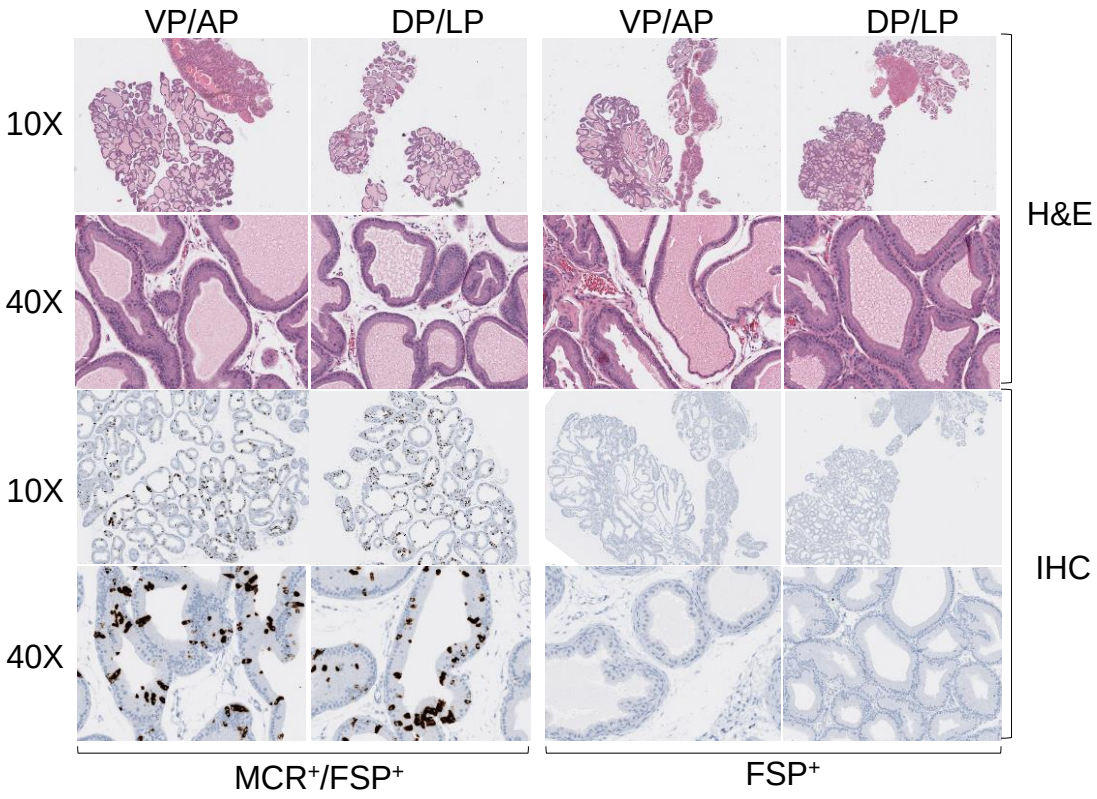

Figure 5. IHC for PSMA showing MCR⁺/FSP⁺ rats express human PSMA in the prostate gland. 5-week-old rat VP/AP and DP/LP of MCR⁺/FSP⁺ prostate shows heterogenous focal PSMA expression (brown) which is membranous and not cytoplasmic or nuclear whereas, no PSMA expression is observed on the epithelial cells in the VP/AP and DP/LP of control FSP⁺ rat.

In the ventral and lateral lobes, expression was highly focal, and generally consisted of clusters of PSMA expressing luminal cells dispersed throughout the secretory epithelium. ~5% of luminal epithelial cells in both the ventral and lateral lobes stained positively for human PSMA protein. The pattern of staining was consistent with membrane localization, however, due to the high signal in the PSMA IHC, cytoplasmic staining cannot be ruled out (Figures 5 & 6). In the dorsal lobe, a few isolated pockets of PSMA expression were also observed, generally consisting of fewer than 3 cells (data not shown).

To determine if the expression of human PSMA persists in MCR^+/FSP^+ rat prostate glands during normal aging, MCR^+/FSP^+ rats and MCR^+ negative controls were analyzed by IHC for PSMA expression at 11 and 25 weeks of age and compared to that in 5-week-old rats. By 11 weeks of age, PSMA expression was observed in ~15-20% of ventral and lateral prostate epithelial cells, indicating an increase in transgene expressivity as a function of age (Figures 6 & 7). MCR^+ rats remained negative for PSMA expression. By 25 weeks of age PSMA expression was observed in ~80% of prostate epithelial cells in both the ventral and lateral lobes of MCR^+/FSP^+ rats, strongly suggesting that the expressivity of the FSP transgene continues to increase with age (Figure 6).

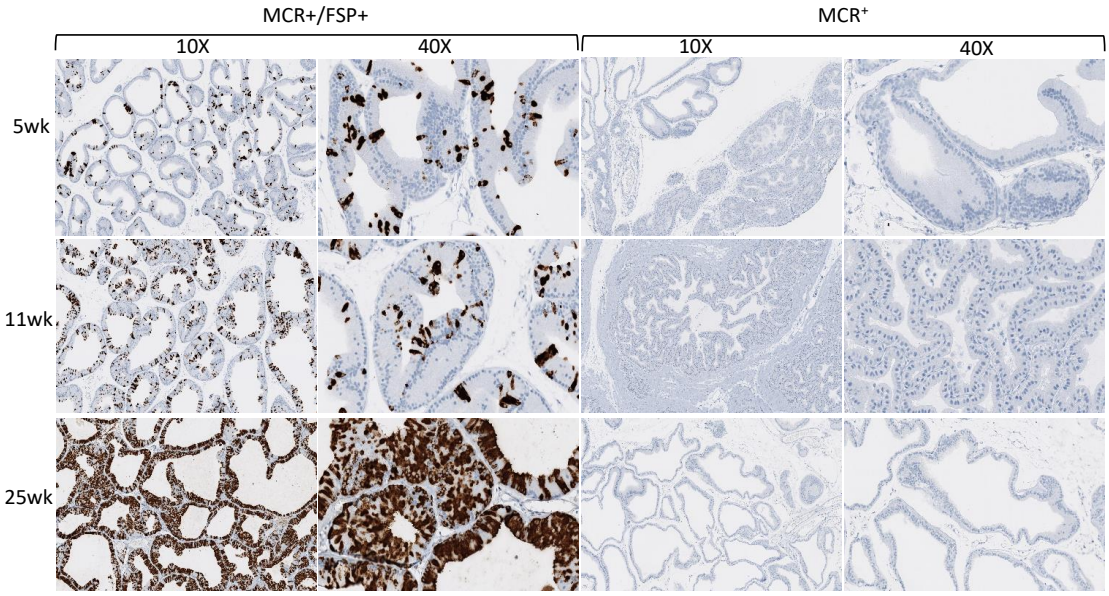

Figure 6. PSMA expression at various timepoints. PSMA expression in VP increases with age of the rats. IHC for PSMA in VP of rat prostate at 5, 11, and 25 weeks of age. The expression of PSMA (brown) goes on increasing with age and from being heterogenous and focal at 5 weeks becomes almost homogeneous by 25 weeks of age with only small areas staining negative for PSMA.

To determine if human PSMA expression alters the morphology of the prostate epithelium, H&E-stained sections from 5-, 11-, and 25-week-old MCR^+/FSP^+

rats were analyzed by a prostate pathologist in a blinded manner to determine whether changes in cellular or glandular architecture could be detected. H&E sections from MCR^+ prostate lobes expressing human MYC but not human PSMA as well as from wild type Sprague Dawley prostates served as controls. As expected, in both MCR^+ and MCR^+/FSP^+ ventral prostates, a few areas with suspected PIN lesions were noted, most likely due to the expression of the human MYC oncoprotein transcribed from the MCR transgene (M. Rubenstein, unpublished). However, no morphological features that distinguished the ventral or lateral prostate epithelia in MCR^+/FSP^+ rats from those of MCR^+ rats were noted. These data demonstrate that stable human PSMA expression can be achieved in rat ventral and lateral prostate epithelial cells in the context of human MYC oncoprotein expression, apparently without morphological consequences observable at the level of light microscopy (Figure 6 & 7).

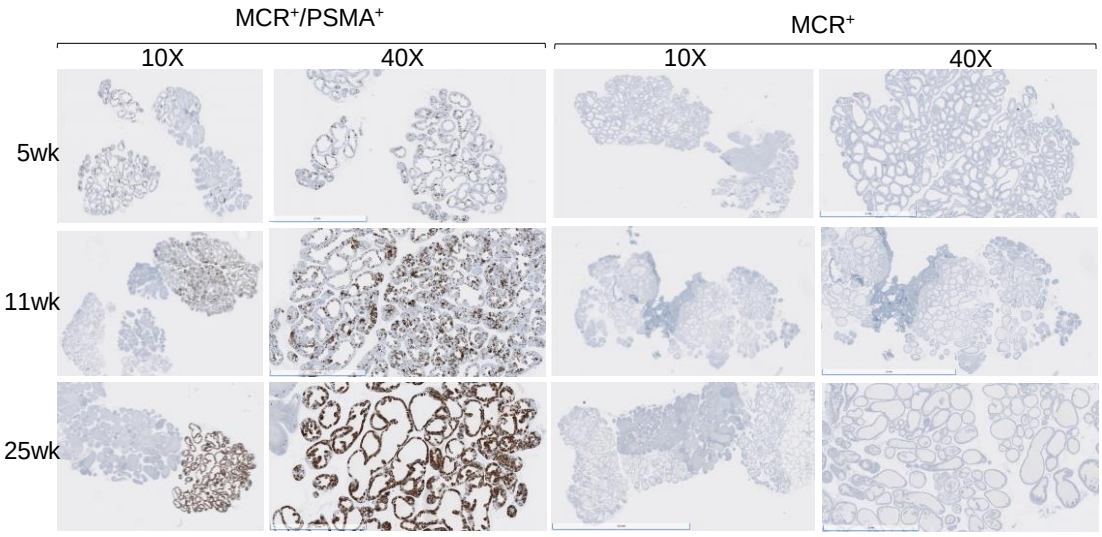

Figure 7. PSMA expression is lobe specific. IHC shows PSMA expression in LP lobes of the prostate increases with age but not DP. IHC for PSMA in LP of rat prostate at 5, 11, and 25 weeks of age. The expression of PSMA (brown) similarly to VP increases with age and from being heterogenous and focal at 5 weeks becomes nearly homogeneous by 25 weeks of age.

Consistent with the known enzymatic activity of human PSMA and the increase in PSMA during cancer progression, Koocheckpour et.al. reported a positive correlation increasing grade of cancer and increased serum glutamate levels in patients (Koochekpour et al. 2012). To determine if the increased PSMA expression in MCR^{+}/FSP^{+} rats would affect serum glutamate level, we performed a pilot experiment to quantify serum glutamate in a 25-week-old MCR^{+}/FSP^{+} rats and an MCR^{+} control. Glutamate-Glo™ Assays were performed in triplicate (Figure 8). Although there was no statistical Our data indicate that overexpression of PSMA does indeed lead to increase in blood serum levels of glutamate when compared to control (Figure 8).

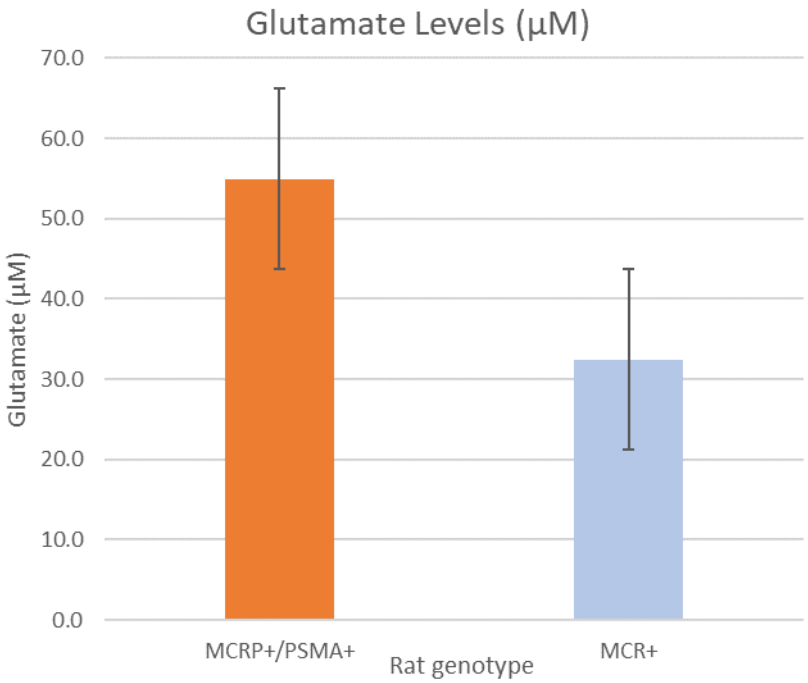

Figure 8. Glutamate-glo assay for estimation of serum glutamate. Blood serum glutamate levels from Glutamate-glo assay show that rats expressing PSMA have higher serum glutamate levels when compared to rats not expressing PSMA.

Discussion

Since PSMA's discovery in 1987, it has been a molecule of great interest in human prostate cancer diagnosis and treatment. PSMA expression cancer increases 100-1000 fold at the protein level when compared to normal healthy prostate (Silver DA, Pellicer I, Fair WR, Heston WD 1997; D. G. Bostwick et al. 1998). The increase in PSMA expression is observed upon the onset of carcinogenesis and continues to increase during disease progression and metastasis (Carter, Feldman, and Coyle 1996; D. G. Bostwick et al. 1998; Sweat et al. 1998). However, despite the fact that it was discovered 33 years ago, the basic physiological functions of PSMA in prostate cancer progression have not been clearly established. A limiting factor in understanding the role of PSMA in prostate cancer is the lack of animal models that express PSMA in the prostate gland. Mice and rats, which are the preeminent species in which human prostate pathology has historically been modeled, do not express endogenous PSMA in their prostate glands (Simons et al. 2019). To gain a better understanding of the role PSMA plays in prostate cancer, we developed the first animal model that expresses human PSMA in the luminal epithelial cells of the prostatic ducts.

Our original intent was to develop a mouse model capable of expressing human PSMA in either a constitutive or conditional manner. Toward this end, we first expended considerable effort to derive transgenic mice carrying a human BAC containing the PSMA locus and surrounding genomic sequences. By and large, there is considerable conservation of gene regulatory mechanisms between humans and mice, such that human transgenes are expressed in mice in a manner that mimics their

pattern of expression in humans. However, this was not the case for BAC CH17-403C8 which, in addition to the 62 kb region spanning the exons and introns of PSMA, contained 40 kb of upstream and 120 kb of downstream sequences. The weak expression of PSMA we observed from BAC CH17-403C8 in transgenic mice could be due to several factors. First, it is possible that the cis-regulatory elements required to direct PSMA expression in the prostate are not captured in BAC CH17-403C8. Unfortunately, the relatively large size of the PSMA RNA coding region severely limited our choices among BACs in extant repositories. BAC CH17-403C8 was chosen because it bears the largest up- and downstream regions of all publicly available PSMA-containing human BACs. Testing a wider swath of the genome for PSMA regulatory sequences would require the development of a customized human BAC, which is technically feasible and worthy of consideration. Second, it is possible that the regulatory apparatus of the human PSMA locus requires transcriptional regulatory proteins that are not expressed in the mouse prostate. Given that the prostate is a secondary sex organ and is highly divergent in morphology between mice and humans, this is a well-founded possibility. Exploring this possibility would require a detailed in vivo comparison of the regulatory regions of the mouse and human FOLH1 loci. A third formal possibility is that robust expression of human PSMA under the direction of human regulatory elements is incompatible with life in mice.

Our second strategy to achieve human PSMA expression in the mouse prostate was to use endogenous mouse Hoxb13 regulatory elements to direct expression of a multifunctional mRNA encoding human MYC, Cre, and human

PSMA separated by 2A peptides. The fact that we were unable to derive transgenic founder generation mice carrying this transgene strongly suggests that human PSMA, when directed by Hoxb13 regulatory sequences, leads to embryonic death. In other work, we have successfully derived multiple transgenic mouse strains expressing human MYC or Cre under the control of Hoxb13 elements and have also combined those two transgenes by breeding. This clearly points to PSMA as the lethal element in the MCP construct. How might human PSMA lead to embryonic death? Hoxb13 is a highly conserved developmental patterning gene, that is strongly expressed in the posterior spinal cord and somite-derived mesoderm. One potential mechanism that could underlie PSMA toxicity is its ability to generate glutamate from polyglutamated folate and N-acetyl-aspartyl-glutamate. In the nervous system, glutamate is an important neurotransmitter, however some neurons are susceptible to glutamate excitotoxicity. It is possible that during development, human PSMA expression in the expression domain of Hoxb13 could elicit neurodegeneration or prevent proper development due to glutamate excitotoxicity in the posterior spinal cord, leading to embryonic death. This potential mechanism may also underlie our failure to develop a conditional model of PSMA expression in mice using the FSP transgene, which would also lead to PSMA expression in the posterior spinal cord during embryonic development. To circumvent this outcome, it may be possible to develop a Tet-On system wherein Cre expression is under Doxycycline (Dox) control. In this scenario, Dox would be administered postnatally to mice carrying a Hoxb13-rtTA transgene, a TetO-Cre transgene, and the FSP construct. This approach is eminently feasible, given that Hoxb13-rtTA transgenic mice already exist.

The increase in expressivity of PSMA in MCR^{+}/FSP^{+} rats is an interesting feature that has several potential explanations. One potential mechanism is entirely technical in nature and is unrelated to the enzymatic activity of PSMA. In this scenario, the activation of the FSP transgene, which is dependent upon a threshold amount of Cre recombinase, is stochastic in nature, such that the number of 'successful' activation events increases over time. Once Cre has removed the floxed-stop cassette in the nucleus of a cell, human PSMA expression becomes constitutive. Provided that PSMA activation has o deleterious consequences, PSMA-expressing cells would accumulate as a function of time. Another potential mechanism recognizes the fact that PSMA has glutamate carboxypeptidase and folate hydrolase activity, cleaving glutamates from its substrates, polyglutamated-folates and N-acetyl-aspartyl-glutamate (NAAG) (Watt et al. 2001; Zhao, Matherly, and Goldman 2009). In this scenario, the production of glutamate and/or folate through the activity of PSMA would provide a cell autonomous and/or focal benefit by providing an increased supply of these key cellular nutrients. In support of the latter scenario, it has been suggested the overexpression of PSMA in cancer plays a role in satisfying the increased metabolic need of cancer cells by providing both glutamate and folate (Hong et al. 2022). Clinical studies in human prostate cancer patients have also revealed a correlation between increased blood serum glutamate levels and advanced disease stage with high Gleason score, which also has higher PSMA expression (Koochekpour et al. 2012). Glutamates and their receptors are themselves thought to play an important role in multiple catabolic pathways in volved in cancer, as glutamate is a major source of cellular energy (Willard and Koochekpour 2013b;

Koochekpour 2013). Folates have also been implicated in supporting the increased metabolic needs of cancer cells and are being explored as potential targets for therapeutic approaches (Hagner and Joerger 2010; Ristau, O 'keefe, and Bacich 2014). Kaittanis et. al. have recently shown that glutamates cleaved from vitamin B9 activate the phosphoinositide 3-kinase (PI3K) signaling axis that plays a pro-tumorigenic role in multiple cancers and is thought to play an important role in prostate cancer progression (Kaittanis et al. 2018b; Palamiuc and Emerling 2018). The MCR^+/FSP^+ rat model may provide a powerful new system in which role of glutamate, folate, their respective receptors and transporters, and their interplay with PSMA can be explored to develop therapeutic approaches for treatment of prostate cancer. Realization of the potential of the MCR^+/FSP^+ rat model to contribute to our understanding of prostate cancer is predicated upon the availability a rat model that faithfully recapitulates key morphological features of the human disease. To date, this goal has been elusive, however, some progress has been made toward its achievement (M. Rubenstein 2022).

PSMA is thought to act as a cell surface receptor, however, its natural ligand or ligands have not yet been identified. Some in vitro experiments using antibodies against the extracellular domain of PSMA have shown that it interacts with membrane scaffold proteins that govern endocytosis of PSMA-bound substrates (S. A. Rajasekaran et al. 2003; A. K. Rajasekaran, Anilkumar, and Christiansen 2005; Anilkumar et al. 2006). This function of PSMA provides ample opportunity for developing precision therapeutics/theranostic approaches for treatment, and the MCR^+/FSP^+ rat model is the platform to explore such approaches. Clearly, the

MCR^+/FSP^+ model fills an important gap in which critical unanswered questions surrounding the molecular basis of prostate cancer initiation and progression, and the efficacy of PSMA-directed diagnostics and therapeutics can be experimentally approached.

References:

Anilkumar, Gopalakrishnapillai, Sonali P Barwe, Jason J Christiansen, Sigrid A Rajasekaran, Donald B Kohn, and Ayyappan K Rajasekaran. 2006. "Association of Prostate-Specific Membrane Antigen with Caveolin-1 and Its Caveolae-Dependent Internalization in Microvascular Endothelial Cells: Implications for Targeting to Tumor Vasculature." https://doi.org/10.1016/j.mvr.2006.03.004.

Bostwick, David G., Anna Pacelli, Michael Blute, Patrick Roche, and Gerald P. Murphy. 1998. "Prostate Specific Membrane Antigen Expression in Prostatic Intraepithelial Neoplasia and Adenocarcinoma: A Study of 184 Cases." *Cancer* 82 (11): 2256–61. https://doi.org/10.1002/(SICI)1097-0142(19980601)82:11<2256::AID-CNCR22>3.0.CO;2-S.

Briceno, N. 2015. "Developing a Hoxb13-Based Mouse Model of Lethal Metastatic Prostate Cancer - ProQuest Dissertations & Theses Global - ProQuest." *ProQuest Dissertations & Theses Global.* https://www.proquest.com/pqdtglobal/docview/1734372920/58FD6F02D86F4F94PQ/1?accountid=14577.

Bubendorf, Lukas, Alain Schopfer, Urs Wagner, Guido Sauter, Holger Moch, Niels

Willi, Thomas C Gasser, and Michael J Mihatsch. 2000. “Metastatic Patterns of Prostate Cancer: An Autopsy Study of 1,589 Patients.” *Human Pathology* 31 (5): 578–83. https://doi.org/10.1053/hp.2000.6698.

Carter, Ruth E, Alexis R Feldman, and Joseph T Coyle. 1996. “Prostate-Specific Membrane Antigen Is a Hydrolase with Substrate and Pharmacologic Characteristics of a Neuropeptidase.” *Neurobiology* 93: 749–53. http://www.pnas.org/content/93/2/749.long.

Feldman, Brian J., and David Feldman. 2001. “The Development of Androgen-Independent Prostate Cancer.” *Nature Reviews Cancer* 1 (1): 34–45. https://doi.org/10.1038/35094009.

Ghosh, Arundhati, and Warren D.W. Heston. 2004. “Tumor Target Prostate Specific Membrane Antigen (PSMA) and Its Regulation in Prostate Cancer.” *Journal of Cellular Biochemistry* 91 (3): 528–39. https://doi.org/10.1002/jcb.10661.

Hagner, Nicole, and Markus Joerger. 2010. “Cancer Chemotherapy: Targeting Folic Acid Synthesis.” *Cancer Management and Research* 2 (November): 293–301. https://doi.org/10.2147/CMR.S10043.

Hong, Xi, Liang Mao, Luwei Xu, Qiang Hu, and Ruipeng Jia. 2022. “Prostate-Specific Membrane Antigen Modulates the Progression of Prostate Cancer by Regulating the Synthesis of Arginine and Proline and the Expression of Androgen Receptors and Fos Proto-Oncogenes.” *Bioengineered* 13 (1): 995. https://doi.org/10.1080/21655979.2021.2016086.

Hubbard, G. K. 2013. “Developing Improved Mouse Models of Prostate Cancer - ProQuest Dissertations & Theses Global - ProQuest.” *ProQuest Dissertations &*

Theses Global. https://www.proquest.com/pqdtglobal/docview/1442583933/31096E8E623485FPQ/1?accountid=14577.

Hubbard, Gretchen K, Laura N Mutton, May Khalili, Ryan P McMullin, Jessica L Hicks, Daniella Bianchi-Frias, Lucas A Horn, et al. 2016. "Combined MYC Activation and Pten Loss Are Sufficient to Create Genomic Instability and Lethal Metastatic Prostate Cancer." *Cancer Research* 76 (2): 283–92. https://doi.org/10.1158/0008-5472.CAN-14-3280.

Hupe, Marie Christine, Christian Philippi, Doris Roth, Christiane Kümpers, Julika Ribbat-Idel, Finn Becker, Vincent Joerg, et al. 2018. "Expression of Prostate-Specific Membrane Antigen (PSMA) on Biopsies Is an Independent Risk Stratifier of Prostate Cancer Patients at Time of Initial Diagnosis." *Frontiers in Oncology* 8 (DEC): 623. https://doi.org/10.3389/FONC.2018.00623.

Israeli, R S, C T Powell, W R Fair, and W D Heston. 1993. "Molecular Cloning of a Complementary DNA Encoding a Prostate-Specific Membrane Antigen." *Cancer Research* 53 (2): 227–30. http://www.ncbi.nlm.nih.gov/pubmed/8417812.

Kaittanis, Charalambos, Chrysafis Andreou, Haley Hieronymus, Ninghui Mao, Catherine A Foss, Matthias Eiber, Gregor Weirich, et al. 2018. "Prostate-Specific Membrane Antigen Cleavage of Vitamin B9 Stimulates Oncogenic Signaling through Metabotropic Glutamate Receptors." *The Journal of Experimental Medicine* 215 (1): 159–75. https://doi.org/10.1084/jem.20171052.

Koh, Cheryl M, Charles J Bieberich, Chi V Dang, William G Nelson, Srinivasan

Yegnasubramanian, and Angelo M De Marzo. 2010. “MYC and Prostate Cancer.” *Genes & Cancer* 1 (6): 617–28. https://doi.org/10.1177/1947601910379132.

Koochekpour, Shahriar. 2013. “Glutamate, a Metabolic Biomarker of Aggressiveness and a Potential Therapeutic Target for Prostate Cancer.” *Asian Journal of Andrology*. Wolters Kluwer -- Medknow Publications. https://doi.org/10.1038/aja.2012.145.

Koochekpour, Shahriar, Sunipa Majumdar, Gissou Azabdaftari, Kristopher Attwood, Ray Scioneaux, Dhatchayini Subramani, Charles Manhardt, et al. 2012. “Serum Glutamate Levels Correlate with Gleason Score and Glutamate Blockade Decreases Proliferation, Migration, and Invasion and Induces Apoptosis in Prostate Cancer Cells.” *Clinical Cancer Research* 18 (21): 5888–5901. https://doi.org/10.1158/1078-0432.CCR-12-1308.

Lauri, Chiara, Lorenzo Chiurchioni, Vincenzo Marcello Russo, Luca Zannini, and Alberto Signore. 2022. “PSMA Expression in Solid Tumors beyond the Prostate Gland: Ready for Theranostic Applications?” *Journal of Clinical Medicine* 11 (21). https://doi.org/10.3390/JCM11216590.

Nelson, William G., Angelo M. De Marzo, and William B. Isaacs. 2003. “Prostate Cancer.” *New England Journal of Medicine* 349 (4): 366–81. https://doi.org/10.1056/NEJMra021562.

Palamiuc, Lavinia, and Brooke M Emerling. 2018. “PSMA Brings New Flavors to PI3K Signaling: A Role for Glutamate in Prostate Cancer.” *The Journal of Experimental Medicine* 215 (1): 17–19. https://doi.org/10.1084/jem.20172050.

Perner, Sven, Matthias D. Hofer, Robert Kim, Rajal B. Shah, Haojie Li, Peter Möller, Richard E. Hautmann, Juergen E. Gschwend, Rainer Kuefer, and Mark A. Rubin. 2007. “Prostate-Specific Membrane Antigen Expression as a Predictor of Prostate Cancer Progression.” *Human Pathology* 38 (5): 696–701. https://doi.org/10.1016/J.HUMPATH.2006.11.012.

Queisser, Angela, Susanne A. Hagedorn, Martin Braun, Wenzel Vogel, Stefan Duensing, and Sven Perner. 2015. “Comparison of Different Prostatic Markers in Lymph Node and Distant Metastases of Prostate Cancer.” *Modern Pathology : An Official Journal of the United States and Canadian Academy of Pathology, Inc* 28 (1): 138–45. https://doi.org/10.1038/MODPATHOL.2014.77.

Rajasekaran, Ayyappan K., Gopalakrishnapillai Anilkumar, and Jason J. Christiansen. 2005. “Is Prostate-Specific Membrane Antigen a Multifunctional Protein?” *American Journal of Physiology-Cell Physiology*. https://doi.org/10.1152/ajpcell.00506.2004.

Rajasekaran, Sigrid A, Gopalakrishnapillai Anilkumar, Eri Oshima, James U Bowie, He Liu, Warren Heston, Neil H Bander, and Ayyappan K Rajasekaran. 2003. “A Novel Cytoplasmic Tail MXXXL Motif Mediates the Internalization of Prostate-Specific Membrane Antigen.” *Molecular Biology of the Cell* 14: 4835–45. https://doi.org/10.1091/mbc.E02–11.

Ristau, Benjamin T, Denise S O ’keefe, and Dean J Bacich. 2014. “The Prostate-Specific Membrane Antigen: Lessons and Current Clinical Implications from 20 Years of Research.” *Urologic Oncology: Seminars and Original Investigations* 32: 272–79. https://doi.org/10.1016/j.urolonc.2013.09.003.

Robinson, Dan, Eliezer M. Van Allen, Yi Mi Wu, Nikolaus Schultz, Robert J. Lonigro, Juan Miguel Mosquera, Bruce Montgomery, et al. 2015. “Integrative Clinical Genomics of Advanced Prostate Cancer.” *Cell* 161 (5): 1215–28. https://doi.org/10.1016/J.CELL.2015.05.001.

Rubenstein, M. 2022. “Molecular Basis of Carcinogenesis in Rodent Models of Prostate Cancer - ProQuest Dissertations & Theses Global - ProQuest.” *ProQuest Dissertations & Theses Global*. https://www.proquest.com/pqdtglobal/docview/2728493074/42958B868B304A6FPQ/1?accountid=14577.

Siegel Mph, Rebecca L, Kimberly D Miller, Nikita Sandeep, Wagle Mbbs, | Ahmedin, Jemal Dvm, and Rebecca L Siegel. 2023. “Cancer Statistics, 2023.” *CA: A Cancer Journal for Clinicians* 73 (1): 17–48. https://doi.org/10.3322/CAAC.21763.

Silver DA, Pellicer I, Fair WR, Heston WD, Cordon-Cardo C. 1997. “Prostate-Specific Membrane Antigen Expression in Normal and Malignant Human Tissues.” *Clin Cancer Res.* 81 (5).

Simons, Brian W., Norman F. Turtle, David H. Ulmert, Diane S. Abou, and Daniel L.J. Thorek. 2019. “PSMA Expression in the Hi-Myc Model; Extended Utility of a Representative Model of Prostate Adenocarcinoma for Biological Insight and as a Drug Discovery Tool.” *The Prostate* 79 (6): 678–85. https://doi.org/10.1002/PROS.23770.

Sommer, Ulrich, Tiziana Siciliano, Celina Ebersbach, Alicia Marie K. Beier, Matthias B. Stope, Korinna Jöhrens, Gustavo B. Baretton, Angelika Borkowetz, Christian

Thomas, and Holger H.H. Erb. 2022. “Impact of Androgen Receptor Activity on Prostate-Specific Membrane Antigen Expression in Prostate Cancer Cells.” *International Journal of Molecular Sciences* 23 (3). https://doi.org/10.3390/IJMS23031046/S1.

Sweat, Susan D., Anna Pacelli, Gerald P. Murphy, and David G. Bostwick. 1998. “Prostate-Specific Membrane Antigen Expression Is Greatest in Prostate Adenocarcinoma and Lymph Node Metastases.” *Urology* 52 (4): 637–40. https://doi.org/10.1016/S0090-4295(98)00278-7.

Taylor, Barry S., Nikolaus Schultz, Haley Hieronymus, Anuradha Gopalan, Yonghong Xiao, Brett S. Carver, Vivek K. Arora, et al. 2010. “Integrative Genomic Profiling of Human Prostate Cancer.” *Cancer Cell* 18 (1): 11–22. https://doi.org/10.1016/J.CCR.2010.05.026.

Watt, Fujiko, Anna Martorana, Diana E Brookes, Thu Ho, Elizabeth Kingsley, Denise S O ’keefe, Pamela J Russell, Warren D W Heston, and Peter L Molloy. 2001. “A Tissue-Specific Enhancer of the Prostate-Specific Membrane Antigen Gene, FOLH1.” https://doi.org/10.1006/geno.2000.6446.

Willard, Stacey S, and Shahriar Koochekpour. 2013. “Glutamate Signaling in Benign and Malignant Disorders: Current Status, Future Perspectives, and Therapeutic Implications.” *International Journal of Biological Sciences* 9 (7): 728–42. https://doi.org/10.7150/ijbs.6475.

Zhao, Rongbao, Larry H Matherly, and I David Goldman. 2009. “Membrane Transporters and Folate Homeostasis: Intestinal Absorption and Transport into Systemic Compartments and Tissues.” *Expert Reviews in Molecular Medicine*

11 (January): e4. https://doi.org/10.1017/S1462399409000969.

Chapter 3: Extending the limits of multi-fragment DNA assembly for synthetic biology applications

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Introduction

DNA assembly plays a pivotal role in the construction of gene expression systems and even entire chromosomes (Ostrov et al. 2019). The ability to manipulate and combine DNA from different sources to create novel gene constructs has greatly propelled biological research and biotechnology for the past four decades (Casini et al. 2015). The introduction of DNA cloning technology revolutionized the field of biological research, facilitating a deeper comprehension of genetic mechanisms across diverse organisms and equipping us with essential tools to genetically engineer cells and even entire organisms (Ellis, Adie, and Baldwin 2011). The pivotal breakthrough enabling this revolution was the discovery of restriction enzymes, bacterial proteins that act as precise molecular scissors, cutting DNA in specific patterns (Pingoud, Wilson, and Wende 2014; Loenen et al. 2014). DNA fragments cut in this manner can be easily reassembled, provided they are cut using restriction enzymes that leave compatible single-stranded overhangs. So-called double digests, wherein one end of DNA molecule has been cleaved with one restriction enzyme, and the other has been cleaved with a different enzyme, provides a strategy to create ordered assemblies of DNA. However, joining more than 4-5 DNA molecules using this strategy is technically difficult.

The invention of polymerase chain reaction (PCR) revolutionized molecular biology by enabling the synthesis of custom double-stranded DNA molecules of user defined sequence and length (Zhu et al. 2020; Rose 1991). The human genome project further accelerated the development of molecular biology techniques that

democratized them to mainstream researchers (Olson 1993; Guyer and Collins 1993; Lanchbury 1998; Greenhalgh 2005; Moraes and Góes 2016).

From the late 1970s until 2009, iterative conventional restriction enzyme-based cloning was essentially the only method available to create complex DNA assemblies. In 2009, Daniel Gibson invented a new approach based on end sequence homology among fragments to be joined to generate a higher order assembly. Gibson DNA Assembly is now the predominant method used for so-called 'scar-less' molecular cloning that enables the specific joining of multiple DNA fragments without the constraint of restriction enzyme sites. The method uses DNA fragments with ~25 nucleotides of end homology generated by PCR, and employs three enzymes a 5` exonuclease, a DNA polymerase, and a DNA ligase. The reactants are subjected to an isothermal reaction to combine the DNA fragments in an ordered fashion (Gibson et al. 2009a; Sayers and Eckstein 1990). Unlike traditional methods, that use restriction enzymes to generate sticky ends, Gibson Assembly uses a different enzyme to trim back the ends of the double-stranded DNA molecules exposing short single-stranded regions that can be designed in a complimentary fashion to connect the DNA fragments in a predefined order (Gibson 2011). This approach generates a scarless DNA assembly that is extremely difficult, if not impossible to achieve with traditional cloning methods. Gibson Assembly is a two-step process, Step 1 entails the preparation of a linear bacterial vector using a restriction enzyme and followed by a purification procedure. Step 2 involves using the three enzyme mixture and oligonucleotides to assemble DNA fragments in defined order with the linear bacterial vector to form a covalently closed DNA circle

(Gibson et al. 2009a; Ostrov et al. 2019). Although this technology is very powerful, Gibson Assembly has technical limitations related to the assembly of DNA fragments, including constraints on the size and number of fragments that can be efficiently joined. Gibson Assembly efficiency reduces profoundly when either using fragments < 200 base pairs in length, or > 5 DNA-fragments, or synthesis of short strands <100 base pairs (Hoose et al. 2023; Avilan 2023). Gibson Assembly efficiency is dependent on the intermolecular ligation of DNA molecules in a proper order, which is influenced by concentration of the fragments being assembled (Czar et al. 2009). However, optimal DNA concentration to achieve the highest efficiency has not been thoroughly explored. Several Gibson Assembly kits are commercially available that claim to address some of the limitations of the original protocol, but their adoption by the wider scientific community remains uncertain (Gibson 2011).

Despite these advancements in molecular biology, the process of assembling DNA parts into complex new constructs continues to be a time-consuming, laborious, and unpredictable tradecraft. There is a need for the development and adoption of new DNA assembly methods that can be scaled up potentially to the point of whole genome assembly by the research community. Cost and efficiency are also other crucial factors that guide scientific end-users in selecting such protocols. To address these limitations, we have developed and improved a DNA assembly method that we term ExSembly, that combines two-step Gibson Assembly into a one-pot, time-and-cost-saving solution. Here, we demonstrate that ExSembly functions with a wide array of commonly used restriction enzymes and quantify its efficiency in assembling up to 12 DNA fragments. We also provide a highly practical and economical solution

to reduce the technical difficulty in achieving the high DNA concentration that supports assembly efficiency.

Materials and methods

PCR amplification of DNA fragments

The human SAP130 cDNA cloned in the vector pcDNA3.1 was established as the standard construct to be assembled. Primers used for amplification of DNA fragments used in 4-fragment ExSembly are in Table 3-1 and for amplification of 12-fragment ExSembly are in Table 3-2. PCR was performed to a final volume of 400 µl using for each DNA fragment using Phusion polymerase (Cat# M0530L), 5X Phusion HF buffer pack (Cat# B0518SVIAL), and 10mM dNTPs from New England BioLabs (NEB), using standard protocol of hot start at 98°C for 3 min, denaturation at 98°C for 30 sec, annealing for 30 sec, extension at 72°C for 30 sec for 30 cycles, and final extension at 72°C for 2 mins. All fragments were generated in bulk to maintain consistency throughout testing.

Table 3-1. List of primers for four fragment ExSembly

Primer name	Sequence (5` to 3`)
Kanamycine 5 KpnI	TCCGCATGCGAGCTCGGTACTGATTGAACAAGATGGATTG
Kanamycine HindIII	AGTCCAAGCTCAGCTAATTATCAGAAGAACTCGTCAAGAA
SAP130 5	TTCTTGACGAGTTCTTCTGAAGTTCTCAACAGTTTCCTCG
SAP130 3’ KpnI	TGCAGGTCGACCCGGGGTACCTAGACTTTTTCCTTTCGCT
SAP130 5R1561	GATACTGTCGGATAGTGGACACGGAACTGGTGATTGGGGT
SAP130 6F 1581	GTCCACTATCCGACAGTATCCAGTTTCAGCTCAGGCTCCA

Table 3-2. List of primers for twelve fragment ExSembly

Primer name	**Sequence**
Kanamycine 5 KpnI	TCCGCATGCGAGCTCGGTACTGATTGAACAAGATGGATTG
Kanamycine HindIII	AGTCCAAGCTCAGCTAATTATCAGAAGAACTCGTCAAGAA
SAP130 5	TTCTTGACGAGTTCTTCTGAAGTTCTCAACAGTTTCCTCG
SAP130 3 KpnI	TGCAGGTCGACCCGGGGTACCTAGACTTTTTCCTTTCGCT
Sap130 1R	CCGAAAATGAAAGTGGCACTGCTGGCGTCAGGTGTGCTG
SAP130 2F	AGTGCCACTTTCATTTTCGGAGGGACTTATGAAGCCGCCC
SAP130 2R	CATTGCTGCGGATGATAGACATTTGCACATTTGTAGTCAT
SAP130 3F	GTCTATCATCCGCAGCAATGCTCCTGGGCCCCCTCTTCAC
SAP130 3R 961	GCCTACTAAGTGCTGAATCAGTAGCATGCGCCGCTGTCGT
SAP130 4F 981	TGATTCAGCACTTAGTAGGCCAACCTTGTCTATCCAGCAT
SAP130 4R 1261	AGGAATGGGAGGGTACTGTCATGGTAACAATGGTACTTGT
SAP130 5F 1281	GACAGTACCCTCCCATTCCTCCCATGCTACTGCTGTGACC
SAP130 5R1561	GATACTGTCGGATAGTGGACACGGAACTGGTGATTGGGGT
SAP130 6F 1581	GTCCACTATCCGACAGTATCCAGTTTCAGCTCAGGCTCCA
SAP130 6R 1861	TCTTTCCTTCAGGCTGAGGCTGCTGAGTACCCATAGGTGC
SAP130 7F 1881	GCCTCAGCCTGAAGGAAAGACTTCAGCAGTGGTGTTGGCA
SAP130 7R2161	GGGCAGTTGGAGGGACGGCAATGGTAGGCTGATCATTATT
SAP130 8F2181	TGCCGTCCCTCCAACTGCCCAGCAGCCCCCACCGACCATT
SAP130 8R2461	TGTTTGCCAGCAATGCAAGAGATGGAGACACAGTGTTGGT
SAP130 9F2481	TCTTGCATTGCTGGCAAACAACTTGTCCATGCCTACAAGT
SAP130 9R2761	TGTACCTCTGAAAGTGGTGGTAAGCAGCTTTCCAGGGGTT
SAP130 10F 2781	CCACCACTTTCAGAGGTACAGTGACGTCCGGGTCAAAGAG

Restriction Enzymes and vectors

All the restriction enzymes and vectors used in ExSembly reactions were purchased from NEB and Addgene respectively and are listed in Table 3-3.

Table 3-3. List of vectors and restriction enzymes tested for ExSembly.

Commercial vector	Restriction enzyme
pQE-80L	BamHI, SalI, HindIII, KpnI, SmaI, XmaI, SacI, PstI
pPROEX	EcoRI, NotI, SpeI, XbaI, StuI, XhoI, EagI
pBluescript II KS(+)	ApaI, EcoRV
pRSET B	NcoI, NdeI, NheI, EagI, StuI

ExSembly master mix and protocol

The ExSembly master mix was made in the lab using the ExSembly buffer from the 2X ExSembly™ Cloning Master Mix (LifeSCT LLC, Cat# M0005). Phusion polymerase (2 units/reaction) (NEB, Cat# M0530L), T5 exonuclease (30 units/reaction) (NEB Cat# M0663L), and Ampligase DNA ligase (5 units/reaction) (Lucigen, Cat# A3210K) were combined in a 10µl volume and added to a final volume of 10µl of all DNA fragments to be assembled. All DNA fragments were added equimolarly, then concentrated using a SpeedVac to the desired concentration. pQE-80L vector was purified by mini-prep and stored at a concentration of 2µg/ml at 4°C and used in all ExSembly reactions. ExSembly conditions were 37°C for 15mins, 50°C for 45mins. The reactions were then purified using column purification and 2µl of ExSembly reaction was transformed in 20µl of DH10B cells using electroporation at 250µFD, 200 ohms. The cells were then inoculated in 1 ml prewarmed SOC at 37°C for 1 hour with shaking at 250 rpm. The cells were then pelleted by

centrifugation at 7000 g for 30 sec, and 200 µl were plated on each Ampicillin (Amp) (100 µg/ml) plates and Ampicillin + Kanamycin (A+K) (50 µg/ml Amp + 125 µg/ml Kan) plates. Plated bacteria were allowed to grow overnight at 37°C.

Colony counting

Colonies were counted manually by dividing the plate in to four quadrants and counting colonies in one quadrant then multiplying by four to determine an estimated total number of colonies on each plate. An improvised method of replica plating was used, replica streaking, to verify true positive colonies. Colonies from Amp plates were picked at random and streaked on A+K plates in specific grid boxes that were superimposed on the bottom of the plate (H DOLD 1951; Zimmermann 1952; Lindstrom 1977).

Colony PCR

10 colonies per plate were picked randomly and colony PCR was performed to determine the correct order of DNA fragments. Colony PCR conditions were hot start 98°C for 3 mins, denaturation at 98°C for 30 sec, annealing at 60°C for 30 sec, extension at 72°C for 30 sec for 25 cycles, and final extension at 72°C for 2 mins.

Results

ExSembly is compatible with commonly used vectors and enzymes.

An important consideration for wide adoption of any cloning technique is its compatibility with commonly used vectors and enzymes to facilitate integration into existing workflows. To determine if ExSembly is compatible with a set of commonly

used commercially available vectors and enzymes, we tested various combinations of vectors and restriction enzyme sites, present in their respective multiple cloning site, by incorporating eGFP (684 bp) into the pQE-80L vector in a two fragment reaction to determine the efficiency of assembly. The vectors and enzyme combinations tested are listed in Table 3-3. ExSembly had an average positivity rate across all vectors and enzyme combinations of >90% as evidenced by the incidence of colonies that exhibited green fluorescence (Figure 1).

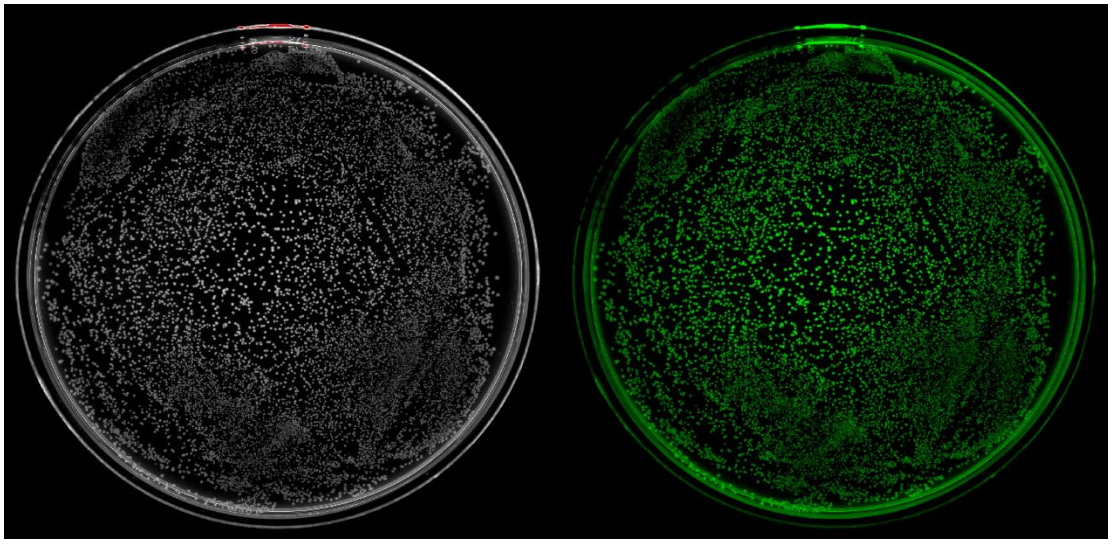

Figure 1. Representative image of pQE-80L ExSembly showing eGFP expressing fluorescent colonies. The fluorescent colonies show the successful integration of eGFP in to the empty vector.

As further confirmation of successful assembly, 10 colonies from each plate were tested by colony PCR for integration of eGFP. To further confirm colony PCR results, restriction digestion and sequencing were performed on DNA prepared from a few representative colonies Together, these data demonstrate that ExSembly is compatible with a wide range of vectors and restriction enzymes that are commonly used by cloning practitioners.

Effect of DNA concentration on cloning efficiency in a four-fragment ExSembly reaction

Having demonstrated that ExSembly functions efficiently in a two-fragment assembly using a variety of commonly used restriction enzymes, we endeavored to determine its efficiency with an increasing number of DNA fragments. Gibson Assembly can be used to assemble >4 fragments, however efficiency falls off dramatically requiring a much higher input of time and resources to achieve success. Standard Gibson Assembly protocol calls for inserts to be present 2-3-fold molar excess when compared to vector when the goal is to assemble two or more inserts into that vector. In practice, this often necessitates achieving a relatively high concentration of insert DNA, for example, when the inserts are of comparable length to the vector (Gibson et al. 2009b).

To determine the optimal insert:vector ratio for a four-fragment ExSembly reaction, pilot experiments were performed using the standard SAP130 insert and pQE80 vector reaction described above, with 3:1, 2:1, and 1:1 ratios. Surprisingly, the 1:1 ratio yielded the highest cloning efficiency (data not shown). Based on this observation, we chose to use the 1:1 insert:vector molar ratio moving forward.

It is well established that the efficiency of Gibson DNA Assembly is DNA concentration dependent. To determine whether the efficiency of ExSembly is also DNA concentration-dependent, and to determine the optimal DNA concentration to achieve the highest efficiency in a four-fragment assembly, the standard SAP130-pQE-80 reaction was performed. In the experimental setup, the insert:vector ratio

was fixed at 1:1, and DNA concentration was varied across a 16-fold range (Table 3-4). Two of the inserts were PCR-amplified fragments of SAP 130 cDNA, one was a PCR-amplified Kanamycin resistance gene, and the vector was pQE-80L. Since pQE-80L carries an ampicillin resistance gene, we used the kanamycin resistance gene as a second selection marker, such that correctly assembly clones would be both Amp and Kan resistant. The experimental set-up for the four fragment is shown in Table 4. Each of the three biological replicates was plated on both Amp alone and Amp plus Kan (A+K) plates, and colony forming units (Cfu's) on each plate were quantified. The mass and number of moles of DNA was added for each precursor fragment in the 4-fragment ExSembly is shown in (Table 3-5).

Table 3-4. ExSembly experimental setup

Sample	**Three biological replicates**
Undiluted DNA	Cfu (Amp) Cfu (A+K)
2-fold dilution	Cfu (Amp) Cfu (A+K)
4-fold dilution	Cfu (Amp) Cfu (A+K)
8-fold dilution	Cfu (Amp) Cfu (A+K)

Table 3-5. Four fragment ExSembly DNA amounts for each insert.

Insert name	SAP left	SAP right	Kan	pQE-80L
Length (bp)	1580	1780	800	4800
Concentration (ng/µl)	184.3	269	158	730
Volume (µl)	20 (3.775 pmol)	15.4 (3.766 pmol)	11.8 (3.771 pmol)	15.3 (3.765 pmol)

Analysis of the resulting data demonstrated that the reaction with 7.5435 pmol total DNA concentration (two-fold dilution from the highest concentration, 15.087 pmol) yielded the most colonies on the A+K plates (Figure 2). The difference between 15.087 pmol and 7.5435 pmol was statistically significant, suggesting that at DNA concentrations above 15.087 pmol, the efficiency of four-fragment ExSembly reactions diminishes (Figure 2). Furthermore, ExSembly reactions with <7.54 pmol DNA yielded significantly fewer colonies (Figure 2). These data demonstrate that 7.54 pmol DNA is optimal for a four-fragment ExSembly reaction.

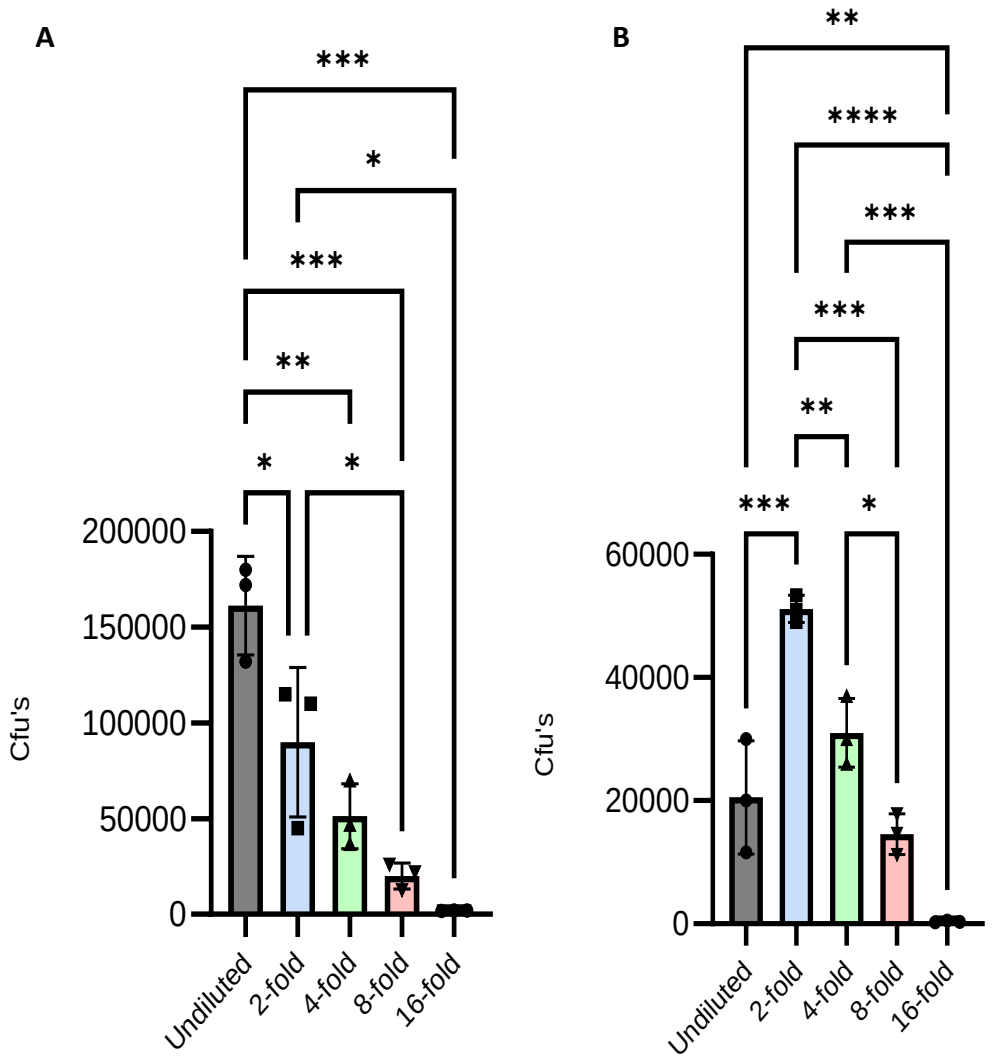

Figure 2. Cloning efficiency of 4-fragment ExSembly: Total number of colony forming unit (Cfu's) on Amp only plate (A) and A+K plate (B) from a four-fragment ExSembly reaction with varying DNA concentrations. Results show that two-fold dilution from the highest concentration yields significantly more positive colonies than other concentrations. Statistical analyses were performed using two-way anova test to perform multiple comparisons on GraphPad Prism. (=p<0.05, **=p<0.01, ***=P<0.001).*

These data above suggest that incorporation of a second selectable marker as an insert in a multi-fragment DNA assembly may be a generalizable strategy to increase the efficiency of the cloning workflow. Typically, both conventional and homology-based cloning workflows incorporate a single selection marker that is an integral component of the cloning vector. Such workflows are subject to the

emergence of false positive colonies that result from self-ligation of the vector. The extent of false positives dictates the overall efficiency of the workflow. For example, a high rate of false positives mandates that a large number of colonies be screened by the practitioner to identify a 'correct' clone. In this regard, it is important to note that ExSembly potentially holds a significant advantage over Gibson Assembly, since the restriction enzyme used to linearize the vector remains in the reaction during DNA assembly. Provided that the restriction enzyme remains active at 50 degrees C, its activity could serve to reduce the number of false positives due to vector self-ligation by 'recutting' those vector molecules as they arise in the reaction. To determine the extent to which false positives arose in the four fragment ExSembly reaction, we also plated the reactants on Amp plates and quantified the resulting colonies (Figure 2A). Analysis of the resulting data revealed that the incidence of false positives was reduced <5-fold by reducing the molarity of the DNA from 15.08 pmol to 7.54 pmol. This observation could be related to the initial incomplete digestion of the vector during the 37 degrees phase of the reaction, or inefficient digestion of self-ligated vector molecules that arise during the 50 degrees phase, or a combination of those events. These data indicate that there is a practical upper limit to the amount of vector in an ExSembly reaction to achieve the highest efficiency.

To quantify the effect of DNA concentration on assembly efficiency, we compared the percent positivity at each concentration using the following formula:

$$\text{Percent positivity rate} = \left(\frac{\text{Total No. of colonies on A+K}}{\text{Total No. of colonies on Amp only}} \right) \text{X } 100$$

The data shown in Figure 3 demonstrate that the four fragment ExSembly reaction is most efficient in a DNA concentration range from 7.54 pmol to 3.77 pmol.

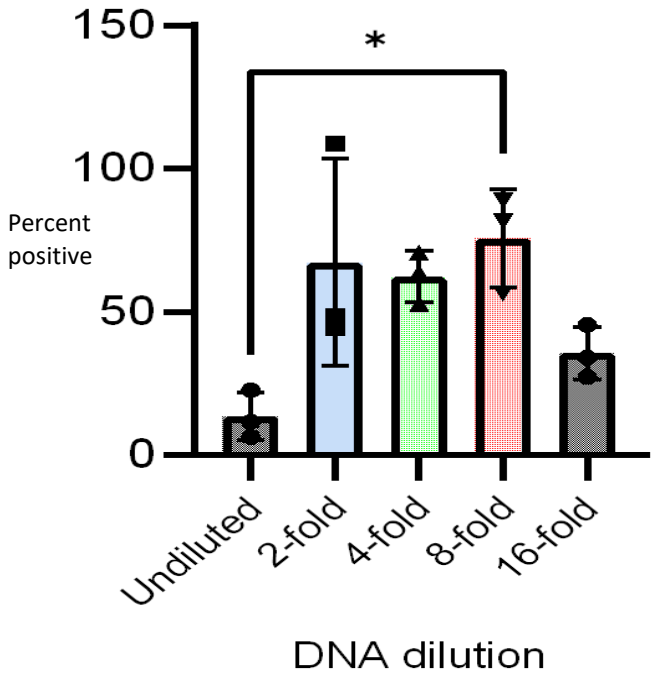

Figure 3. Percent positive rate for the four fragment ExSembly. The highest number of positive clones were observed in the range of 2-fold (7.54pmol) to 8-fold (3.77pmol). Statistical analyses were performed using two-way anova test to perform multiple comparisons on GraphPad Prism. (=p<0.05, **=p<0.01, ***=P<0.001).*

Effect of DNA concentration on cloning efficiency in a twelve-fragment ExSembly reaction

Many synthetic biology applications, including the development of complex constructs to engineer the genome during the creation of animal models, require the assembly of >4-5 DNA 'parts' to generate the desired outcome. One of Gibson Assembly's major limitation has been the low efficiency in synthesis of assembly when >4-5 DNA molecules are to be joined (Roth, Milenkovic, and Scott 2014).

Efficiency is also low when the individual fragment sizes are smaller, as Gibson Assembly was designed to assemble new DNA molecules from precursors that were kilobases in size, but later adapted to accommodate precursors of smaller sizes for general cloning needs of the majority of molecular cloning practitioners (Anderson et al. 2010). Another Gibson Assembly's weakness is its error-prone repair of homologous sequence regions during assembly (Roth, Milenkovic, and Scott 2014). To determine whether ExSembly could be deployed to overcome these limitations, we endeavored to accomplish a 12-fragment assembly using our standard SAP130/Kan/pQE-80 platform.

For the 12 fragment ExSembly, we divided the SAP gene insert into 10 parts, plus kanamycin, and the vector pQE80-L. The shortest part in this experiment was 260 bp and the longest was 4800 bp, thus effectively covering a wide range between the sizes of parts to be assembled in a single reaction. The 10 SAP precursors and kanamycin were PCR amplified and purified as described above. All 12 precursors were combined equimolarly, and the experiment was as shown in Table 4. Table 6 lists the exact amount of DNA that was added for each precursor fragment in the 4-fragment ExSembly.

Table 3-6. Twelve fragment ExSembly DNA amounts for each insert.

Insert name	Length (bp)	Concentration (ng/µl)	Volume (µl)
5'+1R	360	307	15.0 (20.70 pmol)
2F+2R	260	311	10.7 (20.71 pmol)
3F+3R	320	286	14.3 (20.68 pmol)
4F+4R	320	116	35.3 (20.70 pmol)
5F+5R	320	272	15.0 (20.63 pmol)
6F+6R	320	280	14.6 (20.67 pmol)
7F+7R	320	278	14.7 (20.66 pmol)

8F+8R	320	243	16.8 (20.64 pmol)
9F+9R	320	272	15.0 (20.63 pmol)
10F+3'	430	285	19.3 (20.70 pmol)
Kanamycin	800	280	36.5 (20.67 pmol)
pQE-80L	4800	730	84.1 (20.70 pmol)

The 12-fragment assembly was analyzed exactly as described above for the 4-fragment assembly. Although the total number of colonies appearing on the Amp selection plates was similar to that observed in the 4-fragment assembly (Figure 2A & Figure 4A), there was a striking decrease in assembly efficiency as determined by the percentage of positive clones (Figure 2B & Figure 4B; Figure 3 & Figure 5). However, the 12-fragment reaction still yielded a substantial number of correctly assembled clones, as evidenced by the number of colonies appearing on the Amp + Kan plates, and their subsequent confirmation by colony PCR, restriction digestion, and DNA sequencing. For most practitioners, a few, or even a single correct assembly is sufficient to permit advancement to the next phase of experimentation.

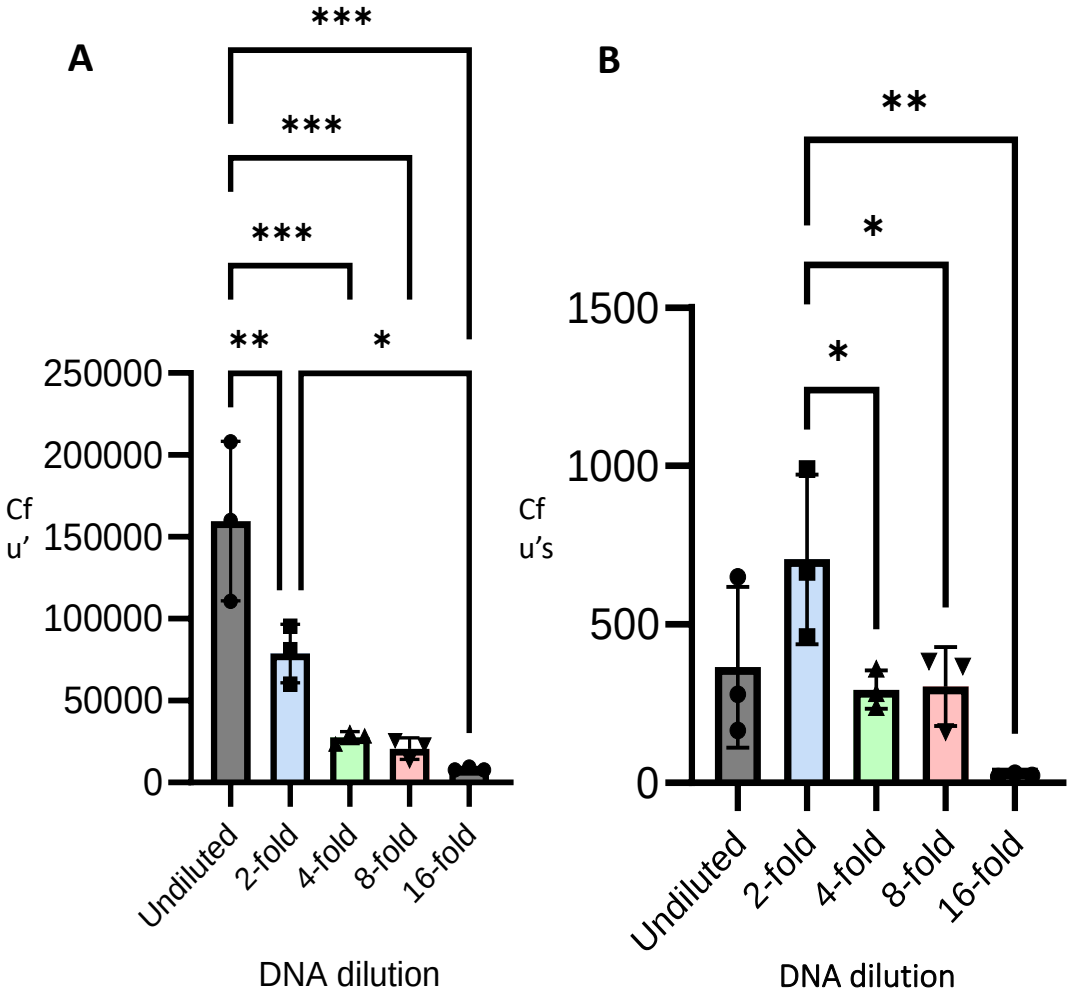

Figure 4. Cloning efficiency of 12-fragment ExSembly: 12-fragment ExSembly follows the same trends as observed in the 4-fragment with regards to DNA concentration. Single selection results in higher colony number and goes on decreasing proportionally with DNA amount (A), double selection shows a bell curve trend with 2-fold have the highest colonies (B). Statistical analyses were performed using two-way anova test to perform multiple comparisons on GraphPad Prism. (=p<0.05, **=p<0.01, ***=P<0.001).*

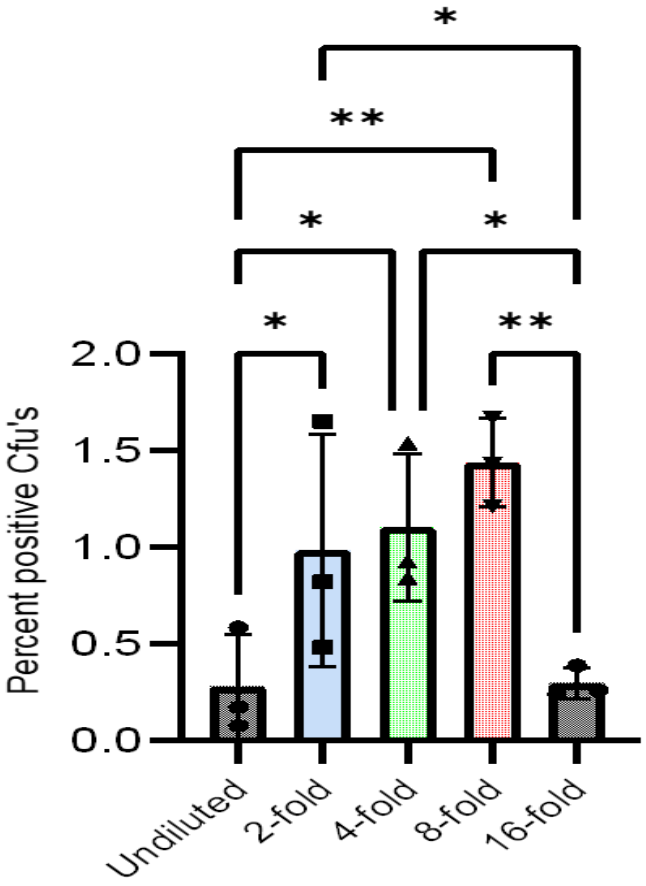

Figure 5. Positivity rate of 12-fragment ExSembly. The highest number of positive clones were observed in the range of 2-fold to 8-fold following the same pattern as 4-fragment ExSembly. Statistical analyses were performed using two-way anova test to perform multiple comparisons on GraphPad Prism. (=p<0.05, **=p<0.01, ***=P<0.001).*

Non-specific "third-party" DNA improves efficiency of DNA assembly by ExSembly

A potential factor that could potentially limit the widespread adoption of ExSembly to achieve complex DNA assemblies is the relatively high concentrations of DNA required to support efficiency. To generate the DNA parts for the four and twelve fragment assemblies described above, relatively large (400 µl) PCR reactions were required. A priori, higher DNA concentrations could support DNA assembly by

increasing intermolecular interactions among the specific DNA parts that are being assembled based on their homologous ends. We hypothesized that DNA concentration *per se* may also be a factor contributing to successful assembly. However, to our knowledge, this possibility has never been explored experimentally for either conventional cloning-based or homology-based DNA assembly. To determine whether the addition of non-specific DNA to ExSembly reactions could improve cloning efficiency, we repeated the four and twelve fragment assemblies described above, adding variable amounts of sheared salmon sperm DNA to the SAP130 + Kan + vector parts. Salmon sperm DNA (SSD), was chosen as it is widely used in molecular biology experiments and upon denaturation has previously been used as a blocking agent in hybridization experiments, and to improve transformation efficiency (J. Lee et al. 2015; Symonds, Walker, and Sin 1994).

We designed an experiment using the lowest molar concentration of DNA that formed colonies in both our 4-fragment and 12-fragment ExSembly experiments and supplemented the reactants with SSD in the range of 0.25 µg to 4 µg in two-fold increments and compared the efficiency of assembly to identical reactions without SSD. We added SSD to the 16-fold diluted 4-fragment ExSembly reaction with 2-fold increments, from 0.25 µg of SSD to a max of 4 µg of SSD, with 16-fold with no SSD acting as a control.

The results of the four-fragment assembly with and without SSD are shown in Figure 6. The addition of 0.25-1.0 µg SSD to the reaction increased the number of colonies arising on Amp + Kan plates, which, as described above, is an accurate indicator of correct assembly. However, statistical significance in total colony yield

was only achieved in the 1.0 µg SSD reaction (Figure 6). Addition of >2.0 µg SSD did not augment the number of colonies, and at 4.0 µg, was significantly inhibitory compared to the 1.0 µg reaction. The effect of SSD on cloning efficiency is shown in Figure 7. SSD supplementation significantly increased efficiency at 0.25, 0.5, and 1.0 µg. These data clearly demonstrate that addition of non-specific DNA to a homology-based DNA assembly reaction can improve the likelihood of successful assembly. To determine if addition of SSD could also improve the outcome of a more complex ExSembly reaction, we supplemented the twelve-fragment reaction described above. The data shown in Figures 8 and 9 demonstrate that the addition of

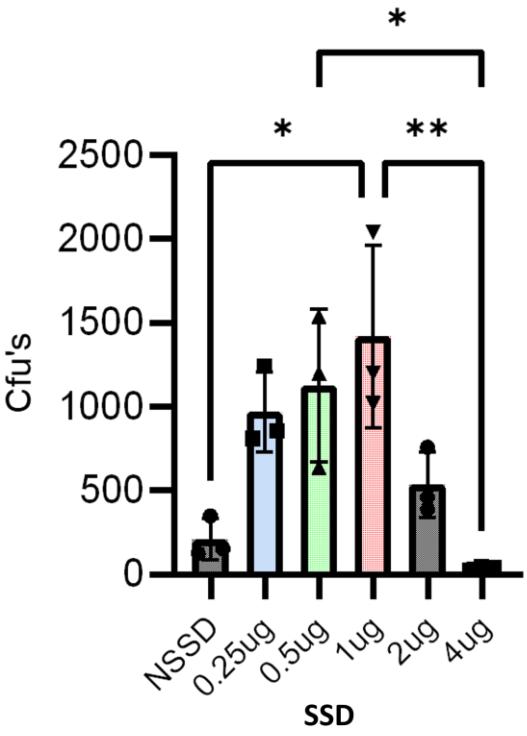

Figure 6. Cloning efficiency 4-fragments ExSembly with SSD: Only 1µg of SSD had any significant impact on colony number yield. Statistical analyses were performed using two-way anova test to perform multiple comparisons on GraphPad Prism. (=p<0.05, **=p<0.01, ***=P<0.001).*

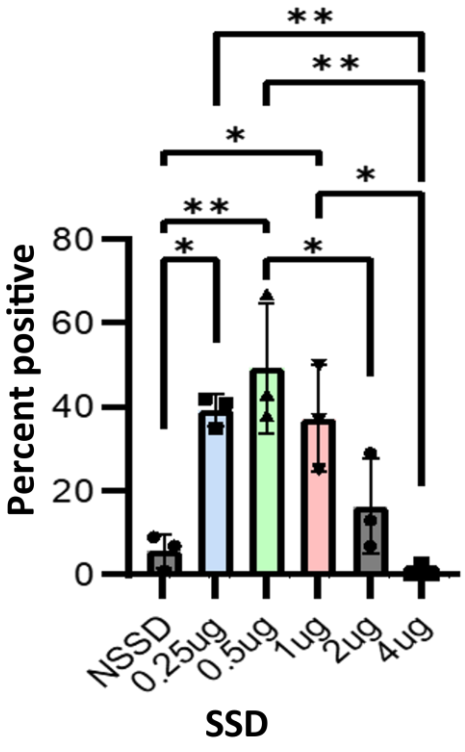

Figure 7. Positivity rate of 4-fragment with SSD: 0.25, 0.5 and 1 µg of SSD had any significant impact on positive clones yield. Statistical analyses were performed using two-way anova test to perform multiple comparisons on GraphPad Prism. (=p<0.05, **=p<0.01, ***=P<0.001).*

SSD led to a higher colony yield and cloning efficiency in a manner similar to that which we observed in the SSD-supplemented four-fragment assembly.

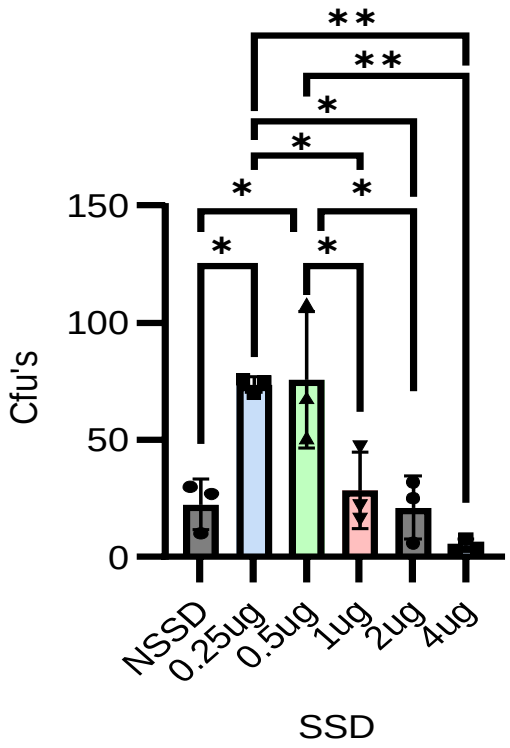

Figure 8. Cloning efficiency of SSD on 12-fragment ExSembly: 0.25 and 0.5 ug of SSD had significant impact on cloning efficiency when compared to other amounts of SSD. Statistical analyses were performed using two-way anova test to perform multiple comparisons on GraphPad Prism. (=p<0.05, **=p<0.01, ***=P<0.001).*

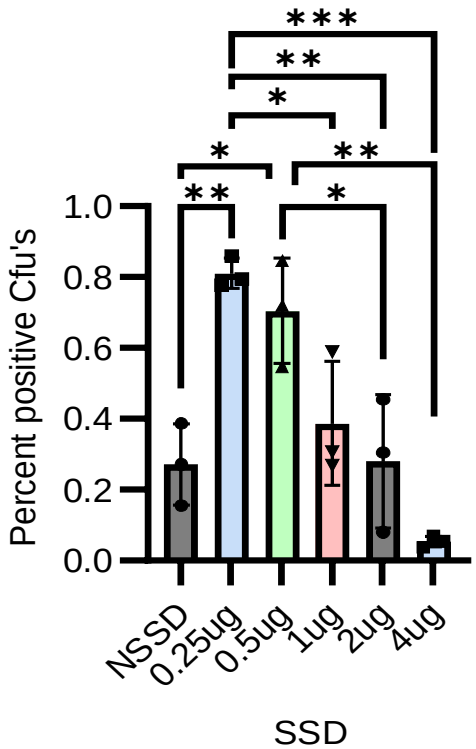

Figure 9. Positivity rate of 12-fragment ExSembly showing highly significant differences between 0.25-0.5ug of SSD with other amounts of SSD. Statistical analyses were performed using two-way anova test to perform multiple comparisons on GraphPad Prism. (=p<0.05, **=p<0.01, ***=P<0.001).*

Discussion

ExSembly is a second-generation homology-based DNA assembly technology for joining DNA parts in an ordered, user defined manner. Like Gibson Assembly, ExSembly also uses DNA polymerase, exonuclease and ligase to assemble DNA precursors into complete DNA constructs *in vitro* (Gibson et al. 2009b). Gibson Assembly requires linearization of vector before being used in the reaction, whereas ExSembly is a one pot method which incorporates vector linearization into the assembly reaction. Here, we demonstrate that ExSembly is compatible with commonly used restriction enzymes and vectors. A limitation of ExSembly is that care must be taken to ensure that the restriction enzyme used for vector linearization does not have a recognition site within the other DNA parts to be assembled. Since the restriction enzyme is present during the reaction vector self-ligation, which contributes to false positives, can be recut during the reaction.

A limitation of first-generation Gibson Assembly is the inefficiency in integrating 5 or more fragments in to a complete construct (Hoose et al. 2023; Roth, Milenkovic, and Scott 2014). The data reported here demonstrate that ExSembly is capable of assembling at least DNA 12 fragments to form an ordered assembly and identify the optimal molar concentration of DNA to achieve high efficiency. This is an important advancement in the field that can potentially enable more practitioners to achieve highly complex DNA assemblies in support of synthetic biology applications.

Our observation that non-specific DNA can improve cloning efficiency provides a facile and practical solution to achieve the high DNA concentration that

supports successful DNA assembly in ExSembly reactions. It obviates the need to produce large quantities of individual DNA parts, in fact, for most assemblies, the amount of DNA produced in a standard PCR reaction would be sufficient with SSD supplementation. Although we have not explored the molecular basis of this phenomenon, it is interesting to speculate on the possibilities. One hypothesis is that DNA of any kind acts a crowding agent by sequestering water molecules, effectively increasing the concentration of the enzymes in the reaction. In addition, it will be interesting the explore the effect of the length of supplemental non-specific DNA fragments, which dictates the molarity of free 5' DNA ends on which the exonuclease is able to act. It is possible that this may affect the average length of 'chew back' that occurs on the specific DNA parts, bringing it into an optimal range. Further experimentation will be required to determine if this is the case.

References

Anderson, J. Christopher, John E. Dueber, Mariana Leguia, Gabriel C. Wu, Adam P. Arkin, and Jay D. Keasling. 2010. "BglBricks: A Flexible Standard for Biological Part Assembly." *Journal of Biological Engineering* 4 (1). https://doi.org/10.1186/1754-1611-4-1.

Avilan, Luisana. 2023. "Assembling Multiple Fragments: The Gibson Assembly." *Methods in Molecular Biology (Clifton, N.J.)* 2633: 45–53. https://doi.org/10.1007/978-1-0716-3004-4_4/COVER.

Casini, Arturo, Marko Storch, Geoffrey S. Baldwin, and Tom Ellis. 2015. "Bricks and Blueprints: Methods and Standards for DNA Assembly." *Nature Reviews*

Molecular Cell Biology 2015 16:9 16 (9): 568–76. https://doi.org/10.1038/nrm4014.

Czar, Michael J., J. Christopher Anderson, Joel S. Bader, and Jean Peccoud. 2009. "Gene Synthesis Demystified." *Trends in Biotechnology* 27 (2): 63–72. https://doi.org/10.1016/J.TIBTECH.2008.10.007.

Ellis, Tom, Tom Adie, and Geoff S. Baldwin. 2011. "DNA Assembly for Synthetic Biology: From Parts to Pathways and Beyond." *Integrative Biology* 3 (2): 109–18. https://doi.org/10.1039/C0IB00070A.

Gibson, Daniel G. 2011. "Enzymatic Assembly of Overlapping DNA Fragments." *Methods in Enzymology* 498: 349–61. https://doi.org/10.1016/B978-0-12-385120-8.00015-2.

Gibson, Daniel G., Lei Young, Ray Yuan Chuang, J. Craig Venter, Clyde A. Hutchison, and Hamilton O. Smith. 2009a. "Enzymatic Assembly of DNA Molecules up to Several Hundred Kilobases." *Nature Methods* 6 (5): 343–45. https://doi.org/10.1038/nmeth.1318.

———. 2009b. "Enzymatic Assembly of DNA Molecules up to Several Hundred Kilobases." *Nature Methods 2009 6:5* 6 (5): 343–45. https://doi.org/10.1038/nmeth.1318.

Greenhalgh, Trisha. 2005. "The Human Genome Project." *Journal of the Royal Society of Medicine* 98 (12): 545–545. https://doi.org/10.1177/014107680509801212.

Guyer, Mark S., and Francis S. Collins. 1993. "The Human Genome Project and the Future of Medicine." *American Journal of Diseases of Children (1960)* 147 (11):

1145–52. https://doi.org/10.1001/ARCHPEDI.1993.02160350019003.

H DOLD. 1951. “A Petri Dish Cover with Incised Grid to Aid in Counting Bacterial Colonies] - PubMed.” https://pubmed.ncbi.nlm.nih.gov/14914098/.

Hoose, Alex, Richard Vellacott, Marko Storch, Paul S. Freemont, and Maxim G. Ryadnov. 2023. “DNA Synthesis Technologies to Close the Gene Writing Gap.” *Nature Reviews. Chemistry* 7 (3): 144. https://doi.org/10.1038/S41570-022-00456-9.

Lanchbury, J. S. 1998. “The Human Genome Project.” *British Journal of Rheumatology* 37 (2): 119–21. https://doi.org/10.1093/RHEUMATOLOGY/37.2.119.

Lee, Jonghwan, Kyung Ju Choi, Youngsok Choi, Bahy A. Ali, Abdulaziz A. Al-Khedhairy, and Soonhag Kim. 2015. “Sperm DNA-Mediated Reduction of Nonspecific Fluorescence during Cellular Imaging with Quantum Dots.” *Chemical Communications (Cambridge, England)* 51 (58): 11584–86. https://doi.org/10.1039/C5CC04503G.

Lindstrom, E. B. 1977. “Alternative Replica Plating Technique.” *Applied and Environmental Microbiology* 34 (2): 225–27. https://doi.org/10.1128/AEM.34.2.225-227.1977.

Loenen, Wil A.M., David T.F. Dryden, Elisabeth A. Raleigh, and Geoffrey G. Wilson. 2014. “Type I Restriction Enzymes and Their Relatives.” *Nucleic Acids Research* 42 (1): 20–44. https://doi.org/10.1093/NAR/GKT847.

Moraes, Fernanda, and Andréa Góes. 2016. “A Decade of Human Genome Project Conclusion: Scientific Diffusion about Our Genome Knowledge.” *Biochemistry*

and Molecular Biology Education : A Bimonthly Publication of the International Union of Biochemistry and Molecular Biology 44 (3): 215–23. https://doi.org/10.1002/BMB.20952.

Olson, M. V. 1993. "The Human Genome Project." *Proceedings of the National Academy of Sciences of the United States of America* 90 (10): 4338–44. https://doi.org/10.1073/PNAS.90.10.4338.

Ostrov, Nili, Jacob Beal, Tom Ellis, D. Benjamin Gordon, Bogumil J. Karas, Henry H. Lee, Scott C. Lenaghan, et al. 2019. "Technological Challenges and Milestones for Writing Genomes." *Science* 366 (6463): 310–12. https://doi.org/10.1126/SCIENCE.AAY0339.

Pingoud, Alfred, Geoffrey G. Wilson, and Wolfgang Wende. 2014. "Type II Restriction Endonucleases--a Historical Perspective and More." *Nucleic Acids Research* 42 (12): 7489–7527. https://doi.org/10.1093/NAR/GKU447.

Rose, Elise A. 1991. "Applications of the Polymerase Chain Reaction to Genome Analysis." *The FASEB Journal* 5 (1): 46–54. https://doi.org/10.1096/fasebj.5.1.1991584.

Roth, Theodore L., Ljiljana Milenkovic, and Matthew P. Scott. 2014. "A Rapid and Simple Method for DNA Engineering Using Cycled Ligation Assembly." *PLoS ONE* 9 (9): 107329. https://doi.org/10.1371/JOURNAL.PONE.0107329.

Sayers, J. R., and F. Eckstein. 1990. "Properties of Overexpressed Phage T5 D15 Exonuclease. Similarities with Escherichia Coli DNA Polymerase I 5'-3' Exonuclease." *Journal of Biological Chemistry* 265 (30): 18311–17. https://doi.org/10.1016/S0021-9258(17)44753-3.

Symonds, J. E., S. P. Walker, and F. Y.T. Sin. 1994. “Electroporation of Salmon Sperm with Plasmid DNA: Evidence of Enhanced Sperm/DNA Association.” *Aquaculture* 119 (4): 313–27. https://doi.org/10.1016/0044-8486(94)90297-6.

Xia, Yongzhen, Kai Li, Jingjing Li, Tianqi Wang, Lichuan Gu, and Luying Xun. 2019. “T5 Exonuclease-Dependent Assembly Offers a Low-Cost Method for Efficient Cloning and Site-Directed Mutagenesis.” *Nucleic Acids Research* 47 (3): e15–e15. https://doi.org/10.1093/NAR/GKY1169.

Zhu, Hanliang, Haoqing Zhang, Ying Xu, Soňa Laššáková, Marie Korabečná, and Pavel Neužil. 2020. “PCR Past, Present and Future.” *BioTechniques* 69 (4): 317–25. https://doi.org/10.2144/BTN-2020-0057.

Zimmermann, Wilhelm. 1952. “Zählschalen Für Keimzahlbestimmungen.” *Zeitschrift Für Hygiene Und Infektionskrankheiten* 135 (5): 421–22. https://doi.org/10.1007/BF02149885.

Chapter 4: Future Directions

Introduction

Prostate cancer is the second most prevalent malignancy in men globally (Siegel Mph et al. 2023; Bray et al. 2018). Prostate cancer patients can present with a localized or advanced disease. Currently there are no effective treatments available to treat an advanced form of the disease termed castration resistant prostate cancer. Identifying novel biomarkers to aid in the development of diagnostics and therapeutics is critical to identify indolent versus aggressive disease. Prostate specific membrane antigen (PSMA) is a protein that is expressed on the prostate cancer cell surface may be able to address these needs (Israeli et al. 1993). To develop novel diagnostic and treatment options, another urgent need is to develop animal models that would recapitulate human disease and can be used to elucidate the molecular mechanisms involved in disease progression. Currently there are no rodent models of prostate cancer expressing PSMA in the prostate gland. Earlier our lab has developed a mouse model of prostate cancer, termed BMPC, that is currently state of the art genetically engineered mouse model for prostate cancer (Gretchen K Hubbard et al. 2016). We now have successfully developed a rat model that overexpresses PSMA in the prostate gland combined with the overexpression of oncogene *MYC*. However, our attempts to make a model with loss of tumor suppressor have yet to be successful. In our attempts to generate these transgenic models we realized there is a need for DNA assembly methods that can generate complex multi-functional DNA constructs to manipulate gene expression *in vivo*. To address this, we have developed a next generation DNA assembly method termed ExSembly that can form circular constructs from 12 precursor fragments.

Characterizing the PSMA rats in context of cancer

In the second chapter we demonstrated a rat model that conditionally overexpresses human PSMA on the surface of epithelial cells in the prostate gland. The PSMA expression initially was heterogenous and focal, but with increasing age it became almost homogenous throughout the ventral prostate of the rat. Despite overexpressing PSMA we did not observe any pathological signs of cancer in the tissue. To develop a rat model of prostate cancer our lab has been attempting to recreate the BMPC mouse model in rats. BMPC mice have a complicated mating scheme and are labor intensive to generate, to simplify and increase the utility of our rat model we have cloned a polycistronic construct termed MCR that is currently being used to drive PSMA expression (M. Rubenstein 2022). We are now in the process of generating a Pten flox rat line that would then be mated to the existing MCR/PSMA rat line to have rat model of prostate cancer expressing PSMA.

Our efforts to generate a mouse line conditionally expressing PSMA in the prostate gland has not yet been successful. As mentioned in the second chapter we have used multiple different approaches to try and over express PSMA conditionally in the mouse prostate gland, all the pups that were born genomically positive for PSMA were stillbirths. During our attempts to generate this mouse line we discovered that overexpressing PSMA under the Hoxb13 promoter could be developmentally lethal. Our hypothesis is that overexpression of PSMA under the Hoxb13 promoter leads to excitotoxicity in the developing embryo due to hydrolysis of glutamate from *N*-Acetyl-aspartyl-glutamate (NAAG). NAAG is an abundant neuropeptide found in vertebrates that is essential for normal functioning by regulating excessive glutamate signaling (Lodder-Gadaczek et al. 2011a; Morland and Nordengen 2022). Levels of NAAG are maintained under tight regulation in the nervous system as altered levels

have been implicated in several neuropsychiatric disorders (Coyle 1997). Since the Hoxb13 promoter begins expressing the transgenes under its control early in development we believe that is what leads to glutamate excitotoxicity in our models (McMullin, Mutton, and Bieberich 2009a). We have observed in our PSMA rat line that when homozygous PSMA pups are born they lose the ability to move their hind legs and usually die by 5 weeks of age (data not shown). In order to overcome this obstacle we propose to make a multi-gene construct that would only express PSMA in an inducible fashion using the Tet-On system that we have used successfully previously in lab (T. Das, Tenenbaum, and Berkhout 2016; Rao et al. 2012; Ashok et al. 2019).

The physiological roles of PSMA in human prostate cancer are not known, and our PSMA rat model provides us an opportunity to gain a better understanding of its functions. There is evidence in literature from in vitro studies that PSMA interacts with multiple other proteins and is involved in the signaling pathways commonly involved in prostate cancer progression (Evans et al. 2016b). Androgen receptor (AR) is a transcription factor that is the most amplified gene involved in prostate cancer initiation (Taylor et al. 2010). Prostate cancer in human patients is initially androgen dependent and becomes independent after androgen ablation therapy. Studies have shown that AR is involved in negative regulation of PSMA expression (Ghosh and Heston 2004; Sommer et al. 2022). MYC is the second most amplified in prostate cancer (Taylor et al. 2010). In future experiments, it will be interesting to analyze the effect on MYC expression in context of PSMA overexpression in our MCR/PSMA rat model. Preliminary, non-quantitative observations suggests that PSMA may be

allowing MYC to accumulate to a higher level in prostate epithelial cells that have both MYC and PSMA when compared to cells expressing MYC alone. In our PSMA rat model we intend to perform immunohistochemical analysis to further explore this possibility, and to perform deeper analyses to understand the mechanism involved in these interactions. In other future experiments, we will seek to determine if overexpression of PSMA is capable of driving proliferation, since in humans, PSMA expression is correlated with increase stage of the disease (Kasperzyk et al. 2013). Glutamates hydrolyzed by PSMA have been shown to activate the PI3k/Akt/mTOR signaling pathway via the glutamatergic signaling (Kaittanis et al. 2018a; Caromile and Shapiro 2017). PTEN, a tumor suppressor gene, is also implicated in the progression of human prostate cancer, and PSMA's secondary interaction with one of its subunits is shown to activate the PI3K/Akt/mTOR signaling pathway (Mateo et al. 2020; Kaittanis et al. 2018a). Our current MCR/PSMA rat model does not have PTEN loss, so it would be interesting to see the effect of PSMA overexpression on PI3K signaling. Investigating these mechanisms would help us gain a better understanding of the complexities involved and develop therapeutic options.

The prostate gland has the highest concentration of Zinc (Zn) amongst all soft tissue and secretes high amounts of Zn in the prostatic fluid (MAWSON and FISCHER 1952; Ho and Song 2009). However, Zn concentrations in the prostate cancer tissue are drastically reduced (Costello and Franklin 2006). Research to elucidate role of Zn in prostate cancer pathophysiology has been contradictory (Ho and Song 2009). PSMA also has a Zn binding moiety site in its catalytic site and

requires Zn for enzymatic activity (Davis et al. 2005). In future experiments we could use the PSMA rat to explore these intriguing hypotheses.

Analyzing effect of PSMA overexpression in rat prostate by RNA-Seq

Prostate cancer has been shown to be dependent on glutamine metabolism for growth and survival, as glutamine is an nonessential amino acid, that is essential for many metabolic processes involved in growth and proliferation especially in cancer cells (Bhowmick et al. 2023). In future experiments, it would be interesting to investigate glutamine metabolism and the related glutamatergic pathways and their relation to PSMA in prostate cancer. Given the robust PSMA expression in our rat model it provides an opportunity to determine if PSMA expression is sufficient to alter gene expression patterns. We have recently submitted tissue samples from six-month-old rats that are expressing PSMA and MCR, MCR only, and Sprague Dawley for bulk RNA sequencing (RNA-Seq) study. In future experiments, we will mine those data to shed light on the potential molecular mechanisms whereby PSMA may support the growth of prostate cancer cells.

Although we did not have access to RNA-Seq data from PSMA-expressing rat prostates at the time of this writing, we had previously performed RNA-Seq in BMPC mouse prostate cancer cases. In pilot experiments, we mined these data to determine if PSMA associated candidate genes are differentially expressed during disease progression in the BMPC model. We looked at the glutamate metabolism related genes in our BMPC mice RNA-Seq data set and found that glutaminase (GLS 1/2), glutamate metabotropic receptor 5 GRM5), Aspartate aminotransferase (GOT1) and Ribosomal modification protein rimK like family member A (RIMKLA) were

consistently overexpressed in tumor and metastases samples but not in samples in which PIN was the only pathology present (Figure 1).

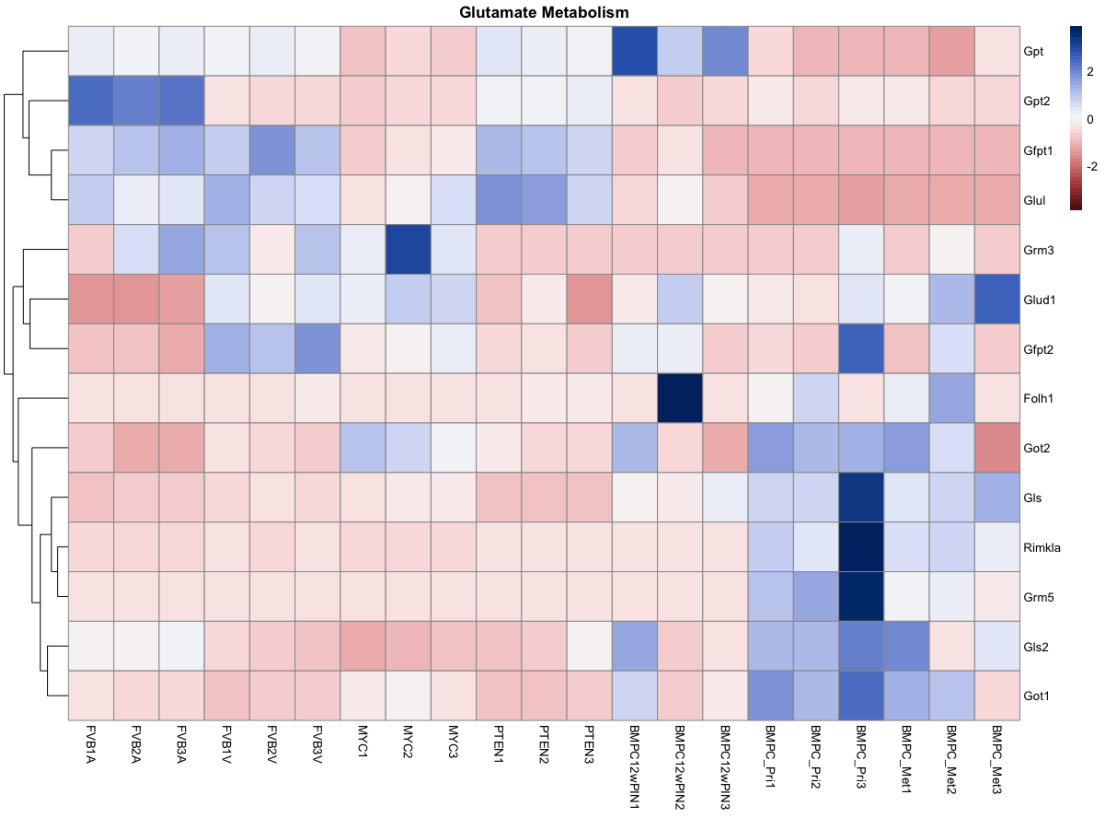

Figure 1. Glutamate metabolism gene expression across all BMPC samples. GLS, Rimkla, GRM5, GLS2 and GOT1 are shown to be overexpressed only in BMPC primary tumor and metastases samples.

For example, we observed that GLS1 and GLS2 to have increased expression in BMPC primary tumors, and metastases but not incases with MYC driven PIN (Figure 2A). GLS is a phosphate-activated amidohydrolase that catalyzes the hydrolysis of glutamine to glutamate and ammonia, the rate limiting step in glutamine pathway, and is also involved in DNA/RNA and amino acid synthesis and contributes to tricarboxylic acid cycle (Myint et al. 2021; Xu et al. 2022). Recently it was shown that GLS, like PSMA, acts as a provider of glutamate by hydrolyzing it of NAAG in

prostate cancer, and blocking both GLS and NAAG simultaneously has therapeutic effect (T. Nguyen et al. 2019). We suspect that in our BMPC model GLS is playing the same role that PSMA does in human prostate cancer, as they both provide free glutamates from their precursors that act via the glutamatergic pathway, and mice do not express PSMA in their prostate gland (Figure 2B). We would like to explore our theory in the PSMA rat model.

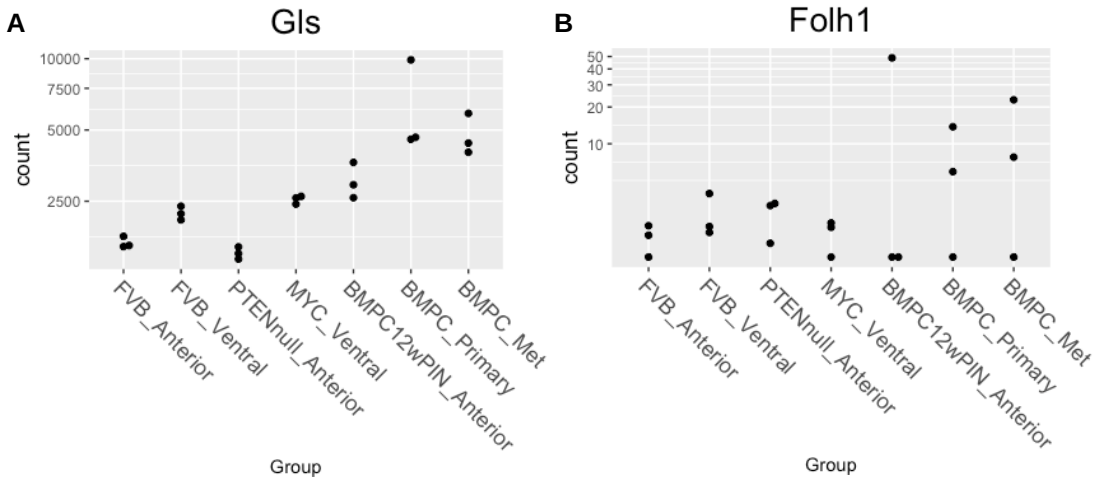

Figure 2. Expression of GLS and PSMA (Folh1) in BMPC mice. GLS expression goes on increasing in MYC expressing mice and BMPC PIN when compared to PTEN null or normal FVB mice, GLS expression goes higher in BMPC primary tumor and metastases sample when compared to BMPC PIN (A). PSMA expression stays unaltered in across all samples as mice do not express PSMA in prostate gland (B).

Another gene family involved in the glutamatergic pathway is the G-protein coupled receptor, glutamate metabotropic receptor (GRM). GRM's have been shown to activate the PI3K signaling pathway via the $p110_{\beta}$ subunit of PTEN (Kaittanis et al. 2018b). Various GRM's, for example, GRM1-8, have been implicated in prostate cancer progression and are shown to have altered expression levels in prostate cancer cell lines (Stepulak et al. 2009; Pissimissis et al. 2009; Yu et al. 2017). From our analysis of BMPC mice RNA-Seq data we have identified GRM5 to be overexpressed

only in the BMPC primary and metastases samples while remaining unchanged in others (Figure 3). Overexpression of GRM5 only in primary tumor and metastases could indicate that its overexpression is correlated with increased cancer cell metabolism in those tissues. In future work, we will use our PSMA model to further investigate the role GRM's might be playing in prostate cancer and identify potential candidate targets as markers or advanced disease and as therapeutic targets.

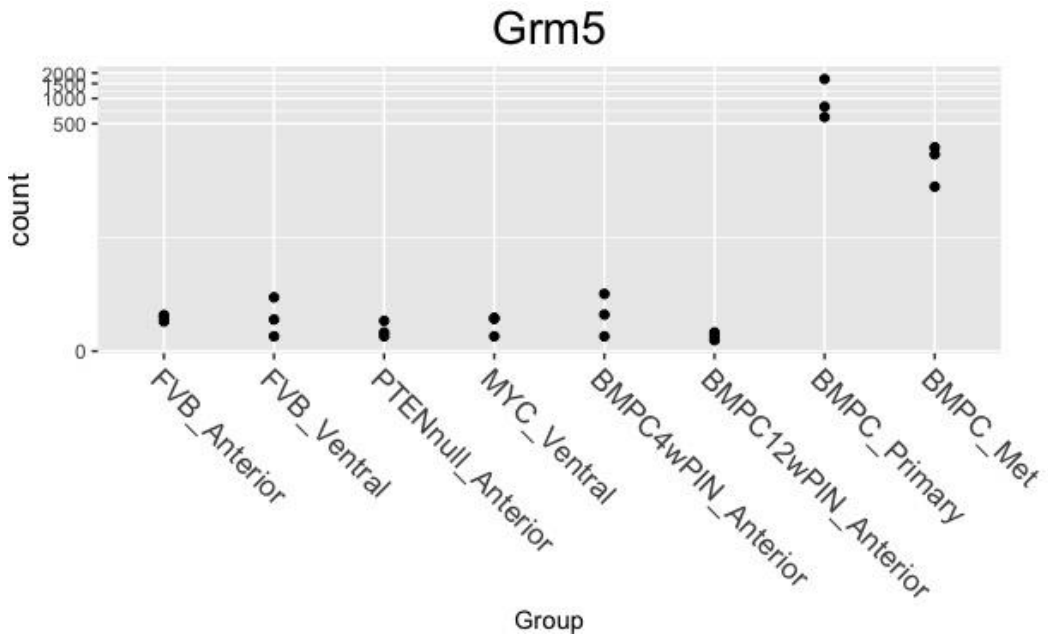

Figure 3. Expression profile of GRM5 in BMPC mice. GRM5 is significantly overexpressed in tumor and metastases tissue in the BMPC mice and not in normal, MYC overexpression or PIN tissue samples.

RIMKLA is also known as NAAG synthase, as it catalyzes the synthesis of NAAG from *N*-Acetyl-aspartyl (NAA) and glutamate, NAAG is the substrate PSMA hydrolyzes glutamate from (Lodder-Gadaczek et al. 2011b). Currently there is no know role for RIMKLA in prostate cancer. However, Grasso et. al. have characterized the transcriptome of lethal castration-resistant prostate cancer to

identify candidate genes, we found RIMKLA to also be overexpressed in lethal cases of human prostate cancer (Figure 4) (Grasso et al. 2012). We have also identified other candidate genes from this dataset that we can examine further for there potential role in prostate cancer.

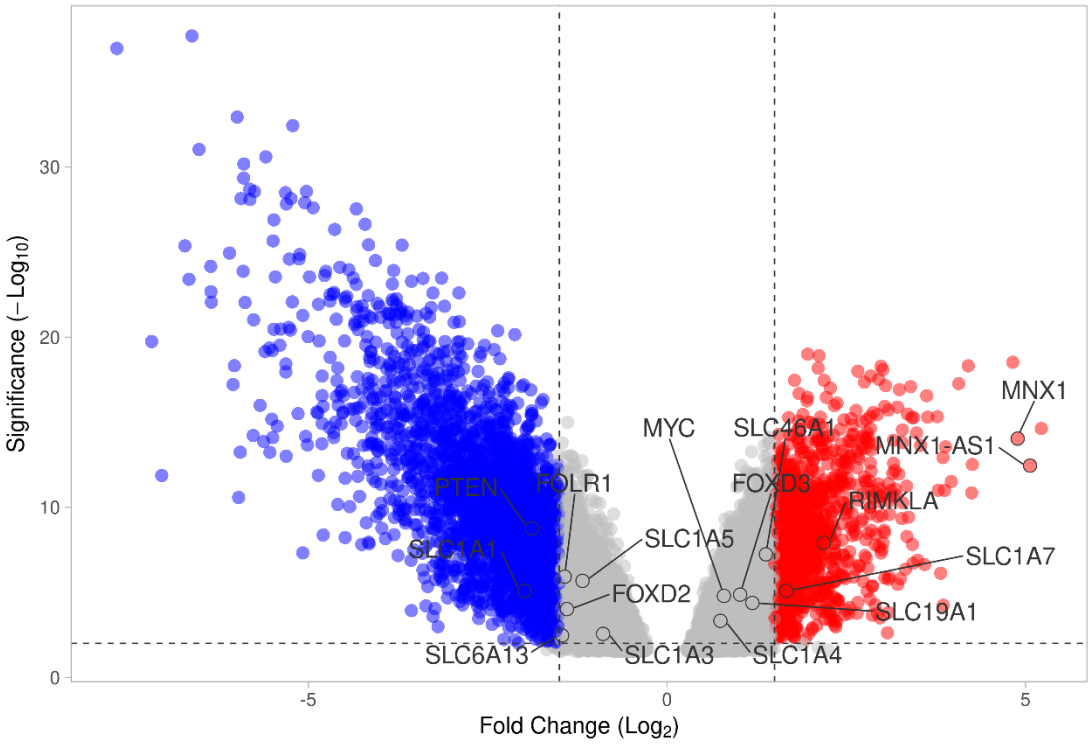

Figure 4. A volcano plot showing differential gene expression in lethal prostate cancer samples when compared to normal prostate tissue. Some of the genes such as SLC's MNX1 have been thought to play a role in prostate cancer, whereas RIMKLA, MNK1 AS1 are newly identified genes observed to be overexpressed.

Generating complex multi-functional DNA construct using ExSembly

In chapter three we demonstrated successful assembly of twelve precursor DNA fragments into a functional DNA construct. We are attempting to make a multifunctional DNA construct in PSMA expression would be inducible by the rtTA-Tet on system driving Cre recombinase. We also would like to use the knowledge we

have gained from optimizing or current protocol to develop a method in which ExSembly can assemble twenty fragment or more as well as fragments < 200 base pairs into a functional construct.

Overall, the work presented here achieves substantial advancements in two distinct areas: discerning the role of PSMA in prostate cancer biology, and in the construction of complex DNA assemblies. However, the latter is highly likely to contribute to the former in future work, given that we anticipate the need to develop ever more complex animal models to study PSMA function, which will require increasingly complex genome engineering using multifunctional transgene constructs.

References

Ashok, Arya, Rebecca Keener, Michael Rubenstein, Stephanie Stookey, Sagar Bajpai, Jessica Hicks, Angela K. Alme, et al. 2019. "Consequences of Interleukin 1β-triggered Chronic Inflammation in the Mouse Prostate Gland: Altered Architecture Associated with Prolonged CD4 $^{+}$ Infiltration Mimics Human Proliferative Inflammatory Atrophy." *The Prostate*, March, pros.23784. https://doi.org/10.1002/pros.23784.

Bhowmick, Neil, Edwin Posadas, Leigh Ellis, Stephen J Freedland, Dolores Di Vizio, Michael R Freeman, Dan Theodorescu, Robert Figlin, and Jun Gong. 2023. "Targeting Glutamine Metabolism in Prostate Cancer." *Frontiers in Bioscience-Elite* 15 (1): 2. https://doi.org/10.31083/J.FBE1501002/PDF.

Bray, Freddie, Jacques Ferlay, Isabelle Soerjomataram, Rebecca L. Siegel, Lindsey A. Torre, and Ahmedin Jemal. 2018. "Global Cancer Statistics 2018:

GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries." *CA: A Cancer Journal for Clinicians* 68 (6): 394–424. https://doi.org/10.3322/caac.21492.

Caromile, Leslie Ann, and Linda H. Shapiro. 2017. "PSMA Redirects MAPK to PI3K-AKT Signaling to Promote Prostate Cancer Progression." *Molecular and Cellular Oncology* 4 (4). https://doi.org/10.1080/23723556.2017.1321168.

Costello, Leslie C., and Renty B. Franklin. 2006. "The Clinical Relevance of the Metabolism of Prostate Cancer; Zinc and Tumor Suppression: Connecting the Dots." *Molecular Cancer* 5. https://doi.org/10.1186/1476-4598-5-17.

Coyle, Joseph T. 1997. "The Nagging Question of the Function of N-Acetylaspartylglutamate." *Neurobiology of Disease* 4 (3–4): 231–38. https://doi.org/10.1006/NBDI.1997.0153.

Davis, Mindy I, Melanie J Bennett, Leonard M Thomas, and Pamela J Bjorkman. 2005. "Crystal Structure of Prostate-Specific Membrane Antigen, a Tumor Marker and Peptidase." *British Journal of Pharmacology* 102 (17): 5981–86. https://doi.org/doi: 10.1073/pnas.0502101102.

Evans, James C, Meenakshi Malhotra, John F Cryan, and Caitriona M O'Driscoll. 2016. "The Therapeutic and Diagnostic Potential of the Prostate Specific Membrane Antigen/Glutamate Carboxypeptidase II (PSMA/GCPII) in Cancer and Neurological Disease." *British Journal of Pharmacology* 173 (21): 3041–79. https://doi.org/10.1111/bph.13576.

Ghosh, Arundhati, and Warren D.W. Heston. 2004. "Tumor Target Prostate Specific Membrane Antigen (PSMA) and Its Regulation in Prostate Cancer." *Journal of*

Cellular Biochemistry 91 (3): 528–39. https://doi.org/10.1002/jcb.10661.

Grasso, Catherine S., Yi Mi Wu, Dan R. Robinson, Xuhong Cao, Saravana M. Dhanasekaran, Amjad P. Khan, Michael J. Quist, et al. 2012. "The Mutational Landscape of Lethal Castration-Resistant Prostate Cancer." *Nature 2012 487:7406* 487 (7406): 239–43. https://doi.org/10.1038/nature11125.

Ho, Emily, and Yang Song. 2009. "Zinc and Prostatic Cancer." *Current Opinion in Clinical Nutrition and Metabolic Care* 12 (6): 640. https://doi.org/10.1097/MCO.0B013E32833106EE.

Hubbard, Gretchen K, Laura N Mutton, May Khalili, Ryan P McMullin, Jessica L Hicks, Daniella Bianchi-Frias, Lucas A Horn, et al. 2016. "Combined MYC Activation and Pten Loss Are Sufficient to Create Genomic Instability and Lethal Metastatic Prostate Cancer." *Cancer Research* 76 (2): 283–92. https://doi.org/10.1158/0008-5472.CAN-14-3280.

Israeli, R S, C T Powell, W R Fair, and W D Heston. 1993. "Molecular Cloning of a Complementary DNA Encoding a Prostate-Specific Membrane Antigen." *Cancer Research* 53 (2): 227–30. http://www.ncbi.nlm.nih.gov/pubmed/8417812.

Kaittanis, Charalambos, Chrysafis Andreou, Haley Hieronymus, Ninghui Mao, Catherine A. Foss, Matthias Eiber, Gregor Weirich, et al. 2018a. "Prostate-Specific Membrane Antigen Cleavage of Vitamin B9 Stimulates Oncogenic Signaling through Metabotropic Glutamate Receptors." *Journal of Experimental Medicine* 215 (1): 159–75. https://doi.org/10.1084/jem.20171052.

Kaittanis, Charalambos, Chrysafis Andreou, Haley Hieronymus, Ninghui Mao,

Catherine A Foss, Matthias Eiber, Gregor Weirich, et al. 2018b. “Prostate-Specific Membrane Antigen Cleavage of Vitamin B9 Stimulates Oncogenic Signaling through Metabotropic Glutamate Receptors.” *The Journal of Experimental Medicine* 215 (1): 159–75. https://doi.org/10.1084/jem.20171052.

Kasperzyk, Julie L, Stephen P Finn, Richard Flavin, Michelangelo Fiorentino, Rosina Lis, Whitney K Hendrickson, Steven K Clinton, et al. 2013. “Prostate-Specific Membrane Antigen Protein Expression in Tumor Tissue and Risk of Lethal Prostate Cancer.” *Cancer Epidemiology, Biomarkers & Prevention : A Publication of the American Association for Cancer Research, Cosponsored by the American Society of Preventive Oncology* 22 (12): 2354–63. https://doi.org/10.1158/1055-9965.EPI-13-0668.

Lodder-Gadaczek, Julia, Ivonne Becker, Volkmar Gieselmann, Lihua Wang-Eckhardt, and Matthias Eckhardt. 2011a. “N-Acetylaspartylglutamate Synthetase II Synthesizes N-Acetylaspartylglutamylglutamate.” *The Journal of Biological Chemistry* 286 (19): 16693. https://doi.org/10.1074/JBC.M111.230136.

———. 2011b. “N-Acetylaspartylglutamate Synthetase II Synthesizes N-Acetylaspartylglutamylglutamate.” *The Journal of Biological Chemistry* 286 (19): 16693–706. https://doi.org/10.1074/JBC.M111.230136.

Mateo, Joaquin, George Seed, Claudia Bertan, Pasquale Rescigno, David Dolling, Ines Figueiredo, Susana Miranda, et al. 2020. “Genomics of Lethal Prostate Cancer at Diagnosis and Castration Resistance.” *Journal of Clinical Investigation* 130 (4): 1743–51. https://doi.org/10.1172/JCI132031.

MAWSON, C. A., and M. I. FISCHER. 1952. “The Occurrence of Zinc in the Human

Prostate Gland." *Canadian Journal of Medical Sciences* 30 (4): 336–39. https://doi.org/10.1139/CJMS52-043.

McMullin, Ryan P., Laura N. Mutton, and Charles J. Bieberich. 2009. "Hoxb13 Regulatory Elements Mediate Transgene Expression during Prostate Organogenesis and Carcinogenesis." *Developmental Dynamics* 238 (3): 664–72. https://doi.org/10.1002/dvdy.21870.

Morland, Cecilie, and Kaja Nordengen. 2022. "N-Acetyl-Aspartyl-Glutamate in Brain Health and Disease." *International Journal of Molecular Sciences 2022, Vol. 23, Page 1268* 23 (3): 1268. https://doi.org/10.3390/IJMS23031268.

Myint, Zin W., Ramon C. Sun, Patrick J. Hensley, Andrew C. James, Peng Wang, Stephen E. Strup, Robert J. McDonald, Donglin Yan, William H. St. Clair, and Derek B. Allison. 2021. "Evaluation of Glutaminase Expression in Prostate Adenocarcinoma and Correlation with Clinicopathologic Parameters." *Cancers* 13 (9). https://doi.org/10.3390/CANCERS13092157.

Nguyen, Tu, Brian James Kirsch, Ryoichi Asaka, Karim Nabi, Addison Quinones, Jessica Tan, Marjorie Justine Antonio, et al. 2019. "Uncovering the Role of N-Acetyl-Aspartyl-Glutamate as a Glutamate Reservoir in Cancer." *Cell Reports* 27 (2): 491-501.e6. https://doi.org/10.1016/j.celrep.2019.03.036.

Pissimissis, Nikolaos, Efstathia Papageorgiou, Peter Lembessis, Athanasios Armakolas, and Michael Koutsilieris. 2009. "The Glutamatergic System Expression in Human PC-3 and LNCaP Prostate Cancer Cells." *Anticancer Research* 29 (1): 371–77. http://www.ncbi.nlm.nih.gov/pubmed/19331175.

Rao, Varsha, Jamie C. Heard, Helya Ghaffari, Aminah Wali, Laura N. Mutton, and

Charles J. Bieberich. 2012. “A Hoxb13-Driven Reverse Tetracycline Transactivator System for Conditional Gene Expression in the Prostate.” *Prostate* 72 (10): 1045–51. https://doi.org/10.1002/pros.22490.

Rubenstein, M. 2022. “Molecular Basis of Carcinogenesis in Rodent Models of Prostate Cancer - ProQuest Dissertations & Theses Global - ProQuest.” *ProQuest Dissertations & Theses Global*. https://www.proquest.com/pqdtglobal/docview/2728493074/42958B868B304A6FPQ/1?accountid=14577.

Siegel Mph, Rebecca L, Kimberly D Miller, Nikita Sandeep, Wagle Mbbs, | Ahmedin, Jemal Dvm, and Rebecca L Siegel. 2023. “Cancer Statistics, 2023.” *CA: A Cancer Journal for Clinicians* 73 (1): 17–48. https://doi.org/10.3322/CAAC.21763.

Sommer, Ulrich, Tiziana Siciliano, Celina Ebersbach, Alicia Marie K. Beier, Matthias B. Stope, Korinna Jöhrens, Gustavo B. Baretton, Angelika Borkowetz, Christian Thomas, and Holger H.H. Erb. 2022. “Impact of Androgen Receptor Activity on Prostate-Specific Membrane Antigen Expression in Prostate Cancer Cells.” *International Journal of Molecular Sciences* 23 (3). https://doi.org/10.3390/IJMS23031046/S1.

Stepulak, Andrzej, Hella Luksch, Christine Gebhardt, Ortrud Uckermann, Jenny Marzahn, Marco Sifringer, Wojciech Rzeski, et al. 2009. “Expression of Glutamate Receptor Subunits in Human Cancers.” *Histochemistry and Cell Biology* 132 (4): 435–45. https://doi.org/10.1007/S00418-009-0613-1.

T. Das, Atze, Liliane Tenenbaum, and Ben Berkhout. 2016. “Tet-On Systems For

Doxycycline-Inducible Gene Expression." *Current Gene Therapy* 16 (3): 156–67. https://doi.org/10.2174/1566523216666160524144041.

Taylor, Barry S., Nikolaus Schultz, Haley Hieronymus, Anuradha Gopalan, Yonghong Xiao, Brett S. Carver, Vivek K. Arora, et al. 2010. "Integrative Genomic Profiling of Human Prostate Cancer." *Cancer Cell* 18 (1): 11–22. https://doi.org/10.1016/J.CCR.2010.05.026.

Xu, Lingfan, Bing Zhao, William Butler, Huan Xu, Nan Song, Xufeng Chen, J. Spencer Hauck, et al. 2022. "Targeting Glutamine Metabolism Network for the Treatment of Therapy-Resistant Prostate Cancer." *Oncogene 2021 41:8* 41 (8): 1140–54. https://doi.org/10.1038/s41388-021-02155-z.

Yu, Lumeng J., Brian A. Wall, Janet Wangari-Talbot, and Suzie Chen. 2017. "Metabotropic Glutamate Receptors in Cancer." *Neuropharmacology* 115 (March): 193. https://doi.org/10.1016/J.NEUROPHARM.2016.02.011.