CANCER DRUG RESISTANCE MECHANISMS

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by Nadia Patterson

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The undersigned, appointed by the dean of the Graduate School,

have examined the dissertation entitled

CANCER DRUG RESISTANCE MECHANISMS

Presented by NADIA R. PATTERSON

A candidate for the degree of

Doctor of Philosophy

And hereby certify that, in their opinion, it is worthy of acceptance.

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To my father Raphael Anthony Stapleton

02/03/1960 - 09/23/2019

Despite your physical absence, your presence is evident throughout every page.

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ABSTRACT

Significant advances have been made toward developing potent agents that selectively target and kill cancer cells. However, achieving durable clinical benefits remains a significant challenge in the management of cancer patients. Some patients either fail to respond or quickly relapse due to intrinsic or acquired drug resistance, respectively. Elucidation of the molecular mechanisms of intrinsic and acquired cancer drug resistance will not only improve our understanding of cancer biology, but it will also lead to the development for therapeutic strategies for achieving sensitization of patients to existing therapy, resulting in broader and more durable clinical benefits for patients.

I will discuss my work related to drug resistance in prostate, lung, and pancreatic cancers.

In prostate cancer (PC), hyperactivation of androgen signaling gives rise to adenocarcinoma, which develop into a treatment-resistant and highly lethal PC subtype, neuroendocrine prostate cancer (NEPC). Chemotherapy and androgen deprivation therapy (ADT) are the cornerstones of PC treatments. However, nearly all NEPC patients eventually relapse and succumb to the disease months after treatment initiation. The underlying mechanism is not fully understood.

Understanding and targeting molecular mechanisms that promote NEPC fate transformation and/or NEPC resistance to ADT, will potentially re-sensitize PC to existing therapies.

We found that the proteosome protein PSMA2 plays a dual role in PC resistance to ADT: on the one hand, treatment induced PSMA2 sensitizes PC cells to residual androgen, thereby limiting the efficacy of hormone therapy. We propose that ectopic PSMA2 achieves this effect by sequestering HSP90 away from the androgen receptor (AR), thereby enabling rapid AR nuclear translocation and activity. Consistent with this model, ADT-induced PSMA2 antagonizes AR-HSP90 protein complex formation and stimulate the expression of AR target genes.

On the other hand, PSMA2 pushes prostate adenocarcinoma into NEPC fate transformation trajectory. Pharmacological inhibition of PSMA2 sensitizes these untreatable PC cells to ADT. In support of our findings PSMA2 blockade dramatically prolongs animal survival in a mouse model of NEPC.

Treatment resistance is not unique to prostate cancer or hormone-targeted therapy; it is a ubiquitous challenge in oncology, as evidenced by the transient response of lung cancer patients to targeted therapy. Below I discuss our work on Non-small cell lung cancer (NSCLC) in the context of resistance to epidermal growth factor receptor (EGFR)-directed therapy. NSCLC accounts for a significant proportion of lung cancer cases, and EGFR-directed therapy has emerged as a targeted treatment option for patients with specific genetic mutations. However, resistance to this therapy often develops, ultimately limiting its long-term effectiveness and highlighting the need for novel strategies to overcome or

circumvent resistance mechanisms. The widespread nature of resistance across various cancer types underscores the urgency of identifying innovative approaches to improve patient outcomes and ensure the durability of therapeutic responses.

The etiology of resistance to molecularly targeted therapy in NSCLC frequently involves genetic mutations that trigger alternative signaling pathways. NSCLC patients harboring sensitizing mutations in the epidermal growth factor receptor EGFR (T790M, L578R) are treated with Osimertinib, a potent tyrosine kinase inhibitor (TKI). However, nearly all patients develop TKI resistance. The underlying mechanisms are not fully understood. We found that plasma extracellular vesicles (EV) and circulating microRNAs fundamentally modulate cancer cell response to Osimertinib. Circulating Hsa-miR-22-3p and EV Hsa-miR-184 and Let-7b-5p are deregulated in NSCLC patients. These miRNAs functionally converge on the WNT/ β-catenin and mTOR/AKT signaling axes, known cancer therapy resistance signals. Targeting Hsa-miR-22-3p and Hsa-miR-184 desensitized EGFR-mutated (T790M, L578R) NSCLC cells to Osimertinib.

In addition to inevitable acquired drug resistance, targeted agents benefit only limited subsets of patients because of their allele-dependent mechanisms of action. There is a need for a shift in cancer targeting paradigm to achieve broad and durable benefits for patients. Below I discuss our efforts toward the development of a bacteria-based biologic to target cancers broadly and durably. I focus on Pancreatic cancer (PanC). Oncogenic KRAS mutations are the primary drivers in 98% of pancreatic cancers. KRAS-driven cancers are highly

desmoplastic and immuno suppressive. Desmoplastic pancreatic tumors impede drug delivery, leading to inadequate intratumoral concentrations of the targeted agents and immune exclusion, rendering these tumors resistant to immunotherapy.

KRAS-targeted approaches are being actively pursued to overcome the physical and immune barriers of pancreatic cancers. Oncogenic mutations in the KRAS gene occur at specific codons (12, 13, and 61). Existing KRAS directed agents (Sotorasib, Adagrasib) selectively target the KRAS(V12C), which account only for 1-2% of pancreatic cancers. Further, patients eligible for these agents develop robust resistance, limiting the long-term benefits for this treatment modality. Furthermore, efforts to combine KRAS(V12C) directed agents with checkpoint immunotherapy reveal significant toxicity.

We show that a genetically engineered strain of Salmonella typhimurium (CRC2631) safely penetrates PanC tissues, stimulates effector T cells, and correspondingly reduces tumor burden in mouse models of KRAS PanC.

Our work highlights a potential to achieve re-sensitization of cancer patients to existing therapies, including immunotherapy.

CHAPTER1- INTRODUCTION

1.1 Prostate Cancer

1.1a Prostate Cancer Incidence & Etiology

Prostate cancer (PC) is the second largest cause of cancer-related fatalities in men. In 2022 the Surveillance, Epidemiology, and End Results (SEER) Program anticipated a total of 268,490 newly diagnosed cases of PC in the United States, with an associated mortality rate of around 34,500 (Siegel et al., 2022). The etiology of PC is not fully understood . However, factors such as age, genetics, race or ethnicity, and lifestyle factors, play a role in PC development and patient outcome. More than 20% of men aged 50–59 and 33% of men aged 70–79 had PC in autopsy investigations of individuals who died of other causes (US Preventive Services Task Force et al., 2018). Patients with a family history of PC have a higher risk of developing PC. PC driver mutations include Retinoblastoma gene (Rb), P53, Breast Cancer 1/2 (BRCA1/2) gene, Homeobox B13 (HOXB13), and DNA mismatch repair genes (Chung et al., 2020; Ewing et al., 2012; Rawla, 2019). PC cancer incidence and mortality rates in the US are on the decline, some populations continue to be at a higher risk of contracting or passing away from the disease. Notably, African American men (AA) had incidence rates that were higher than those of White men, with 183.4 new cases being discovered for every 100,000 men. Among all US population groups, AA men continue to have the highest PC mortality rate, with a death rate that is approximately twice that of White men. (Giaquinto et al., 2022; Panigrahi et al., 2019; Siegel et al., 2022; Vidal et al., 2017

1.1b Hormones and Prostate Cancer

Steroid signaling is a crucial determinant in the initiation and progression of PC. Steroid hormones, including androgens, estrogens, progestins, and glucocorticoids, modulate cell proliferation, survival, and differentiation. Dysregulation of these steroid signaling pathways within PC cells can facilitate tumorigenesis and engender resistance to therapeutic interventions (Ganguly et al., 2021; Heinlein & Chang, 2004). Below I discuss the role of the sex homones estrogen and androgen in PC.

Estrogen in Prostate Cancer Progression and Development

PC growth and progression are significantly influenced by estrogen signaling. Both healthy and malignant prostate tissues express estrogen and its receptors, estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) (Di Zazzo et al., 2018; V. S. Wu et al., 2015). Estrogen receptors (ERs) are nuclear transcription factors that mediate the biological effects of estrogen in target tissues. Both ERα and ERβ have been shown to be expressed in PC, with unique and sometimes antagonistic effects on tumor development and therapeutic response (Dey et al., 2014; Leung et al., 2006). ERα activity has been associated with has been linked to PC growth and invasion, whereas ERβ has been linked to tumor suppression (Hartman et al., 2009; Leav et al., 2001). One of the primary targets of estrogen signaling in PC is the androgen receptor (AR), which is essential for the development and survival of PC. It has been shown that estrogen increases AR expression and stimulates its activity through crosstalk with many signaling pathways (Jefferi et al., 2023; Mak et al., 2010; Zellweger et al., 2013).

Androgen in Prostate Cancer Progression and Development

Androgens stimulate normal prostate cell development through the androgen receptor (AR). However, cancer cells become reliant on androgen signaling for survival, and the AR pathway becomes constitutively active, resulting in uncontrolled cancer cell growth and proliferation. In PC, the AR is overexpressed and activated, promoting the growth and proliferation of cancer cells. AR and estrogen receptor (ER) are Type I nuclear hormone transcription factors. Inactive AR is linked to heat shock proteins (HSPs) in the cytoplasm. HSPs inhibit misfolding and preserve 3D protein structure under cellular stress. Like other family members, AR is activated by androgen binding to its ligand binding domain (LBD). AR homodimerizes and translocate into the nucleus, binding to androgen response elements (AREs) to activate and transcribe downstream genes. Androgen binding to AR activates it, promoting the transcription of genes involved in the cell cycle and metabolic pathways. Furthermore, AR activation may block apoptosis while promoting cell motility and invasion (Michmerhuizen et al., 2020).

Although not as extensively studied as androgen and estrogen signaling, progesterone signaling is also involved in PC. The expression of progesterone receptor (PR) is associated with advanced disease and unfavorable prognosis in subset of PCs. Furthermore, the signaling of PR has the potential to impact the expression of multiple growth factors, including vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF), which play in a role in tumor growth and angiogenesis(Y. Yu et al., 2013). Direct PR binding to the VEGF

promoter region has been demonstrated to upregulate VEGF expression in PC cells, increasing angiogenesis and tumor growth (Tong, 2022). Progesterone signaling interact with androgen signaling in the context of PC. It has been proposed that Progesterone may stimulate the expression of androgen receptor (AR) target genes, or it may regulate the activity of AR coregulators, thereby modulating AR-dependent cellular processes (Latil et al., 2001). As we explore the role of androgens in the progression and development of prostate cancer, it becomes increasingly clear that hormone therapy plays a crucial part in the management and treatment of this disease.

1.1c Hormone Therapy in Prostate Cancer

Androgen Deprivation therapy (ADT) is the mainstay treatment for advanced PC. It functions by decreasing the level of androgens in the body, which limits the development and spread of cancer cells. ADT can be achieved by surgical castration, where the testicles are removed, and medical castration, where drugs are used to decrease the level of androgens in the body. A surgical castration reduces testosterone levels rapidly and permanently.

The most used drugs for medical castration are luteinizing hormone-releasing hormone (LHRH) agonists and antagonists.(Huggins & Hodges, 1972) LHRH agonists, such as leuprolide and goserelin, can suppress the production of luteinizing hormone (LH) in the pituitary gland. This results in a reduction of testosterone production in the testes. Luteinizing hormone-releasing hormone (LHRH) antagonists, such as degarelix, act by directly blocking the release of LH, resulting in a swift reduction in testosterone levels without an initial surge in

testosterone(Klotz et al., 2008). Anti-androgens, such as enzalutamide and abiraterone, are another form of ADT that prevents androgens' effects on cancer cells. These second-line hormones therapeutic can overcome some mechanisms of resistance to first-line therapy. The primary means of action of enzalutamide is suppressing the AR signaling pathway. Enzalutamide binds to AR's ligand-binding domain (LBD) and inhibits its activation and nuclear translocation, reducing AR-mediated transcription and the signaling cascades that contribute to cell proliferation and survival. . Enzalutamide blocks AR signaling at three critical points: its inhibit AR's ability to bind to androgen, to translocate to the nucleus, and to bind to transcription coactivator, thereby shutting down AR transcriptional program and resulting in tumor regression (Crawford et al., 2018; Scher et al., 2012; Tran et al., 2009). LHRH agonists or antagonists are frequently combined with enzalutamide to achieve maximum AR signaling blockade. LHRH agonists and antagonists operate differently, their result is the same: both decrease the levels of testosterone in the bloodstream. This reduction of circulating testosterone is essential in curtailing androgen receptor (AR) signaling within prostate cells(Crawford et al., 2018). The therapeutic effects of hormone therapy are transcient, with most patients ultimately encountering a resurgence of the disease.

1.1d Prostate Cancer Resistance to hormone therapy

Although there have been notable improvements in the identification and management of PC, the emergence of resistance to treatment continues to pose a significant obstacle. The mutational landscape and histological transformation of

PC promote intrinsic and adaptive drug resistance mechanisms. ADT aims to disable the androgen signaling pathway. Nevertheless, a significant proportion of patients ultimately progress to an advanced stage of castration-resistant prostate cancer (CRPC) and neuroendocrine cancer (NEPC) (Beltran et al., 2011a; Mu et al., 2017a; Taplin & Balk, 2004a). CRPC-NEPC are characterized by the development of resistance to hormone therapy. CRPC is defined as PC that continues to progress despite castrate levels of testosterone (<50 ng/dL) achieved through ADT(Cornford et al., 2017). In CRPC, the reactivation of androgen receptor signaling is a major contributor to drug resistance, leading to resistance to androgen deprivation therapy and AR-targeted therapies (Abida et al., 2019; Yamada & Beltran, 2021).NEPC is an infrequent yet aggressive form of prostate cancer that is identified by the existence of neuroendocrine markers and a lack of androgen receptor expression. This type of cancer may emerge de novo or develop due to treatment-induced trans-differentiation of adenocarcinoma cells (H. T. Wang et al., 2014a; Yamada & Beltran, 2021). Due to its independence from androgen receptor signaling, NEPC is highly resistant to conventional prostate cancer treatments such as ADT (Aggarwal et al., 2014). CRPC and NEPC are aggressive forms of prostate cancer that exhibit drug resistance through distinct mechanism. Understanding the molecular mechanisms that trigger therapy resistance in PC is essential to devise innovative therapeutic approaches that can surmount these challenges and enhance patient outcomes.

Known mechanisms of resistance include AR amplification, constitutively active AR variants, activation of compensatory growth signals, stimulation of

androgen production in surrounding tissues. This phenomenon entails the amplification of the AR gene, leading to a heightened production of the AR protein. remarkably low androgen levels resulting from hormone therapy, the amplified androgen receptors (ARs) can bind to remaining androgens and initiate downstream signaling pathways, which ultimately stimulate cell survival and proliferation. The amplification of AR is linked to the emergence of CRPC and the insusceptibility to antiandrogen therapies (Visakorpi et al., 1995)

Mutations in the AR gene have been identified as another significant contributor to the development of drug resistance in PC. Mutations can arise in the ligand-binding domain (LBD) or other areas of the AR protein, leading to modifications in ligand-binding specificity, increased ligand-independent activation, or the receptor's constitutive activation(Scher & Sawyers, 2005). Some mutations in the AR gene can result in the receptor displaying sensitivity to non-canonical ligands, including adrenal androgens, glucocorticoids, and antiandrogens. This phenomenon may lead to a paradoxical activation of AR signaling and consequent facilitation of tumor proliferation, despite the treatment of hormone therapy (Joseph et al., 2013; Taplin et al., 1995) .Overexpression of androgen receptor (AR) variants lacking the ligand-binding domain leads to constitutively active AR signaling and resistance to ADT.(Arora et al., 2013; Watson et al., 2015) . Activation of alternative signaling pathways, such as the PI3K/Akt/mTOR pathway, which can bypass AR signaling and promote PC cell survival and growth under low androgen conditions.(H.-K. Lin et al., 2003) . Loss of tumor suppressors, such as Phosphatase and tensin homolog (PTEN) or Rb which can enhance AR signaling

and contribute to ADT resistance PC cancer cells may still synthesize androgens locally, adding to resistance. PC cells may potentially gain the capacity to create androgens via several different mechanisms, including the overexpression of enzymes involved in androgen production (Attard & Antonarakis, 2016; Labbé & Brown, 2018).

PC and stromal cells can compensate for the limited supply of androgen following ADT by producing and releasing androgens in the PC microenvironment. This response maintains residual level of androgens. The production of androgens by tumor cells can originate from different precursors, including adrenal androgens, cholesterol, or other steroid hormones (K.-H. Chang et al., 2013; de Bono et al., 2011). Moreover, various enzymes that participate in the biosynthesis of androgens, such as CYP17A1, AKR1C3, and HSD3B1, are frequently overexpressed in CRPC, thereby promoting the transformation of precursor compounds into functional androgens(Locke et al., 2008; Stanbrough et al., 2006).

The involvement of additional steroid hormone receptors, name ER, PR, and glucocorticoid receptor (GR), has been observed in the development of resistance to hormone therapy. The activation of ERα has been found to have the potential to increase the survival of PC cells. As a result, targeting ERα has been proposed as a possible therapeutic strategy (Tilley et al., 1996). The interaction between PR signaling and AR signaling has the potential to modulate the activity of AR target genes or coregulators (Coutinho et al., 2016). Furthermore, the progression of prostate cancer and resistance to AR-targeted therapies have been linked to glucocorticoid signaling, which is facilitated by the GR. The activation of

GR can facilitate the ligand-dependent transcriptional regulation of genes that play a role in cellular survival, inflammation, and metabolism. When antiandrogens like enzalutamide are present, such as in the case of PC, it has been demonstrated that GR activation can circumvent AR inhibition and increase tumor cell survival (Arora et al., 2013) . PC patients' tumor microenvironment (TME) can also be altered by glucocorticoids, which block angiogenesis, and reduce the synthesis of pro-inflammatory cytokines and chemokines(Ronchetti et al., 2015).

The role of the TME is critical in the emergence of therapy resistance in PC. TME comprises various elements, including Cancer-Associated fibroblasts, tumor-associated macrophages, and myeloid-derived suppressor cells. These components can secrete growth factors, cytokines, and chemokines that facilitate cancer cell survival, proliferation, and resistance to therapy, including immunotherapy. In addition to cytokines, PC avoid immune surveillance through the upregulation of immune checkpoint molecules, including programmed death-ligand 1 (PD-L1). Immune checkpoints are regulatory pathways that play a vital role in sustaining self-tolerance and preventing autoimmune reactions. They modulate the activation and inhibition of T cells and other immune cells (Postow et al., 2015; P. Sharma & Allison, 2015). In some instances, cancer cells can exploit these checkpoints to avoid detection and elimination by the immune system, enabling the tumor to develop and spread. Immune checkpoint inhibitor therapy, also known as checkpoint blockade therapy, is an immunotherapy strategy that targets immune checkpoints with the aim of reinstating the immune system's ability to recognize and eliminate cancer cells. Monoclonal antibodies are utilized to block

checkpoint proteins such as PD-1, PD-L1, and CTLA-4. The obstruction of these checkpoint proteins leads to an increase in the immune system's potential to attack and eradicate cancer cells (L. Chen & Han, 2015; Pardoll, 2012; Postow et al., 2015; P. Sharma & Allison, 2015). Prostate cancer has traditionally been considered immunologically "cold," with limited infiltration of immune cells and low response rates to immunotherapies (Stultz & Fong, 2021). The limited effectiveness of checkpoint inhibitors in treating prostate cancer can be partly attributed to the epithelial-mesenchymal transition (EMT) process, which plays a role in the complex immunosuppressive nature of the tumor microenvironment (TME) (L. Chen et al., 2014; Kudo-Saito et al., 2009; Noman et al., 2017).

In the epithelial-to-mesenchymal transition (EMT), epithelial cells lose their ability to adhere to other cells and instead take on a more migratory and invasive character. Prostate cancer is one of the tumors for which this pathway has been implicated in therapeutic resistance. EMT has the potential to impede the effectiveness of hormone therapies through two mechanisms: the downregulation of androgen receptor (AR) expression and the activation of alternative signaling pathways that facilitate cell survival and proliferation (Sun et al., 2012; Zhu & Kyprianou, 2010). The downregulation of AR expression or activation of alternative signaling pathways, such as the Wnt/ β-catenin and TGF-β pathways, have been demonstrated to contribute to therapy resistance through EMT (Sun et al., 2012; Zhu & Kyprianou, 2010). Cancer cells can also undergo EMT and acquire stem cell-like characteristics, rendering them resistant to therapy (Mani et al., 2008).

Despite advances in early detection and treatment, drug resistance remains a significant challenge in managing this disease. Understanding the molecular mechanisms driving therapy resistance in PC is critical for developing new therapeutic options to overcome these barriers and enhance patient outcomes.

Resistance to hormone deprivation therapy in PC is a common phenomenon that leads to treatment failure and disease progression. However, it is important to note that drug resistance is not limited to hormone therapy alone, and cancers can also develop resistance to non-hormone-based signal interruption. This is exemplified in lung cancer, where resistance to chemotherapy and targeted therapy can arise via complex genetic and non-genetic mechanisms.

1.2 Lung Cancer

1.2a Lung Cancer Incidence & Etiology

Lung cancer remains the main cause of cancer-related fatalities worldwide (Bray et al., 2018a). 85% of cases are associated with smoking cigarettes (Thun et al., 2008). Exposure to secondhand smoke, also known as passive smoking, is a noteworthy risk factor for lung cancer. Non-smokers who live with a smoker are estimated to have a 20-30% increased risk (Öberg et al., 2011; Thun et al., 2008). In addition to tobacco use, various environmental factors play a role in the development of lung cancer. For example, radon is a naturally occurring radioactive gas that is considered as the second leading cause of lung cancer. It is accountable for almost 10% of cases (Darby et al., 2005)). Additional occupational and environmental exposures, including asbestos, silica, and air pollution, have been linked to an elevated likelihood of developing lung cancer

(Alberg et al., 2013). Genetic factors also influence lung cancer susceptibility and various genes and genetic variants have been identified as risk factors. Lung cancer risk has been linked to polymorphisms in genes that are involved in DNA repair, carcinogen metabolism, and cell cycle control (Hung et al., 2008).Studies on familial aggregation also indicate a hereditary factor in the risk of lung cancer, particularly in individuals who have never smoked (Coté et al., 2012). Histological classification of lung cancer comprises two primary subtypes: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). Non-small cell lung cancer (NSCLC) comprises nearly 85% of all lung cancer cases and encompasses adenocarcinoma, squamous cell carcinoma, and large cell carcinoma (Molina et al., 2008a; Travis et al., 2015a). Small cell lung cancer (SCLC), which is a more aggressive variant of the disease, constitutes the remaining 15% of instances. The treatment approach for lung cancer depends on the stage of the disease, the patient's overall health, and the histological subtype.

1.2b Lung Cancer Targeted Therapy and Resistance

The most common treatment method for early-stage NSCLC (stages I and II) is typically surgery, provided that the tumor is confined to a specific area and has not metastasized to other organs. The surgical interventions comprise lobectomy, pneumonectomy, and segmentectomy. These procedures involve the removal of a lung lobe, the entire lung, and a segment of a lung, respectively. The selection of the procedure is contingent upon the dimensions and position of the tumor(Goldstraw et al., 2016). Include clinical benefits of this approach.

The identification of driver oncogenes in NSCLC has resulted in the innovation of targeted therapies, specifically tyrosine kinase inhibitors (TKIs), which have transformed the management of NSCLC. TKIs target mutant EGFR by binding to the ATP-binding pocket located in the kinase domain of the receptor. This binding impedes the receptor's autophosphorylation, which is essential for its activation, thus blocking the downstream signaling pathways. The specificity of TKIs for the mutant EGFR is due to structural disparities in the ATP-binding pocket between the wild-type and mutant forms of the receptor. These differences enable TKIs to preferentially bind and inhibit the mutant receptor without significantly affecting the wild-type EGFR (Mok et al., 2009a; Pao & Chmielecki, 2010; Rosell et al., 2012).The Epidermal Growth Factor Receptor (EGFR) is classified as a transmembrane receptor tyrosine kinase that is a component of the ErbB receptor family. This family of receptors comprises ErbB2 (HER2), ErbB3 (HER3), and ErbB4 (HER4). The Epidermal Growth Factor Receptor (EGFR) is a crucial factor in the regulation of various cellular processes, including but not limited to proliferation, differentiation, and survival. Upon binding to its ligands, such as epidermal growth factor (EGF) or transforming growth factor-alpha (TGF-α), EGFR undergoes dimerization and autophosphorylation, resulting in the activation of downstream signaling pathways, including the phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt), rat sarcoma viral oncogene homolog (Ras)/rapidly accelerated fibrosarcoma (Raf)/mitogen-activated protein kinase (MAPK), and Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathways.

EGFR mutations have been observed in around 10-35% of NSCLC cases, mainly in adenocarcinomas. The most common EGFR mutations are exon 19 deletions and exon 21 L858R point mutations, which together account for 85-90% of all EGFR mutations in NSCLC (Mok et al., 2009b). The clinical benefit of first-generation EGFR TKIs, including gefitinib and erlotinib, has been demonstrated in patients with EGFR-mutant NSCLC (Maemondo et al., 2010; Mok et al., 2009b). However, the acquired resistance to these agents is a common phenomenon, which is due to the emergence of secondary EGFR mutation (T790M). The T790M mutation occurs as a result of the substitution of methionine for threonine at position 790 in the EGFR kinase domain. This mutation has been found to significantly decrease the efficacy of first- and second-generation TKIs through two main mechanisms. Firstly, the T790M mutation enhances the affinity of the ATP-binding pocket for ATP, which leads to a decrease in the potency of the TKIs. Secondly, the T790M mutation alters the conformation of the EGFR kinase domain, making it more challenging for TKIs to bind to the ATP-binding pocket. These two mechanisms work together to ultimately reduce the inhibitory effect of TKIs on EGFR, leading to treatment failure and disease progression (Oxnard et al., 2011). The improved efficacy of second-generation EGFR TKIs, such as afatinib and dacomitinib, has been observed in comparison to first-generation TKIs. However, resistance still emerges despite this advancement (Y.-L. Wu et al., 2017; Yang et al., 2017). The third-generation EGFR TKI Osimertinib selectively target the T790M mutation, leading to improved overall survival for these patients (Mok et al., 2017; Soria et al., 2018).

Although EGFR mutations are widely recognized as a factor in the emergence of TKI resistance in NSCLC , it is important to note that ALK mutations can also play a significant role in TKI resistance. The activation of the ALK signaling pathway in NSCLC can lead to tumor growth and the development of resistance to targeted therapies such as TKIs.

Anaplastic Lymphoma Kinase (ALK) is a tyrosine kinase receptor from the insulin receptor superfamily. The ALK gene encodes it and has been discovered as an oncogenic driver in cancer, which means it plays a critical role in the genesis and advancement of certain forms of cancer. ALK rearrangements have been identified as one of the primary genetic changes generating NSCLC. The echinoderm microtubule-associated protein-like 4 (EML4)-ALK fusion is the most prevalent ALK rearrangement in NSCLC, occurring in around 3-7% of cases. These rearrangements are more common in younger patients, those with adenocarcinoma histology, and nonsmokers (Soda et al., 2007). Crizotinib, a first-generation ALK TKI, has shown considerable clinical benefit when compared to chemotherapy in ALK-rearranged NSCLC (Shaw et al., 2013). Despite the initial response, crizotinib resistance is common. Second-generation ALK TKIs, such as ceritinib, alectinib, and brigatinib, have shown increased effectiveness and intracranial activity when compared to crizotinib (Shaw et al., 2014; Peters et al., 2017; Camidge et al., 2018). Lorlatinib, a third-generation ALK TKI, has shown encouraging benefits in patients with ALK-rearranged NSCLC who had previously failed ALK TKIs (Solomon et al., 2018).

The introduction of TKIs that target distinct molecular changes in NSCLC has revolutionized treatment approaches and enhanced patient outcomes. In fact, Osimertinib is now approved as a first-line therapy for EGFR T790M lung cancer patients. However, survival benefits to 1st line or 2nd line Osimertinib are transient as these patients inevitably develop resistance to Osimertinib. Resistance to osimertinib ensues either due to the acquisition of a mutation (C797S) that reduces the sensitivity to Osimertinib or by triggering intricate compensatory mechanisms that counteract the drug-induced cell death and foster the proliferation of cancer cells (Jänne et al., 2015; Piotrowska & Sequist, 2015; Ramalingam et al., 2018). However, not all resistance mechanisms are genetic. Approximately are non-genetic and can involve changes in the tumor microenvironment, activation of alternative signaling pathways, or the role of EVs in intercellular communication (Leonetti et al., 2019; Y. Zhang et al., 2018).

1.2c. Extracellular vesicles and their roles in cancer drug resistance

Recent evidence suggests that EVs contribute to drug resistance in various cancer types, including lung cancer (Bebelman et al., 2018; Muralidharan-Chari et al., 2016; X. Zhang et al., 2018). EVs are a diverse collection of membranous structures derived from cells, encompassing exosomes, microvesicles, and apoptotic bodies. Exosomes and microvesicles are the main forms of signaling EVs. Exosomes originate from the endosomal compartment and possess a diameter ranging from 30 to 150 nm. Microvesicles are generated through a process of direct budding from the plasma membrane and exhibit a

larger size range compared to exosomes (~100-1000nm). EVs are released by neoplastic cells, stromal cells, and immune cells, and have the capacity to regulate diverse cellular mechanisms, such as cellular proliferation, programmed cell death, and the formation of new blood vessels. EVs are essential components in facilitating intercellular communication through the transfer of their protein, lipid, and nucleic acid cargo, which includes messenger RNA (mRNA), microRNA, and other non-coding RNA molecules, to the cells that receive them (Tkach & Théry, 2016).

EVs have been reported to play an important role in acquired cancer drug resistance. Cancer cells can transfer drug resistance to other cells in the tumor microenvironment. For instance, EVs originating from drug-resistant cancer cells can transfer resistance to chemotherapy drugs such as doxorubicin, cisplatin, and paclitaxel to drug-sensitive cancer cells (H. Rashed et al., 2017). EVs can also contribute to drug resistance by triggering various signaling pathways. For instance, EVs obtained from cancer-associated fibroblasts can stimulate the Wnt/β-catenin signaling pathway, resulting in drug resistance in breast cancer cells (Boelens et al., 2014). The underlying molecular details are not fully understood. Further, EVs can significantly impact the efficacy of cancer immunotherapy. For instance, EVs released by melanoma cells have been shown to impede the functioning of T-cells and facilitate evasion of the immune system (Peinado et al., 2012). Furthermore, EVs can transfer oncogenic molecules to neighboring cells, which can lead to the growth and spread of tumors. For example, EVs originating

from metastatic PC cells can stimulate differentiation of osteoclasts, ultimately leading to the development of bone metastases (Rana et al., 2013).

The significance of EVs in cancer drug resistance and metastasis cannot be overstated. Therefore, targeting EVs has emerged as a promising therapeutic approach to enhance the efficacy of cancer therapy. To this end, various strategies have been developed, including inhibiting EV biogenesis and release, targeting specific EV contents, and modulating EV uptake by recipient cells. However, further research is needed to gain a more comprehensive understanding of the intricate roles of EVs in cancer and to devise more effective EV-targeted therapies.The role of EV in acquired resistance to Osimertinib in lung cancer has not been fully explored. In chapter X, I will discuss our findings on the role of plasma EV in the resistance of EGFR T790M lung cancer cells to Osimertinib.

The observation that cancer cells inevitably mount resistance to targeted therapy calls for a paradigm shift in drug design. Strategies that target molecular or cellular characteristics that are shared broadly across cancers of diverse mutational landscapes will generate deeper and more durable benefits for patients. In chapter 4 I will discuss our ongoing effort to develop a technology that meets these characteristics in the setting of pancreatic cancer.

1.3 Pancreatic Cancer

1.3a Pancreatic Cancer Incidence & Etiology

Pancreatic cancer (PanC) is a very aggressive disease with a dismal prognosis often identified at late stages owing to its asymptomatic nature in the early stages. PanC ranks seventh among the primary causes of cancer-related

fatalities globally, representing roughly 4.5% of all cancer-related deaths (Bray et al., 2018b; Siegel et al., 2022) The frequency of pancreatic cancer has demonstrated a consistent rise, exhibiting a worldwide age-standardized incidence rate of roughly 5.5 per 100,000 persons. Regional variations in incidence have been observed, with comparatively higher rates in high-income nations, including North America and Western Europe, in contrast to low- and middle-income countries(Siegel et al., 2020).

Pancreatic cancer is triggered by a sequence of genetic and epigenetic changes that cause normal pancreatic cells to convert into malignant cells. The KRAS gene is classified as a proto-oncogene and is responsible for encoding a small GTPase protein that acts as a molecular switch in various cellular signaling pathways. The KRAS gene is a crucial regulator of various cellular processes, including but not limited to cell growth, proliferation, differentiation, and survival. Normally, KRAS undergoes a transition between a state of inactivity, where it is bound to GDP, and a state of activity, where it is bound to GTP. The activation of cell surface receptors, specifically receptor tyrosine kinases (RTKs), results in the activation of KRAS. This activation subsequently triggers downstream signaling cascades, which include the RAF-MEK-ERK and PI3K-AKT pathways (Cox et al., 2014) Activating KRAS mutations are present in around 90% of pancreatic cancer cases, with a higher prevalence at codon 12 and lower prevalence at codons 13 and 61. The KRAS GTPase activity is impaired by these mutations, resulting in a constitutively active GTP-bound state and ongoing activation of downstream

signaling pathways, which promote uncontrolled cell proliferation and survival (Jones et al., 2008).

1.3b Pancreatic Cancer Treatment and Resistance

Surgical resection is the only potentially curative treatment for PanC. The surgical procedure is contingent upon the precise location of the tumor within the pancreas. However, only 15-20% of patients are eligible for surgery at the time of diagnosis due to advanced stage disease(Neoptolemos et al., 2018). Several therapeutic strategies have been proposed to target mutant KRAS or its downstream effectors, given the crucial role of KRAS mutations in pancreatic cancer. Chemotherapy is an essential component in the management of pancreatic cancer for patients with locally advanced or metastatic disease. The combination of gemcitabine and nab-paclitaxel, along with FOLFIRINOX, which is a blend of 5-fluorouracil, leucovorin, irinotecan, and oxaliplatin, has demonstrated enhanced survival outcomes in comparison to gemcitabine alone, as evidenced by randomized clinical trials (Conroy et al., 2011; Von Hoff et al., 2013). Immune checkpoint inhibitors, such as anti-PD-1 and anti-CTLA-4 monoclonal antibodies, have demonstrated exceptional effectiveness in reactivating T cell-mediated antitumor immunity in numerous cancer types. These current systemic treatments exhibit limited efficacy, resulting in a median survival rate of 6 months for individuals with metastatic ailment(Neoptolemos et al., 2018). Pancreatic cancer typically exhibits a low tumor mutational burden (TMB), resulting in fewer neoantigens that can be recognized by the immune system. This may partially explain the poor response to immune checkpoint inhibitors, as cancers with high

TMB are more likely to respond to these therapies (Goodman et al., 2017). TME is a critical factor in the development of drug resistance in pancreatic cancer. Factors such as a dense desmoplastic stroma, hypoxia, and inadequate vascularization impede drug penetration, while immune evasion and interactions between cancer cells and stromal cells facilitate survival and resistance. Overcoming drug resistance is critical for improving PanC treatment results. Greater understanding of the mechanism driving medication resistance will allow for development of innovative therapeutic tactics and individualized treatment approaches.

1.3c. A Bacteria-based immunotherapy approach to achieve broader and more durable benefits

The standard modes of cancer treatment, including chemotherapy, radiation therapy, and targeted therapy, are frequently confronted with challenges such as drug resistance, toxicity, and limited duration of response. Consequently, there is a growing emphasis on formulating inventive approaches to augment the efficacy of cancer treatment. The utilization of specific bacterial strains to activate the immune system, resulting in more extensive and long-lasting therapeutic advantages, is a developing technique in cancer treatment known as bacteria-based immunotherapy. The approach utilizes the immunomodulatory and tumoricidal properties of bacteria to selectively target cancer cells and circumvent the limitations of conventional cancer treatments. The application of bacteria in cancer treatment can be traced back to the early 1900s, when Coley (Coley, 1910) made the observation that bacterial infection led to tumor regression.

Due to its ability to specifically colonize and multiply inside the tumor microenvironment, the Salmonella genus of Gram-negative bacteria has become a prospective cancer therapeutic alternative. The potential of attenuated Salmonella strains in cancer therapy is being investigated due to its ability to elicit antitumor immune responses and serve as carriers for cancer-targeting agents (Kazmierczak et al., 2020; C.-H. Lee et al., 2008; Xiang et al., 2006; M. Zhao et al., 2006). Salmonella-based immunotherapy is a promising strategy for obtaining broader and more durable cancer treatment benefits. This strategy has the potential to surmount the limitations of conventional cancer therapies by exploiting the unique properties of attenuated Salmonella strains to stimulate the immune system and target cancer cells. Continued research and development in this field, including the optimization of bacterial strains, the development of combinatorial treatment strategies, and the translation of preclinical findings into clinical practice, will be necessary for realizing the full potential of Salmonella-based immunotherapy in improving the outcomes of cancer patients.

1.4 Board Overview

Cancer is a detrimental disease that has a high mortality rate, causing millions of fatalities worldwide annually. The incidence rate of prostate, lung, and pancreatic cancers has been on the rise in recent years, making them some of the most prevalent cancer types. Cancer research has made significant progress in understanding the molecular foundation of cancer cells as well as developing therapeutic approaches to target them. Nevertheless, neoplastic cells undergo

constant mutations acquiring resistance mechanisms against anticancer therapies, presenting major challenges in the management of these malignancies. Understanding the mechanisms of drug resistance in cancer is crucial for enhancing therapeutic effectiveness and patient outcomes. Although prostate cancer, lung cancer, and pancreatic cancer originate from distinct tissues, they exhibit commonalities in the emergence of drug resistance. These shared features may offer novel avenues for targeted therapeutic strategies.

PC is a prevalent neoplasm, with a projected incidence of one in nine males globally. This neoplasm exhibits a low rate of proliferation and remains localized within the confines of the prostate gland for a protracted duration. The conventional therapeutic modalities for PC encompass surgical intervention, radiation therapy, hormone manipulation, and chemotherapeutic agents. Nevertheless, the adaptive mechanisms of PC cells against these therapies are well-documented, resulting in their inefficacy. One of the fundamental mechanisms underlying the development of resistance to hormone therapy, specifically androgen deprivation therapy (ADT), is the emergence of mutated androgen receptors that retain their functionality even in the absence of androgens. In Chapter 2, I describe the role of proteasome subunit alpha 2 (PSMA2) in treatment resistant PC. I demonstrate that in response to enzalutamide, ADT-resistant NEPC cells significantly upregulate the PSMA2 and PSMA2 inhibition re-sensitized NEPC to ADT. data reveal that blocking PSMA2 reduces AR nuclear translocation and transcriptional targets, including NEPC fate markers. Moreover, PSMA2 inhibition reduced AR transcriptional output to androgen.

The phenomenon of treatment resistance transcends the boundaries of prostate cancer or therapies specifically targeting hormones. It is a widespread conundrum in the field of oncology, as corroborated by the short-lived responses observed in lung cancer patients undergoing targeted therapeutic interventions. Lung cancer is the primary contributor to cancer-related fatalities on a global scale, resulting in approximately 1.6 million deaths each year. Non-small cell lung cancer (NSCLC) is the most prevalent form of lung cancer, representing 80-85% of all cases. The therapeutic modalities available for NSCLC encompass surgical intervention, chemotherapy, radiation therapy, targeted therapy, and combination therapy. The implementation of targeted therapy, aimed at precise molecular modifications in malignant cells, has enhanced the outlook for individuals with NSCLC. However, cancer cells exhibit resistance mechanisms towards these therapies, resulting in the failure of treatment. The primary cause of resistance to targeted therapy is the activation of bypass signaling pathways that enable the targeted therapy's effects to be circumvented. Chapter 3 reveals that Hsa-miR-22-3p, Hsa-miR-184, and Let-7b-5p have a functional convergence on the WNT/ β-catenin and mTOR/AKT signaling axes, which are recognized as cancer therapy resistance signals. Targeting the Hsa-miR-22-3p and Hsa-miR-184 molecules resulted in the desensitization of NSCLC cells with EGFR mutations (T790M, L578R) to Osimertinib.

Pancreatic cancer (PanC) is a highly lethal form of cancer, exhibiting a mortality rate of 93%. The therapeutic interventions for PanC encompass surgical procedures, radiation therapy, chemotherapy, and targeted therapy. Nevertheless,

pancreatic neoplastic cells are widely recognized for their remarkable resistance to conventional therapeutic approaches. In Chapter 4, I demonstrate that PanC cells containing oncogenic KRAS are preferentially killed by a genetically modified strain of Salmonella typhimurium (CRC2631). In addition to inducing cytotoxicity in cancer cells, CRC2631 effectively permeates PanC tissues, activates effector T-cells associated with tumors, and consequently diminishes tumor load in murine KRAS PanC models.

In Chapter 5, I suggest a working model and further investigations to refine my findings.

CHAPTER 2 – CASTRATION INDUCED-PROTEASOME SUBUNIT ALPHA 2 SENSITIZES ANDROGEN RECEPTOR SIGNALING TO RESIDUAL ANDROGEN

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2.1 Abstract:

Despite advances in effective, tailored anti-cancer medicines, drug resistance remains a serious barrier in cancer therapy. It is vital to understand the molecular basis of cancer medication resistance to provide patients with long-term survival advantages. My study focuses on malignancies of the prostate, lung, and pancreas, with a special emphasis on neuroendocrine prostate cancer (NEPC), a resistant and deadly subtype of prostate cancer (PC). Because of PC cancer, NEPC develops alternate growth-promoting pathways. Chemotherapy and androgen deprivation therapy (ADT) are presently the standard therapies for PC, although NEPC patients often relapse and die months after treatment begins. When treated to ADT, ADT-resistant NEPC cells markedly upregulate the proteasome protein Proteasome Subunit Alpha 2 (PSMA2). PSMA2 inhibition re-sensitized NEPC cells to ADT, suggesting that PSMA2 enhances ADT resistance by sensitizing NEPC cells to residual androgens. PSMA2 is thought to inhibit HSP90, which typically sequesters the androgen receptor (AR) in the cytoplasm. This causes rapid AR nuclear translocation and expansion, ultimately leading to the destiny of the NEPC. PSMA2 inhibition affects AR nuclear translocation and transcriptional targets, including NEPC fate markers, according to our findings. Furthermore, blocking PSMA2 desensitizes AR transcriptional output to androgens, extending animal life in a NEPC mouse model. This study has the potential to re-sensitize current medicines and enhance NEPC patient survival rates.

2.2 Introduction:

PC progresses into therapy-resistant subtypes: castration-resistant prostate cancer (CRPC) and neuroendocrine cancer (NEPC). Androgen signaling drives PC, which ultimately acquires mixed carcinoma/CRPC-NEPC histology as part of the disease's normal progression; or because of androgen deprivation therapy (e.g., enzalutamide, abiraterone). CRCP-NEPC incidence is expected to climb in the next decade as more potent ADT agents are deployed as first-line therapy options(Beltran et al., 2011a; Heinlein & Chang, 2004; Taplin & Balk, 2004a, 2004b).

CRPC-NEPC is highly lethal, and there is no effective therapy available. According to clinicaltrial.gov, there are more than 500 Phase I/II ongoing trials for PC therapeutics. Only 12 new drugs, including Enzalutamide (AZT), have been approved for patients with advanced PC. However, none of these approved drugs are efficacious against NEPC. The molecular mechanisms of CRPC-NEPC drug resistance remain elusive. Without this knowledge, it is impossible to develop rationally designed and efficacious therapies against CRPC-NEPC(Heinlein & Chang, 2004; Mu et al., 2017b; H. T. Wang et al., 2014b).

The heat shock protein (HSP) family, including chaperones such as HSP90 and HSP70, play a crucial role in managing the maturation and nuclear translocation of Androgen Receptors (ARs). These chaperones are part of a conserved family that assists in the proper folding, transportation, and degradation of proteins. For stability and defense against proteasomal degradation, studies have shown that the androgen-bound AR first interacts with heat shock proteins

(HSPs), such as HSP90, HSP70, and p23 (De Leon et al., 2011; Heinlein & Chang, 2004). Heat Shock Protein 90 (HSP90) plays a critical role in AR signaling. HSP90 interacts with and sequesters AR in the absence of androgen. Androgen binds to AR and dissociates from HSP90, allowing AR to translocate into the nucleus. It activates a transcriptional program that promotes cell survival and proliferation (De Leon et al., 2011) and increases the expression of AR downstream targets such as Prostate Specific Antigen (PSA) and NXK3. The regulation of HSP90-AR interaction varies in different settings, including the presence of mutations and therapy resistance. Additionally, it has been observed that mutations in AR are common in CRPC and can affect the way it interacts with HSP90 and other chaperones. This can lead to changes in AR's stability and activity, independent of ligand presence(Clegg et al., 2012; Hu et al., 2009).

Proteasome Subunit Alpha (PSMA2) is a 20S core proteasome complex component. PSMA2 is vital in controlling many cellular activities, such as cell cycle progression(Qi et al., 2021), and is highly expressed in numerous cancers, such as PC. However, the role of PSMA2 in PC remains unknown. The development of ADT resistance has been linked to the proteasome. The degradation of AR and its co-regulators is facilitated explicitly by the proteasome system. Proteasome inhibition has been found in preclinical trials to specifically prevent the degradation of AR and its co-regulators, hence delaying the onset of CRPC. Additionally, both preclinical and clinical investigations have shown that proteasome inhibitors and ADT have synergistic benefits when administered together (Kuroda & Liu, 2019; O'Reilly et al., 2019; Zhang et al., 2021).

In this study, we sought to understand the role of PSMA2 in AR signaling, PC treatment resistance, and the potential to use PSMA2 as a therapeutic target for CRPC-NEPC. Our mechanistic studies revealed that ADT-resistant NEPC cells dramatically upregulate the proteasome protein Proteasome Subunit Alpha 2 (PSMA2) in response to ADT. Pharmacological Inhibition of PSMA2 re-sensitized NEPC to ADT. This study also revealed that PSMA2 blockade inhibits AR nuclear translocation and transcriptional targets, including NEPC fate markers. Further, PSMA2 blockade desensitized AR transcriptional output to androgen. Finally, PSMA2 blockade prolongs animal survival in a mouse model of NEPC. Information gleaned from this work may help understand the role of PSMA2 in fundamental AR signaling human neoplasia and be used in future clinical trials focusing on preventing relapse in PC.

2.3 Methods:

Cell lines maintenance - All cell lines were obtained from ATCC (Manassas, VA, USA). LNCAP cell line was maintained in RMPI (Gibco #11875101) supplemented with a final concentration of 10% Fetal Bovine Serum (FBS). LASCPC-01 and NCI-H660 cell lines were maintained in Custom RMPI (ATCC #30-2001) supplemented with 5% FBS, 10 nM hydrocortisone, 10 nM β-estradiol, 1x Insulin-Transferrin-Selenium, 1x GlutaMAX. All cells were maintained at 37°C with 5% CO2.

Cell Proliferation Assay/ Drug Sensitivity Assays:

MTT Assay - Cell viability was assessed using an MTT Assay kit (Sigma #CT02) LNCAP, NCI-H660, and LASCPC-01 cells (5 $x10^4$) in a 96-well plate (100µl per well) and grown for 24hrs, followed by treatment with Enzalutamide 1µm, R1881(0.05, 0.1, 0.5 and 1 nM), and Bortezomib 200nM (LC LABS #B-1408) for 48hrs. 5mg/ml of MTT was added and incubated for 4hrs. 200ul Acidic isopropanol was prepared as a 1.5% (v/v) solution of hydrochloric acid in isopropanol. Absorbance was read at 490nm on a microplate reader (BioTek Epoch).

Trypan Blue Assay - Following treatment, cells were collected and centrifuged at 450g for 8 min, then resuspended in 1ml media. 1-part cells were mixed with 1-part 0.4% trypan blue and incubated at room temperature for 3 mins. Viable cells were measured using Thermofisher automated cell counter.

Short hairpin RNA interference Transfection - Cells were seeded in a six-well plate at 5x10^5 cells per well for 24hrs followed by shPSMA2 or non-specific control shRNA (Horizon Dharmacon, GIPZ PSMA2 lentil shRNA Transfection Starter kit # RHS11851-EG5683) transfected using Dharmafect 2 according to manufacturer's instruction. The concentration of shPSMA2 was 1.75µg. The efficiency of RNA was checked by immunoblot and QPCR analysis, respectively.

PSMA2 Overexpression - Cells were seeded in a six-well plate at 5x10^5 cells per well for 24hrs followed by PSMA2OE using Precision LentiORF PSMA2 Viral Particle Starter Kit, viral particles kit (Horizon # OHS5900-202626736) following manufacture protocol.

QPCR analysis - Total RNA was extracted using an RNAeasy miniprep kit (Qiagen #74104) according to the manufacturer's instructions. cDNA synthesis was performed using an Applied Biosystem High-Capacity RT kit (#4368814). Quantitative PCR was performed using Applied Biosystem SYBR Green PCR Kit. as per manufacturer's instruction on BioRad CFX96™ System in 96-well plates in 3–6 repeats. A two-step thermal cycling protocol, i.e., 95 °C for 2 min followed by 50 cycles at 95 °C for 10 s and 56 °C for 60 s, was used. Fold change was calculated using the $2^{-\Delta\Delta Ct}$ method.

Western blot - Cells were lysed using Pierce RIPA Lysis and Extraction Buffer (ThermoFisher Scientific #89900) containing 1 × Halt™ Protease Inhibitor Cocktail (Thermofisher #78425) (Processed for western blotting). Blots were stained against PSMA2 (Origene#CF505474), HSP90(ThermoFisher Scientific # MA110372), PSA/KLK3 (Proteintech#60338-1), AR (Invitrogen # MA5-13426), and GAPDH (DSHB-hGAPDH-2G7) as a loading control (1:1000). Secondary horseradish peroxidase (HRP) antibodies were obtained from Invitrogen. Pierce ECL Chemiluminescence kit (ThermoFisher Scientific #32106) and the ChemiDoc Imaging System (Bio-Rad) were used to detect protein bands.

Immunoprecipitation - Following with cell lysis explained in materials and method section (Westernblot), Cell lines (LASCPC, H660) used for the treatments with (shPSMA2, AZT, R1881) under the experimental conditions. For immunoprecipitation, total of 4mg protein was used. PBS prewashed 100 µL A/G bead slurry (pierce Protein A/G agarose, Cat# 20421, Thermo Scientific) was used for 1mg/mL bacterial cell lysate. Clarified bacterial cell lysates were pre-incubated

with AG beads for 1 h at 4°C to remove non-specific proteins. The preclear lysate obtained after this step were incubated overnight at 4°C on a rotator with primary antibodies. Antibodies used at 0.5 µg/1mg protein ratio (anti-AR, Cat No MA5-13426, anti HSP90, Cat no MA110372, anti-GAPDH, cat no- DSHB-hGAPDH-2G7. FBS used as nonspecific antibody control for the same lysate. At end of incubation centrifuged at 1,000g for 2 min at 4°C, supernatant removed carefully and washed three times with wash buffer (10mM Tris-pH 7.4, 1mM EGTA, 150mM NaCl, 1%Triton X-100, with protease inhibitor cocktail) the end of the incubation, continue with wash steps given below. The Ag-Ab complex is eluted from the beads by heating or boiling samples in loading buffer with denaturant SDS, proceeded for SDSPAGE and western blotting.

EdU staining - Cells were seeded for 24 at in a 6-well plate at 5x 10^5 per well for 24 h, followed by treatment with Enzalutamide 1µm, R1881(0.05, 0.1, 0.5, and 1 nM), and Bortezomib 200nM for 48hrs. After treatment, cells were stained for EDU, using Click-iT™ Plus EdU Alexa Fluor™ 647 Flow Cytometry Assay Kit (Molecular Probes C10634) following manufacturer protocol.

Flow Cytometry - After, Stained cells were analyzed using a FACS flow cytometer (BD Biosciences). Data were analyzed using FlowJo software (Tree Star; https://www.flowjo.com/).

Analysis of PSMA2 expression in PC patients

mRNA expression of PSMA2 in TCGA Patients - Gene Expression Profiling Interactive Analysis (GEPIA2), http://gepia2.cancer-pku.cn/#analysis (Tang et al., 2019) were applied to compare

the PSMA2 expression in Prostate cancer tissue samples with the threshold of P value < .05 and |Log2FC| > 0.1. The mRNA level of PSMA2 between normal tissues and Prostate cancer [PRAD]) (The Cancer Genome Atlas (TCGA) and The Genotype-Tissue Expression (GTEx)) was analyzed.

Survival analysis of PC patients Expressing amplification of PSMA2- The cBioPortal for Cancer Genomics (http://cbioportal.org) is an open-access online platform that enables exploration, visualization, and analysis of multi-dimensional cancer genomic data, including data from The Cancer Genome Atlas (TCGA). In this study, we utilized TCGA datasets specific to Prostate Adenocarcinoma (PRAD) to further investigate any mutations or copy number alterations (CNA) in the PSMA2 gene and PSMA2 correlation with NEPC markers (MYC,SPINK1, ONECUTE, REST, EZH2, FOXA2, AURKA, AKT1, LIN28B and SOX2. We employed the OncoPrint and survival tabs, following the step-by-step instructions provided by cBioPortal's online user guide(Cerami et al., 2012; Gao et al., 2013).

2.4 Results

PC cells upregulate PSMA2 in Response to ADT - From previous studies, we noted that murine and human PC cells stimulate anti-apoptotic programs in response to standard-of-care enzalutamide and cancer targeting biologic (data not shown and (Kazmierczak et al., 2020)), Treatment-induced transcriptomic analyses of PC cells, revealed that PSMA2 was dramatically upregulated in murine and human cells in response to therapy, suggesting a role for PSMA2 in PC

adaptive response to therapy challenges (data not shown). To explore a potential relationship between PSMA2 and Enzalutamide (AZT) resistance in PC, we first analyzed the expression of PSMA2 in Carcinoma cells (LNCAP), NEPC-Like cells (LASCPC), and NEPC cells (NCI H660). These cells were treated for 48h with a low dose of AZT. QPCR analysis revealed that these PC cells transcriptionally upregulate PSMA2 in response to enzalutamide (Fig 1a). Consistent with elevated PSMA2 transcription, Enzalutamide treatment elevated PSMA2 protein levels in LASCPC cells compared to the control (Fig 1b). Further, prospective analyses of PSMA2 mRNA levels in clinical samples revealed that PSMA2 is upregulated in PCa patients compared to non-diseased (Fig 1c). Thus, PCa cells transcriptionally upregulate PSMA2 in response to ADT.

Enzalutamide-resistant cells are sensitive to PSMA2 Inhibition - The above finding suggested that PSMA2 is part of an adaptive molecular response that promotes PC resistance to ADT. To directly test a functional role for PSMA2 in ADT resistance we tested the effect of PSMA2 inhibition on PC sensitivity to ADT. Pharmacological inhibition of PSMA2 using the small molecule inhibitor Bortezomib (BTZ) killed ADT resistant PC cells. AZT generated 2% NEPC killing, however when combined with BTZ be there is ~ 74% cell death, (Fig 2a). We investigate whether inhibition of PSMA2 block androgen- mediated PC cell growth in the context of residual androgen. That is comparing the efficacy of bortezomib and AZT in inhibiting cell growth within the situation of low androgen levels. We treated the PC cells with BTZ-AZT combination for 48h in the presence of minimal androgen, here R1881. Consistently, viability assay showed that BTZ blocks

androgen-mediated PC growth in both carcinoma and neuroendocrine cells, suggesting that BTZ may be a treatment in combination with ADT for PC (Fig 2b). We also demonstrated that by genetically inhibiting PSMA2 with a short hairpin RNA (shRNA), (Fig 2c) RNA interference significantly overrides resistance mechanisms and suppresses AZT-resistant cells in NEPC cell lines (Fig 2d). We concluded that the Inhibition of PSMA2 with AZT may be an effective means of targeting NEPC, prompting us to investigate further the mechanism of PSMA2 involvement in AR activation threshold.

PSMA2 lowers the AR activation threshold - Next, we sought to elucidate whether there is a correlation between PSMA2 upregulation and AR activation. We first compared the growth rate assay on PC Knockdown of control cells treated with AZT and titration of synthetic androgen. Data showed that it took more androgen in the absence of PSMA2 to reach the maximum cell count compared to the control (Fig 3a,b). Next, to understand how AZT-induced PSMA2 may impact AR activity, we analyzed the protein level of AR expression in LASCPC nuclear lysates. To verify that treatment induced PSMA2 influence AR activation, we investigated the correlation between PSMA2 and AR transcript NXK3A. QPCR analysis revealed PSMA2 Inhibition impeded androgen stimulation and Transcription (Fig 3c,d). Importantly, PSMA2 didn't affect NXK3A transcript level in the absence of androgen stimulation. Our findings indicate that PSMA2 promoted ADT resistance in vitro and highlight the PSMA2 signaling pathway as being crucial and active in enzalutamide-resistant PC.

PSMA2 lowers AR activation threshold by sequestering HSP90 - Next, we investigated whether PSMA2 promotes AZT resistance by inhibiting AR chaperone protein, heat shock protein 90 (HSP90). We hypothesized that ADT-stimulated PSMA2 activity accelerates HSP90 protein turnover, thus sensitizing AR to low androgen levels in tissues following ADT, leading to persistent AR-driven NEPC cell survival and tumor growth (Fig 3a,b). Alternatively, PSMA2 supports ADT resistance via HSP90-independent mechanisms. Co-immunoprecipitation (Co-IP) assay was carried out on PC knockdown and control cells treated with AZT and R1881 for 48 h to determine the interaction between PSMA2 and HSP90 and AR complex. The results obtained from the Western blots of AR-immunoprecipitates indicated an increase in the HSP90 content of AR-pulldowns upon androgen exposure while comparing the knockdown of PSMA2 with the control (as shown in Fig. 3 e, f). The results indicate that the expression of HS90-AR complexes was reduced in both LASCPC and H660 cells upon treatment with R1881, while the opposite effect was observed in PSMA2KD cells (Fig. 3 e, f). The addition of AZT resulted in a significant increase in HSP90 levels.

Treatment induced-PSMA2 contributes to NEPC transformation. Previous studies demonstrated that ADT upregulates SPINK1 and potentiates cellular plasticity, promoting stemness and chemoresistance in PC (Tiwari et al., 2020). Congruent with this, previous studies (Beltran et al., 2011b; Montironi et al., 2020; Puca et al., 2019) suggest that, with prolonged AR suppression, AZT resistance can be mediated by trans-differentiation of carcinoma cells into treatment-emergent NEPC. QPCR analyses revealed an increase in SPINK1 (Fig

4a, c) and NEPC biomarker Chromogranin A (CGA) and Synaptophysin (SYP) (Fig 4b, d) with treatment AZT. Interestingly, with the Inhibition of PSMA2, SPINK1, and NEPC, biomarkers were significantly reduced. Congruent to immunohistochemistry, treatment shPSMA2 and AZT was sufficient in reducing SYP expression in LASCPC cells (Fig4 e-p). This data suggests that PSMA2 promotes convergent enzalutamide resistance NEPC. Next, we want to test whether PSMA2 gain is sufficient to promote the transition from carcinoma to NEPC fate. LNCAP treated with PSMAORF viral particle causes an increase in NEPC marker CGA and SYP (Fig 5). In alignment with these findings, analysis of prostate cancer patient data from Cbioportal (Cerami et al., 2012; Gao et al., 2013) demonstrated a strong correlation between high NEPC marker expression and PSMA2 amplification (Fig 6a). Additionally, it was observed that PSMA2 amplification is associated with reduced survival in prostate cancer patients (Fig 6b).Together this data suggests that PSMA2 contributes to ADT resistance and transition to NEPC fate.

2.5 Discussion

Despite many progresses in ADT resistance, the molecular mechanism by which carcinoma develops into CRC-NEPC and acquires resistance to ADT is not fully understood. This study presents evidence highlighting the dual function of PSMA2 in contributing to PC resistance to ADT. Through mechanistic investigations, it was discovered that treatment-induced PSMA2 increases the sensitivity of PC cells to residual androgens, thereby undermining the efficacy of

hormone therapy. The research suggests that ectopic PSMA2 mediates this effect by sequestering HSP90 from AR, promoting accelerated AR nuclear translocation and activation, and consequently leading to the expression of AR target genes. Furthermore, the study reveals the involvement of PSMA2 in promoting the transition of prostate adenocarcinoma towards neuroendocrine prostate cancer (NEPC) fate. The pharmacological inhibition of PSMA2 renders previously unresponsive NEPC cells susceptible to ADT, and PSMA2 blockade notably extends animal survival in an NEPC mouse model. These observations offer valuable insights into the role of PSMA2 in PC resistance to ADT and propose a potential therapeutic approach for treating NEPC.

2.6 Figure

Figure 1

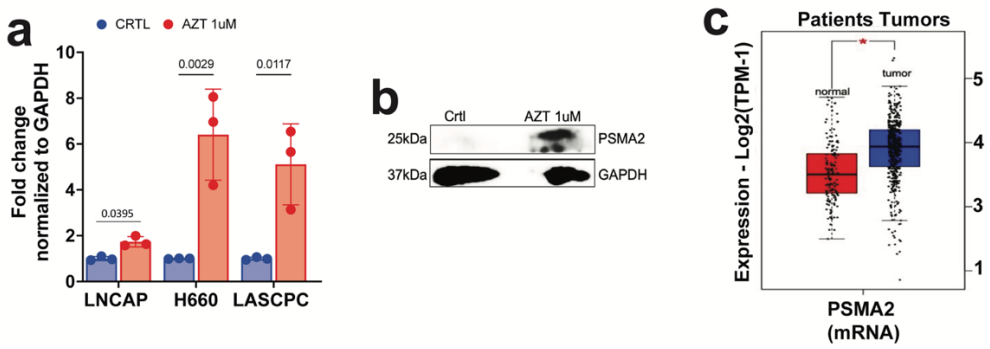


Figure1. PC cells upregulates PSMA2 in Response to ADT. (a) LNCAP, LASCPC And H660 Non-treated cells (blue), treated for 48hrs with Enzalutamide 1uM (red). Quantitative PCR used to assess PSMA2 expressions. Fold change normalized to GAPDH. (b)Western blot analysis of PSMA2 protein levels in

LASCPC lysate treated with/without AZT1uM.GAPDH used as loading control. (C) mRNA expression of PSMA2 in TCGA and GTEx Patients.

Figure 2

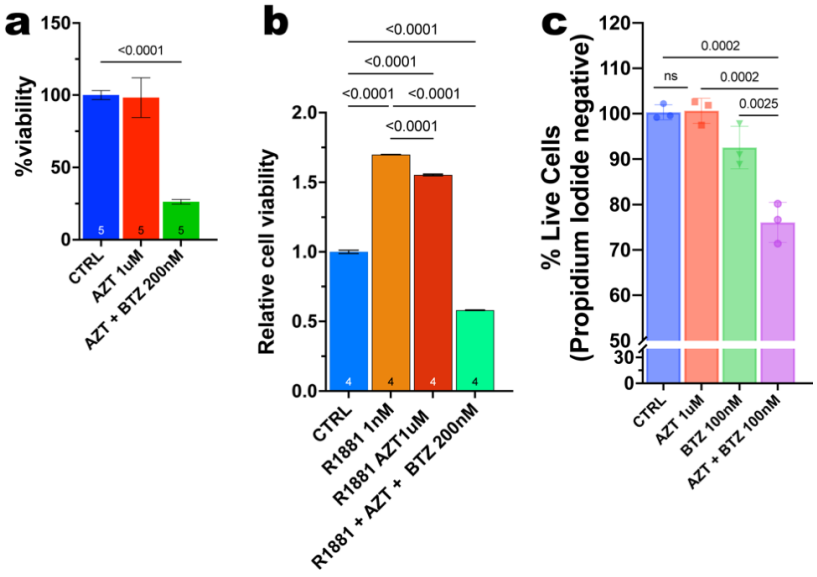


Figure 2. PSMA2 Suppression sensitizes LASCPC to AZT. (a)Viability Assay (MTT) of LASCPC cells non-treated, AZT 1um, AZT+ (Bortezomib) BTZ 200nm and (b)In the presence of Androgen (R18811nM). (c) Percentage Live cells analyzed using propidium iodide (PI) binding by flow cytometry.

Figure 3

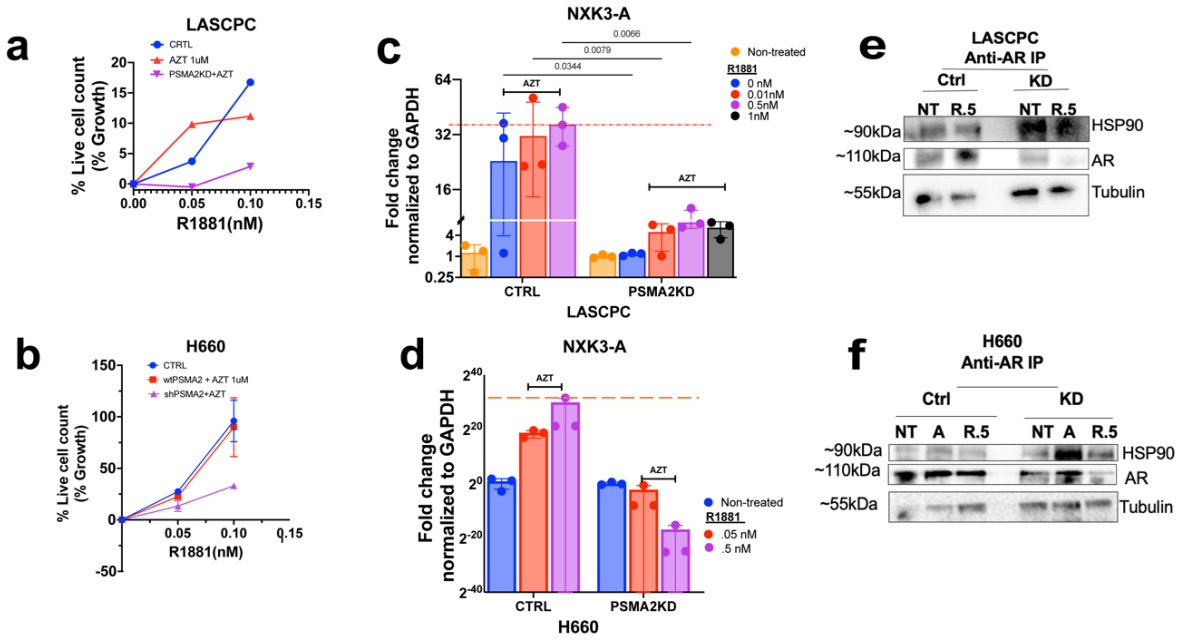


Figure 3. PSMA2 lowers AR activation threshold by sequestering HSP90. Total the count of live cells of LASCPC wtPSMA2 (Ctrl) and ShPSMA2 (KD) treated with or without AZT 1uM observed under Trypan blue (a) LASCPC cells (b) H660. (c)LASCPC Cells and (d) H660 cells transfected with PSMA2KD or Crtl and treated with AZT and increasing concentration of R1881 (0.05,0.1,0.5 nM) Quantitative PCR used to assess NXK3-Aexpression. Fold change normalized to GAPDH. Western blot of Protein Expressions of HSP90, AR, and tubulin on (e) LASCPC (f) H660 CTRL and Knockdown cells AR pulldown lysates treated with Enzalutamide1uM and/ R1881 0.5uM.

Figure 4

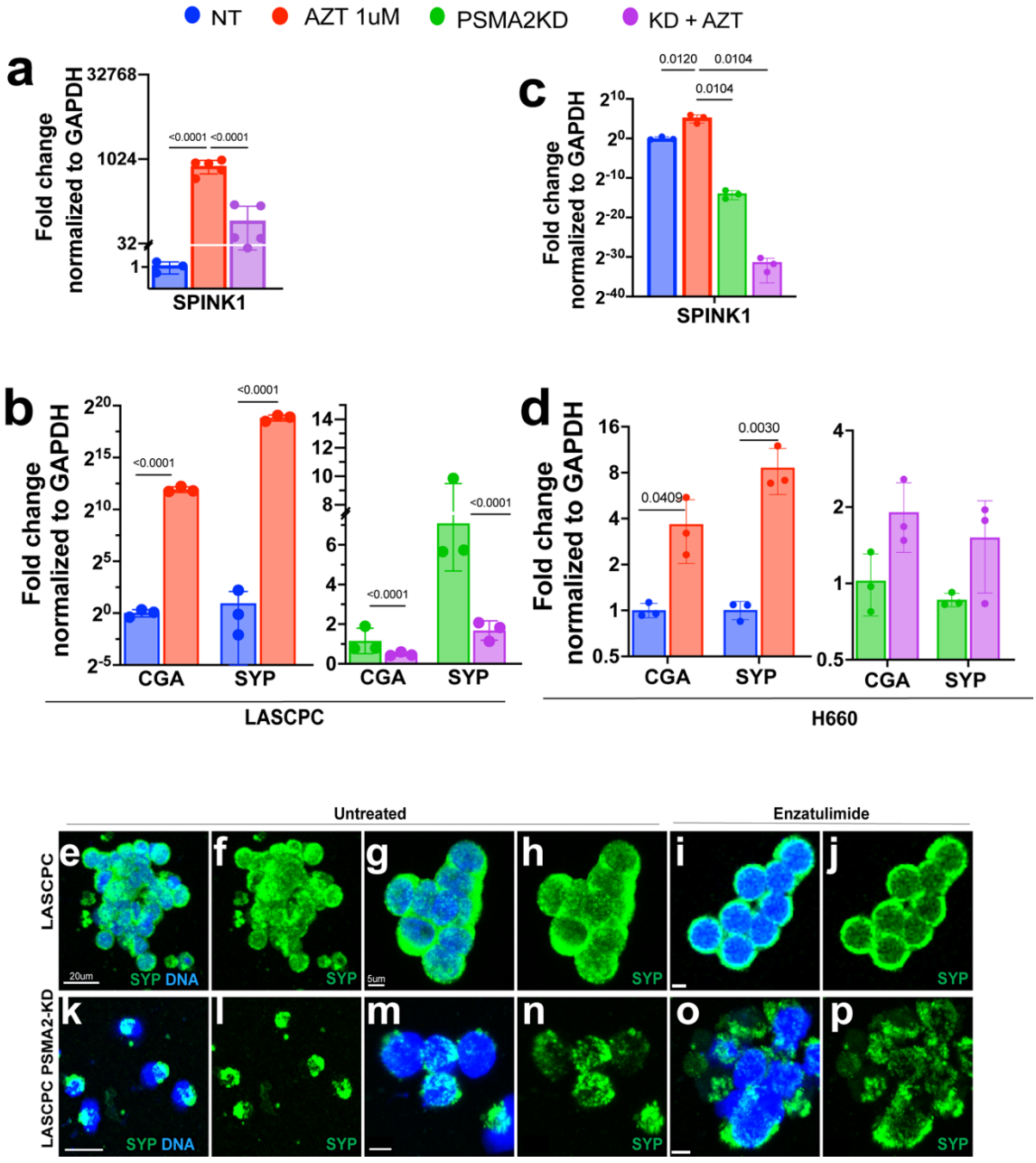


Figure 4. Treatment induced-PSMA2 contributes to NEPC fate Maintenance. LASCPC and H660 Non-treated and treated for 48hrs with Enzalutamide 1ug and LASCPC cells transfected with PSMA2 short hairpin RNA vector with AZT. Quantitative PCR used to assess (a, c) SPINK1 (b, d) Chromogranin A (CGA) and Synaptophysin (SYP) expressions. Fold change normalized to GAPDH.

Immunohistochemistry of LASCPC cells stained with SYP antibody. (a-h) untreated control cells (Imp) control cell s treated with AZT. (k-n) LASCPC PSMA2KD cell and (o, p) LASCPC PSMA2KD treated with AZT.

Figure 5

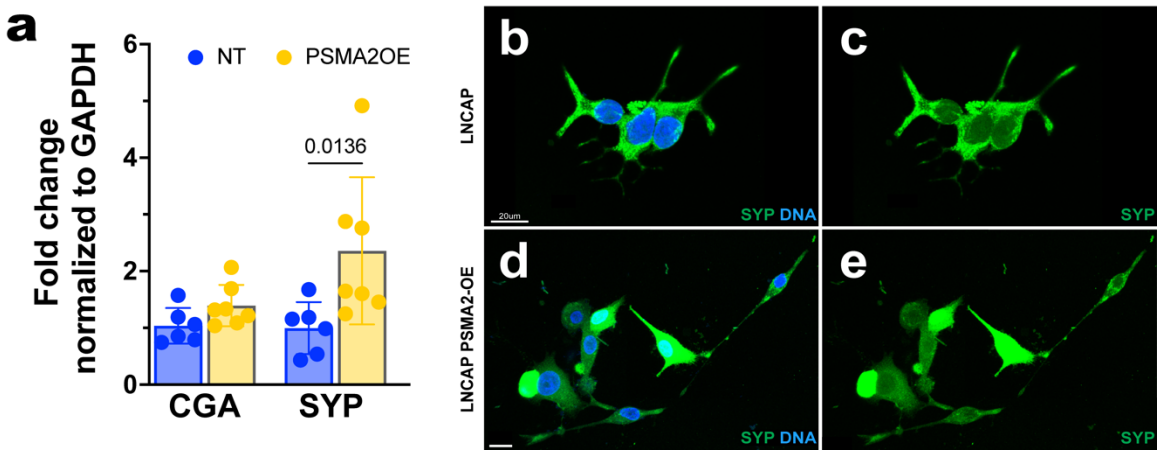
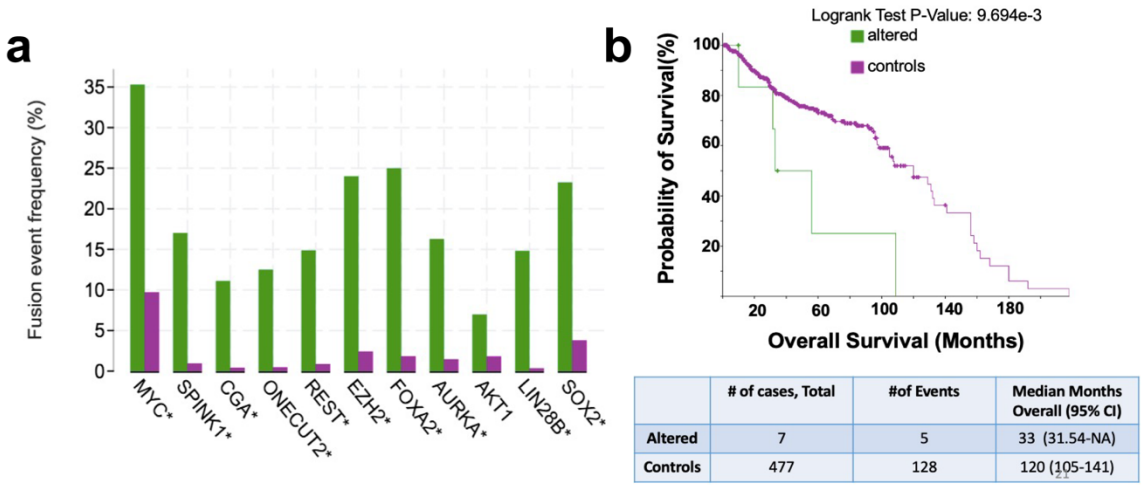


Figure 5. PSMA2 gain is Sufficient to promote NEPC fate transition. Quantitative PCR used to assess (a) Chromogranin A (CGA) and Synaptophysin (SYP) expressions. Fold change normalized to GAPDH**.** Immunohistochemistry of LASCPC cells stained with SYP antibody. (b, c) control cells (d, e) LNCAP PSMA2OE cells.

Figure 6



	# of cases, Total	#of Events	Median Months Overall (95% CI)
Altered	7	5	33 (31.54-NA)
Controls	477	128	120 (105-141)

Figure 6. PSMA2 Amplification Correlates with Poor Survival in PC Patients. (a) Correlation on PSMA2 expression and NEPC fate markers (MYC,SPINK1, ONECUTE, REST, EZH2, FOXA2, AURKA, AKT1, LIN28B and SOX2) in PC patient tissues (b) Kaplan–Meier analysis of PC patients with high NEPC markers and Hight PSMA2 expressions.

CHAPTER 3 – EXTRACELLULAR VESICLE LET-7B-5P, MIR-184 AND CIRCULATING MIR-22-3P MEDIATE RESISTANCE AGAINST TARGETED THERAPY IN LUNG CANCER

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This chapter and the content thereof have been published by Scientific Reports, Springer Nature. Titled Combining plasma extracellular vesicle Let-7b-5p, miR-184 and circulating miR-22-3p levels for NSCLC diagnosis and drug resistance Predictions.

3.1 Abstract:

Drug resistance not only poses a significant challenge in prostate cancer (PC). Like PC, non-small cell lung cancer (NSCLC) is one of the leading causes of cancer-related deaths worldwide. Despite the progress made in treatment modalities, the issue of therapy resistance still poses a considerable hurdle in the management of these malignancies. The etiology of resistance to molecularly targeted therapy in NSCLC frequently involves genetic mutations that trigger alternative signaling pathways. NSCLC patients harboring sensitizing mutations in epidermal growth factor receptor EGFR (T790M, L578R) are treated with Osimertinib, a potent tyrosine kinase inhibitor (TKI). However, nearly all patients develop TKI resistance. The underlying mechanisms are not fully understood. We found that Hsa-miR-22-3p, Hsa-miR-184, and Let-7b-5p functionally converge on WNT/ β-catenin and mTOR/AKT signaling axes, known cancer therapy resistance signals. Targeting Hsa-miR-22-3p and Hsa-miR-184 desensitized EGFR-mutated (T790M, L578R) NSCLC cells to Osimertinib.

3.2 Introduction:

Lung cancer causes the most cancer-related deaths worldwide(Molina et al., 2008b), with 85% of lung cancer patients present with non-small cell lung cancer (NSCLC) and 70% of the cases are diagnosed as late-stage disease (Lemjabbar-Alaoui et al., 2015; Molina et al., 2008a; Travis et al., 2015b). The 5-year survival rate for late-stage NSCLC is 2-5% compared to 92% for early-stage

disease (Molina et al., 2008a), underscoring the importance of early detection. Whilst chemotherapy and targeted therapy have demonstrated enhanced patient outcomes, drug resistance poses a significant obstacle in the treatment of NSCLC.

Patients who are diagnosed with NSCLC are stratified to chemotherapy and/or immunotherapy, or targeted therapy based on the presence or absence of known NSCLC driver mutations in tissue biopsy analyses (Haslam & Prasad, 2019; Hirsch et al., 2017). Depending on patients' ethnicity, activating mutations of epidermal growth factor receptor (EGFR) tyrosine kinase is found in 15-50% of NSCLC (Y.-M. Chen, 2013; Mok et al., 2009b; Zhou et al., 2011). Patients harboring drug sensitizing EGFR mutations are treated with tyrosine kinase inhibitors or TKI. Some patients initially respond to tyrosine kinase inhibitors (TKI) but ultimately develop resistance: cancers acquire TKI-desensitizing EGFR mutations or activate compensatory signals, including WNT/ β-catenin, the mechanistic target of rapamycin mTOR, and AKT signaling to drive cancer recurrence(Arcila et al., 2011; Gini et al., 2020; Jänne et al., 2015; Ludovini et al., 2011; Sequist et al., 2011; H. A. Yu et al., 2013, 2015). Screening for genetic alterations that activate these signaling pathways provides a rationale for prioritizing treatment options that maximize the probability of achieving durable outcomes. However, it is becoming increasingly evident that cancer cells develop drug resistance via complex non-mutational mechanisms that involve emergent cell-cell interactions mediated by microRNAs (miRNAs) (Li et al., 2014; Y. Zhang et al., 2018; Y. Zhang & Wang, 2017). miRNAs are short (19-23) non-coding nucleotides that degrade protein transcripts and fundamentally impact signaling

events (Lagos-Quintana et al., 2001; R. C. Lee et al., 1993; Reinhart et al., 2000).In this study, we identified miRNAs converge on WNT/ β-catenin and AKT/mTOR signaling pathways, suggesting a role in drug-resistance.

3.3 Methods:

Isolation of extracellular vesicles and circulating micro-RNA - Patients' blood was double-centrifuged to remove platelets and the platelet-poor plasma was used for extracellular vesicles (EVs) analysis. Purification of the EVs was done through size exclusion chromatography (SEC) using qEV70s single columns (ddIzon, Netherlands). These columns were filled with 150 µl of plasma and used based on recommendations from the manufacturer; fraction 8 through 11 were collected and pooled to obtain the fraction of purified EVs. The particle size and concentration for all EV samples were determined using nanoparticle tracking analysis (NTA).

Total RNA extraction was performed as previously described (Priglinger et al., 2020) using 200 µl of plasma or 200 µl of pooled EV fractions and the miRNAs mini kit (Qiagen). Samples were thawed at room temperature followed by centrifugation at 12,000×g for 5 minutes at 4°C to remove any debris. Extraction was conducted by use of the miRNA easy Qiagen kit. For homogenization, 200 µL of plasma/EV suspension were mixed with 1000 µL Qiazol and 1 µL of a mix of 3 synthetic spike-in controls (Qiagen, Germany). After a 10-minute incubation at room temperature, 200 µL chloroform were added to the lysates followed by cooled centrifugation at 12,000×g for 15 minutes at 4°C. Precisely 650 µL of the upper

aqueous phase were mixed with 7 µL glycogen (50 mg/mL) to enhance precipitation. Samples were transferred to a miRNeasy mini column, and RNA was precipitated with 750 µL ethanol followed by automated washing with RPE and RWT buffer in a QiaCube liquid handling robot. Finally, total RNA was eluted in 30 µL nuclease free water and stored at -80°C until further use.

Electron microscopy - Exosomes were processed for negative staining as described in M. Rames, et al. Briefly, 5 µL of purified EV sample was applied to a freshly glow discharged (Pelco Easiglow, Ted Pella Redding CA) carbon coated TEM grid (Electron Microscopy Sciences, Hatfield PA) over ice. Samples were washed three times with distilled water, incubated 2 min on 2% paraformaldehyde (Electron Microscopy Sciences), then incubated for 30 sec on 2% uranyl acetate (aqueous, Electron Microscopy Sciences) and backblotted with filter paper (Whatman P1, Fisher Scientific) and allowed to dry. Images were collected on a JEOL JEM 1400 transmission electron microscope operated at 120V equipped with a Gatan Ultrascan 1000 CCD camera.

Small RNA sequencing - Small RNA sequencing was performed as described previously 52. Equal volumes of total RNA (2 µL) were used for small RNA library preparation using the Clean Tag small RNA library preparation kit (TriLink Biotechnologies, US). that utilizes chemically modified adapters to prevent formation of adapter dimers(Shore et al., 2016). Adapter-ligated libraries were amplified using barcoded Illumina reverse primers in combination with the Illumina forward primer. A pool consisting of 40 plasma samples, and a second pool consisting of 40 EV samples was prepared by mixing samples at equimolar rates

based on a DNA-1000 bioanalyzer results (Agilent, CA). The DNA library pool underwent size-selection (BluePippin, SageScience, US) to enrich for microRNAs with an insert size of 18-36 nt, corresponding to a library size of approximately 145 bp.

Sequencing was performed on an Illumina NextSeq 550 with 75 bp single end runs. Overall quality of the next-generation sequencing data was evaluated automatically and manually with FastQC v0.11.8 and MultiQC v1.7. Reads from all passing samples were adapter trimmed and quality filtered using Cutadapt v2.3 and filtered for a minimum length of 17nt. Mapping steps were performed with bowtie v1.2.2 and miRDeep2 v2.0.1.2, whereas reads were mapped first against the genomic reference GRCh38.p12 provided by Ensemble allowing for two mismatches and subsequently miRBase v22.1, filtered for miRNAs of hsa only, allowing for one mismatch. For a general RNA composition overview, non-miRNA mapped reads were mapped against RNAcentral and then assigned to various RNA species of interest.

Statistical analysis of preprocessed NGS data was done with R v3.6 and the packages pheatmap v1.0.12, pcaMethods v1.78 and genefilter v1.68. Differential expression analysis with edgeR v3.28 used the quasi-likelihood negative binomial generalized log-linear model (GLM) functions provided by the package. False discovery rate (FDR) correction was performed to adjust for multiple testing, and a cut-off of FDR < 5% was applied.

Target network analysis - miRNA target network analyses and genes ontology enrichments analyses (KEGG and Reactome) were conducted using miRNet (www.mirnet.ca).

Cell lines and cell culture - The human lung cancer cell line A549 were grown in Dulbecco's Modified Eagle Medium (DMEM, 11965-092), with L-Glutamine, and high glucose supplemented with 10% FBS. Cells were grown in the nutrient medium as suggested by ATCC. Cells were incubated in a humidified incubator with 5% CO2 at 37°C. H1975 cells were grown in ATCC-formulated RPMI-1640 Medium (ATCC, Cat number 30-2001), with 10% FBS (fetal bovine serum) at 37 C, 5% CO2.

Peripheral blood mononuclear cells (PBMC) isolation - 10 mL of blood from healthy individual was collected into EDTA coated anti-coagulant vacutainer tubes (Cat #367899, BD Biosciences). Transferred onto 50 mL sterile 50 mL centrifuge tube and added equal volume with of ice cold DPBS pH 7.4 and gently mix by inversion. Using transfer pipette, carefully transferred diluted blood to sterile centrifuge tube containing 10 mL of Ficoll-Paque plus (Amersham #17144003,) then centrifuge at 2000 rpm for 25 min. after centrifuged from the fractionated phases, carefully collect the PBMCs fraction between ficol-paque and plasma layer. Collected PBMCs washed with ice cold PBS and centrifuge at 1700 rpm for 10 min. pellet re-suspended with 2 ml of Pharm Lyse lysing buffer (Biosciences #555899), mix well incubate at 37^{o}C for 4 min and adjust volume with PBS to 50 mL and proceed with centrifuge at 1700 rpm for 10 min. PBMC pellet re suspend in PBS with 1×106 cells/mL for further experimental purpose.

EV/PBMC supernatant transfer experiments - Isolated human PBMCs were seeded and grown in 6 well culture dish with hybridoma-(SFM) serum free medium (Cat #12045076, Gibco). PBMCs were treated with patient EVs under serum free conditions for 24 hrs. After incubation cell free PBMC or EV/PBMC conditioned media were collected and used for culturing A549 cells. After 24 hours A549 cells were collected for cell counting or lysed for Western blotting.

Western blotting - A549 cells were cultured with similar loads of EV derived either from high-risk controls or confirmed lung cancer patients. Lysates were prepared in lysis buffer (20 mM Tris-HCl pH-7.5, 150 mM NaCl, 1 mM Na2 EDTA, 1 mM EGTA, 1%TritonX-100) and processed for Western blotting. Blots were stained against β-catenin to determine WNT signaling levels and GAPDH as a loading control (1:1000, Cell signaling #5174S). Secondary horseradish peroxidase (HRP) antibodies were obtained from Invitrogen. Pierce ECL Chemiluminescence kit (Thermo Fisher scientific #32106) and the ChemiDoc Imaging System (Bio-Rad) were used to detect protein bands.

A549 cells were washed with PBS and lysed in a lysis buffer (20 mM Tris-HCl pH-7.5, 150 mM, NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1%Triton, 2.5 mM sodium pyrophosphate, 1 mM b- glycerophosphate, 1 mM Na3VO4, 1µg/ml leupeptin) supplemented with protease and a phosphatase inhibitor cocktail (Cell Signaling #9803S). Proteins were electrophoresed on SDS- PAGE using 4-20% Mini-PROTEAN® TGX™ precast gel (Biorad #456-1094,), transferred onto methanol pretreated PVDF membrane. PVDF membranes were probed overnight with mouse anti β-catenin (DSHB, #PY489), mouse anti-mTOR (1:1000, Santa

Cruz, #517464) and mouse anti-GAPDH (DSHB, #2G7) at 4°C and after washing 3 times, membranes were incubated with anti-rabbit mouse horseradish peroxidase (HRP) (1:5000, #31460, Invitrogen). Primary or secondary antibodies were diluted in 5% BSA-TBST. Anti-mouse horseradish peroxidase (HRP) (1:5000, Invitrogen #31430,), anti-rabbit horseradish peroxidase (HRP) (1:5000, Invitrogen #31460,) used to develop respective blots. Membranes were developed using Pierce ECL western blotting substrate (Thermo Fisher scientific #32106,) and imaged on ChemiDocTM MP Imaging System, Bio-Rad Laboratories Inc.

H1975 cells. H1975 cells were lysed in 1× RIPA buffer (Thermofisher #89900) containing 1× Halt™ Protease Inhibitor Cocktail (Thermofisher #78425). Proteins were separated on a 4-12% Bis-Tris gel (Invitrogen, Cat# NP0321) and transferred to PVDF membranes. Membranes were incubated with primary antibodies against Phospho-AKT (1:5000, Proteintech #66444-1-Ig,) or GAPDH (Sigma #G8795) overnight at 4oC. Secondary antibodies were purchased from Cell Signaling (Cat#7076). Blots were developed with Immobilon Western Chemiluminescence Kit (Millipore, #WBKLS0500).

miRNA Transfections and Osimertinib treatments miRNA stock was prepared by suspending in RNAse-free water. Cells were seeded such that they were 70-80% confluent at the time of transfection. Cells were allowed to adhere for 24 hrs and then transfected with 25 nM of miRNA inhibitors against hsa-miR184 (Sigma #HSTUD0282), hsa-miR22-3p (Sigma #HSTUD0393) or 100 nM of hsa-let7b-5p miRNA mimic (Sigma #HMI0007) using Lipofectamine 3000 (Invitrogen #L3000001). Osimertinib (100 nM) was added an hour after transfection to cells.

Cells were allowed to incubate with transfection mix for 24 hrs at 37 oC, 5% CO2 and then washed with 1× PBS and trypsinized to be used for cell count and Western blotting.

cDNA synthesis and qRT-PCR - cDNA synthesis was carried out using the miRCURY LNA RT Kit (Qiagen #339340) from human purified EVs or plasma RNA (10 ng) according to manufacturer's instructions. The synthesized CDNA diluted in nuclease free water and stored until further use at -80oC as per the kit instructions. Quantitative polymerase chain reaction was conducted using miRCURY LNA miRNA SYBR PCR kit (Qiagen #339345) as per manufacturer's instruction on BioRad CFX96TM System in 96-well plates in 3-6 repeats. A two-step thermal cycling protocol i.e., 95°C for 2 min followed by 40 cycles at 95°C for 10 sec and 56°C for 60 sec, was used. A no-reverse transcriptase (NRT) and no-template control (NTC) were included in each reaction to check for primer specificity and any non-specific amplification. miRNA targets include miRNA-184 (Qiagen #YP00204601), miRNA-21-5p (Qiagen #YP00204230), Let-7b-5p (Qiagen #YP00204750), miRNA 22-3p (Qiagen #YP00204606). The expression levels of each miRNA target were normalized to calibrators U6-snRNA or GAPDH. Fold changed of miRNA was calculated by ΔΔCt and 2 -ΔΔCt method. ΔCt was calculated by subtracting the average of Ct values of calibrator from Ct values of target miRNA. ΔΔCt was computed by subtracting ΔCt of the screening control from ΔCt of RADS IV group.

Patient Data Analysis - MicroRNA expression (miRNAseq) and clinical data from Lung adenocarcinoma (LUAD) were collected from the publicly

accessible TCGA database using the Bioconductor tool TCGA Biolinks RTCGA R packages. The "surv_cutpoint" function of the “survminer” R package was used to identify high versus low expressing patients’ samples for survival analysis. Survminer uses selected rank statistics to determine the optimal cut-point of a continuous variable in an unbiased manner. Kaplan–Meier (KM) survival plots and related statistics were generated using the Survival R package.

3.4 Results

Plasma Let-7b-5p, miR-184, and miR-22-3p levels differentiate NSCLC patients from high-risk individuals - We used next generation sequencing (NGS) approaches to profile EV miRNAs from Lung-RADS4 confirmed cancer patients or over-diagnosed Lung-RADS4 individuals or high-risk screening controls (Lung-RADS2). We included circulating plasma miRNA because combining multiple analytes from diverse biological sources have the potential to identify robust biomarkers. We identified 58 differentially expressed miRNAs, including miRNAs widely known to be deregulated in cancers. To identify a set of miRNAs that can robustly discriminate between NSCLC and cancer-free individuals, we prioritized miRNAs that were differentially expressed in at least two of the following comparisons: Lung-RADS2 versus Lung-RADS4; Lung-RADS4 false positive versus confirmed cancer patients; Lung-RADS2 combined with false-positive Lung-RADS4 patients versus confirmed cancer patients; any of the preceding groups versus patients who rapidly progressed (LDCT imaging and/or death shortly after sampling). We then focused on miRNAs showing significant

performance (P and area under the curve/AUC values) in receiver operating characteristic (ROC) analyses for further examination. This approach led to the discovery of let-7b-5p, miR-184, and miR-22-3p as potential biomarkers for discriminating cancer patients from high-risk controls.

Let-7b-5p, miR-184, and miR-22-3p converge on treatment-resistance mechanisms-We considered the possibility that these EV and circulating plasma miRNAs (let-7b-5p, miR-184, and miR-22-3p) mediate cell–cell communication events that support NSCLC disease progression. First, we used the miRNA target proteins analysis platform MIRNET to identify experimentally validated protein targets of let-7b-5p, miR-184, and miR-22-3p. We prioritized proteins that are targeted by at least two of the three miRNAs from experimental data (MIRNET miR2gene)(L. Chang et al., 2020). This approach identified 43 proteins (Fig.1a), which were subsequently interrogated in Gene Ontology analyses using Kyoto Encyclopedia of Genes and Genomes (KEGG) or Reactome classifications to derive signaling pathways. Cancer was the most highly enriched KEGG term (Fig. 1b, c), underscoring the robustness of our experimental pipeline and the relevance of these miRNAs to cancer disease. Interestingly, KEGG and Reactome signaling maps revealed that let-7b-5p, miR-184, and miR-22-3p converge on the activation of WNT and PI3K-AKT-mTOR signaling (Fig. 1c, d), suggesting that circulating and EV miRNAs cooperatively regulate WNT and PI3K-AKT-mTOR activity in NSCLC. Activation of WNT or PI3K-AKT-mTOR signaling in NSCLC tissues is associated with aggressive and therapy resistant disease(L. Liu et al., 2020; Stewart, 2014). Considering that miR-184 and miR-22-3p are

downregulated in cancer patients, this suggests that plasma from high-risk, yet cancer-free individuals contain EV and circulating miRNAs that suppress WNT and the AKT signaling axis and that these mechanisms are restrained in NSCLC patients.

Further, the uptake of EV by immune cells results in paracrine signaling loops that ultimately accelerate disease progression via complex mechanisms(Popēna et al., 2018; Shinohara et al., 2017; Whiteside, 2017). We performed supernatant transfer experiments and asked whether cancer patients EV stimulate AKT/mTOR in A549 cells either directly or via immune cells. A549 cells were cultured in media conditioned by peripheral blood mononuclear cells (PBMC) left untreated or treated with EV either from controls or from cancer patients. Cancer patients EV/PBMC media dramatically stimulated phospho-AKT and phospho-mTOR levels in A549 cells, compared to controls (Fig.2a, b). Note that EV from the high-risk controls also stimulated mTOR, possibly reflecting a NSCLC priming state (see “Discussion”). Consistent with this AKT/mTOR stimulating potential, EV/PBMC conditioned media accelerated the growth of A549 cells (Fig.2c). Taken together, the above data argue that plasma EV act directly or via immune cells to activate AKT in NSCLC.

Next, we sought to determine whether let-7b-5p, miR-184, and miR-22-3p mediate the AKT activating effect of cancer EV and what implication this might have on NSCLC treatment outcomes. Activating mutations in Epidermal Growth Factor Receptor (EGFR) signaling represent one of the most known genetic alterations associated with NSCLC(S. V. Sharma et al., 2007; Tetsu et al., 2016).

Patients harboring sensitizing EGFR mutations (exon 19 deletion and L858R) respond favorably to first- and second-generation Tyrosine Kinase Inhibitors/TKI (gefitinib, erlotinib, afatinib, and dacomitinib). However, patients acquire TKI-desensitizing EGFR mutations (T790M) and become resistant to these TKIs. Osimertinib, a third generation TKI selectively targets EGFR T790M and generates significant clinical benefits in EGFR T790M patients (Maemondo et al., 2010; Mitsudomi et al., 2010; Mok et al., 2009b; Pao et al., 2005; Sequist et al., 2013). Unfortunately, all patients ultimately develop resistance to Osimertinib because they acquire an Osimertinib-desensitizing mutation (C797S) or activate complex compensatory signals to resist drug-induced cell death and to promote cancer cell proliferation (Jänne et al., 2015; C.-C. Lin et al., 2018; Piotrowska & Sequist, 2015; Ramalingam et al., 2018). Understanding the nature of these signals and how they are activated have the potential to inform new treatment strategies for re-sensitizing patients to existing TKIs.

We investigated a role for let-7b-5p, miR-184, and miR-22-3p in NSCLC response to Osimertinib using H1975 NSCLC cells, which harbor L858R and T790M EGFR mutations. First, H1975 cells were transfected with miR-184 and miR-22-3p inhibitors, mimicking their reduction in cancer patient plasma. We then assessed the effect of miR-184/miR-22-3p inhibition on AKT activity. The inhibitors reduced miR-184 and miR-22-3p levels in qPCR assays (Fig.2 e, f, respectively) and cooperatively elevated pAKT levels in H1975 cells (Fig. 2g, h).

In reciprocal experiments we found that Lung-RADS2 EV, which overexpress miR-184), inhibit Osimertinib-induced AKT activity in H1975 cells (Fig.

2h). Importantly, inhibition of miR-22-3p in this setting was sufficient to dramatically unleash AKT (Fig. 2h), further highlighting the cooperation between these two miRNAs in modulating AKT activity. Finally, we investigated the effect of miR-184/miR-22-3p inhibition on Osimertinib-induced cell death. Consistent with AKT stimulation, miR-184/miR-22-3p inhibition significantly suppressed Osimertinib-induced cell death Fig. 2i). Similar results were observed when miR-184/miR-22-3p co-inhibited cells were treated in the presence of a let-7b-5p mimic (Fig. 2i).

AKT activation is associated with NSCLC resistance to TKI, leading to reduced patient survival time (L. Liu et al., 2020; Stewart, 2014). Thus, we sought to determine to what extent reduced miR-184/miR-22-3p tumor expression correlates with reduced patient survival using the cancer genome atlas (TCGA) LUAD patient tumor miRNA expression and survival data. Let-7 could not be combined with miR-22 or miR-184. Only a small number of patients (12) in the LUAD TCGA had combined expression data for Let-7 and miR-22/miR-184, making it difficult to reliably perform the analysis.

We did not detect any significant survival difference between patients whose tumors express low miR-22-3p (Fig. 3a). However, patients with low miR-184 tumor expression had a significantly shorter survival time compared to patients with higher miR-184 tumor expression (Fig. 3bo Hazard Ratio 2.09, 95% CI: 1.13, 3.84, $p < 0.018$). Interestingly, patients with miR-184/miR-22-3p co-repressed tumors experienced even shorter survival time compared to patients with high miR-184/miR-22-3p tumor expression (Fig. 3c, Hazard Ratio 3.43, 95% CI: 1.26, 9.32, $p < 0.016$). Note that the survivorship of miR-184/miR-22-3p tumor low

patients is significantly lower than that of patients with tumor low miR-184 alone or miR-22-3p alone. This is consistent with AKT activation in miR-184/miR-22-3p co-inhibited NSCLC patients' plasma and treatment resistance.

Thus, EV (let-7b-5p, miR-184) and circulating (miR-22-3p) plasma miRNA likely modulate NSCLC response to Osimertinib, highlighting a novel mechanism of resistance and suggesting that these biomarkers may assist in the selection of patients that will likely benefit from Osimertinib/AKT blockade combination treatments.

3.5 Discussion

Confirmed NSCLC patients are stratified to diverse treatment options, including chemotherapy, immunotherapy, and targeted therapies based on histological and genetic mutations profiles obtained from tissue biopsies. Due to positional constraints, however, tissues biopsies often fail to capture the broader complexity of genetic driver mutations, leading to incomplete targeted therapy responses. The profiling of cancer-derived EV from patients' plasma may provide deeper insights into the overall cancer mutational landscape of the tumor and thus better guide treatment decisions in the future. Indeed, pathway analyses of let-7b-5p, miR-184, and miR-22-3p target proteins revealed that these miRNAs converge on therapy resistance signals, including AKT. We propose that circulating and EV miRNAs functionally cooperate to modulate oncogenesis or patients' clinical outcomes (Figure 4a, b). The AKT-suppressing miRNAs miR-184/miR-22-3p function as tumor suppressors and delay oncogenesis (Figure 4a). Cancers downregulate the expression of these miRNAs and/or reduce their systemic

abundance via an unknown mechanism, leading to high baseline AKT activity and potentially resulting into accelerated cancer growth and drug resistance (Figure 4b). EV and plasma miRNAs may act directly or via tumor-interacting immune components to modulate tumor cell signaling and behavior. Mimicking the expression profile of miR-184 (EV) and miR-22-3p (plasma) in NSCLC patients' blood using miRNA inhibitors cooperatively activated AKT and desensitized NSCLC cells (H1975) to Osimertinib. AKT inhibition re-sensitizes TKI-resistant NSCLC cells to erlotinib and gefitinib (Jacobsen et al., 2017). A clinical trial evaluating the efficacy of combining Osimertinib with aspirin (an AKT inhibitor) in advanced NSCLC patients is pending (NCT04184921). Further, aberrant activation of AKT/mTOR and WNT/β-catenin signaling are associated with therapy resistance across different cancer types, suggesting broad translatability for these markers and their underlying mechanisms of action.

One limitation for this study is the limited size of the discovery cohorts (cases and controls). Future validation studies will include determining to what extent blood levels of Let-7b-5p, miR-184, and miR-22-3p detect NSCLC and predict disease relapse in larger patient cohorts.

We noted that EV derived from high-risk control patients triggered mTOR activation in A549 cells. We cannot rule out the possibility that this EV-induced mTOR activation is specific to A549 cells, but it is possible that this effect reflects a priming state that precedes NSCLC onset in these high-risk patients.

These findings demonstrate that drug resistance in lung cancer cells arises from intricate non-mutational pathways that entail emergent intercellular

communications facilitated by microRNAs (miRNAs). I also show that I demonstrated that the resistance mechanisms in prostate and lung cancer are complex and multifactorial. The occurrence of genetic modifications, epigenetic modifications, and modified signaling pathways are prevalent mechanisms observed in both diseases. This is further in illustrated in PanC (Chapter 4).

Acknowledgment

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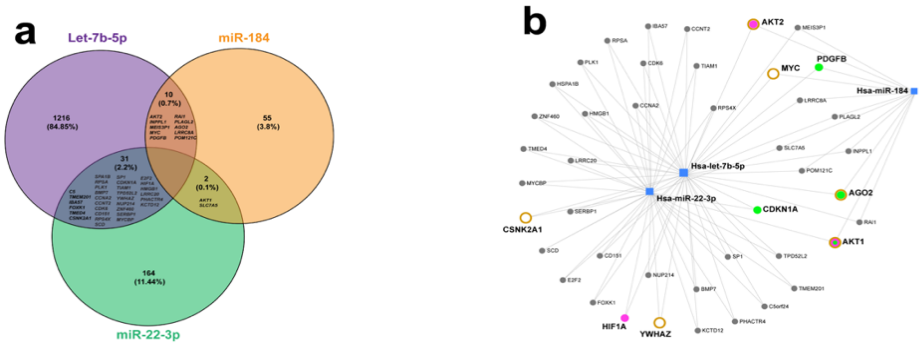
3.6 Figures

Figure 1



c KEGG

Name	Hits	Pval	adj.Pval
Glioma	6	1.34e-7	4.55975E-06
Melanoma	6	1.7e-7	4.55975E-06
Chronoc myeloid leukemia	6	2.7e-7	4.55975E-06
Cell cycle	7	2.99e-7	4.55975E-06
Small cell lung cancer	5	0.0000124	0.000136233
Pathways in cancer	8	0.0000134	0.000136233
Prostate cancer	5	0.000187	0.000162957
Epstein-Barr virus infection	5	0.0000233	0.000177663
Non-small cell lung cancer	4	0.0000465	0.000315167
Renal cell carcinoma	4	0.000082	0.0005002
Pancreatic cancer	4	0.000142	0.000787455
ErbB signaling pathway	4	0.000349	0.001774083
Endometrial cancer	3	0.000677	0.003150214
mTOR signaling pathway	3	0.000723	0.003150214
Colorectal cancer	3	0.000929	0.003595188

d Reactome

Name	Hits	Pval	adj.Pval
Cellular responses to stress	7	0.0000278	0.0022
SMAD2/3/4 trimer transcription	3	0.000138	0.0022
Oncogene Induced Senescence	3	0.000171	0.0022
Cyclin D events in G1	3	0.000171	0.0022
G1 Phase	3	0.000171	0.0022
Signaling by Wnt	6	0.000213	0.0022
Regulation of mRNA stability	4	0.000265	0.0022
Cell cycle	8	0.000292	0.0022
PI3K events in ERBB4 signal	4	0.000357	0.0022
PIP3 activates AKT	4	0.000357	0.0022
PI3K events in ERBB2 signal	4	0.000357	0.0022
PI3K cascade:FGFR1	4	0.000357	0.0022
PI3K cascade:FGFR2	4	0.000357	0.0022
PI3K cascade:FGFR3	4	0.000357	0.0022
PI3K cascade:FGFR4	4	0.000357	0.0022

Figure 1. Mir-184 and Mir-22 Converge on Drug Resistance Signals. (a) Venn diagram showing the number unique of shared protein targets between the selected miRNAs. (b) MIRNET star-network showing proteins that are targeted by at least two of the three miRNAs (blue squares). (c, d) KEGG (c) or Reactome (d) classification analyses of the identified proteins show a convergence onto PI3K-AKT-mTOR (c, magenta label, and d green label) and WNT/β-catenin (d, brown label) signaling pathways. The underlying target genes are shown in corresponding color in “b”.

Figure 2

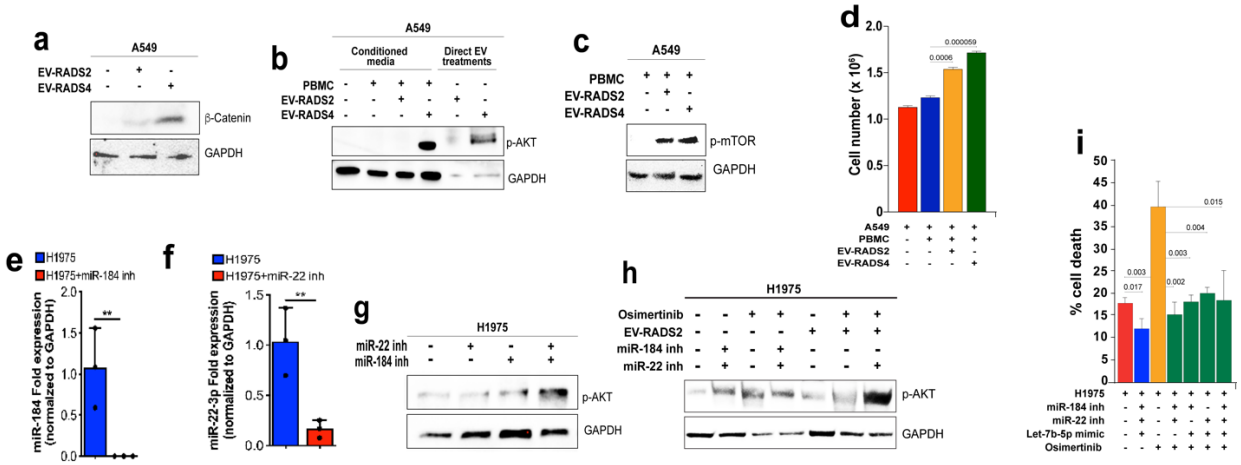


Figure 2. Inhibition of miR-184/miR-22-3p Activates AKT and Desensitizes NSCLC Cells to Osimertinib. (a) Western blot image from A549 cells treated with equal quantity of EV from cancer patients or from high-risk screening controls. Blots were stained against β-catenin to detect WNT signaling levels or GAPDH as a loading control. (b, c) Western blot images from A549 cells cultured in standard media or media conditioned with PBMC in the absence or presence of patients EV (b, lanes 1–4). Also, A549 cells were treated directly with cancer patients or control EV (e, lanes 5 and 6). Blots were stained against phospho-AKT1 (b) or phospho-mTOR (c) or GAPDH as a loading control (b, c). A549 cell numbers from supernatant transfer experiments (b, lanes 1–4) are shown in "d" as average cell numbers from triplicate experiments. Error bars denote SD values. P values are derived from student t-test analyses. (e, f) qPCR data showing mean expression fold changes of miR-184 (e) or miR-22-3p (f) in H1975 cells transfected with miR-184 (f) or miR-22-3p inhibitors (f)(blue bars) compared to untreated H1975 control cells (red bars). Expression was normalized to GAPDH. (g) Western blot images

from H1975 cells left untreated or treated with miR-22-3p or miR-184 inhibitors and blotted against phospho-AKT1 or GAPDH (loading control). (h) Image of a Western blot from untreated H1975 cells or H1975 cells treated with equal portions of EV from screening controls (RADS-II) or with inhibitors against miR-22-3p and/or miR-184 inhibitors followed with Osimertinib (100 nM) treatments. Western blots were stained against phospho-AKT or GAPDH (loading control). (i) Graph showing the proportion of H1975 cell death across the indicated conditions using Trypan blue exclusion assays. These cell death assays were performed in triplicates and the results are shown as the average proportion (percentage) of dead cell across replicates for each treatment conditions. Error bars denote standard deviations and p values were derived from t-tests.

Figure 3

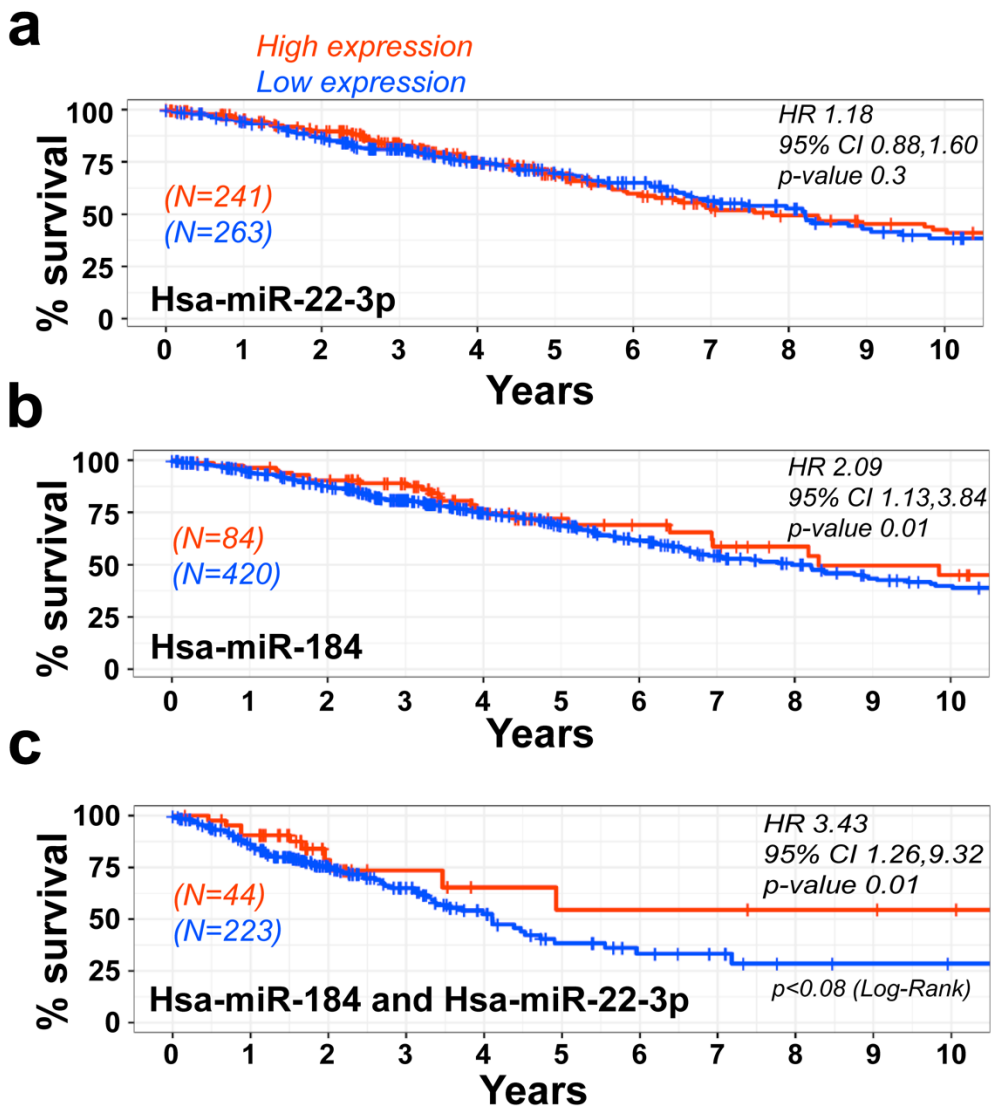


Figure 3. miR-184/miR-22-3p Tumor Co-repression Correlates with Poor Clinical Outcome. (a-c) Survivorship comparison data using miR-184 and/or miR-22-3p expression data in TCGA-LUAD and the Bioconductor tool TCGA Biolinks RTCGA R packages. The “surv_cutpoint” function of the “survminer” R package was used to identify high versus low expressing patients’ samples for miR-22-3p (a) or miR-184 (b) or both (c) in Cox regression analyses. Survminer uses selected

rank statistics to determine the optimal cut-point of a continuous variable in an unbiased manner.

Figure 4

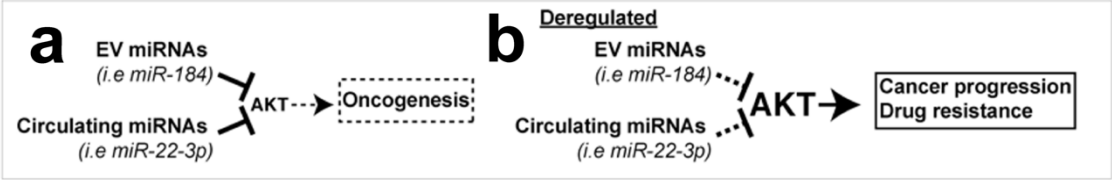


Figure 4. Proposed model summarizing the role of mir-184/mir-22 in Osimertinib drug response. (EV (mir-184) and circulating (mir-22) plasma miRNAs cooperatively target and modulate AKT activity. (a) Cancer-free high-risk individuals up-regulate EV mir-184 and circulating mir-22, keeping AKT levels generally low. In the context of genetic driver mutations, this low AKT activity delays oncogenesis. Similarly, cancer patients with high EV mir-184 and circulating mir-22-3p maintain AKT below an activity threshold required for AKT-mediated drug resistance, leading to positive drug response. However, AKT baseline activity is elevated in patients with low mir-184/mir-22 levels such that even a modest stimulation of AKT drives AKT above the drug-resistance activity threshold, leading to relapses and poor clinical response (b).

CHAPTER 4 – CRC2631 SAFELY TARGETS AND ELIMINATES IMMUNOTHERAPY RESISTANT *KRAS*-DRIVEN PANCREATIC CANCER

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4.1 Abstract

In the Chapter 2 and Chapter 3, I demonstrated that managing prostate (PC) and lung cancers presents significant challenges, mainly due to the development of resistance mechanisms against current therapeutic options though genetic and non-genetic mutation—this challenge in also common in Pancreatic Cancer (Panc).

Oncogenic *RAS* mutations are associated with aggressive PanC that resist existing treatments, including immunotherapy (IO). Over 70% of patients are diagnosed when the disease has progressed past the operable stage. Instead of curative surgery, these patients receive standard of care chemotherapy. Unfortunately, most patients quickly develop chemoresistance. Similarly, checkpoint blockade IO agents have demonstrated impressive survival benefits in several other cancers, but this treatment modality is efficacious only for 1% of PanC patients with well-defined tumor genetic features, further limiting treatment options for this disease. The inefficacy of IO is due to the fibrous and immune-suppressive nature of the PanC milieu, which limits the recruitment and activity of cancer-killing immune cells. Breaking PanC physical and immune barriers to unleash anti-cancer immune activity remains the shortest and most likely path to generating deep and durable clinical benefits in PanC.

Here we show that a genetically engineered strain of Salmonella typhimurium (CRC2631) safely penetrates PanC tissues and preferentially kills PanC cells harboring oncogenic KRAS, which is associated with drug resistance. In addition to direct cancer cell killing, CRC2631 stimulates tumor-associated

effector T-cells, and correspondingly reduces tumor burden in mouse models of KRAS PanC. Our work highlights a potential for bacterial cancer therapy to achieve immune re-potentiation and to re-sensitize panC to IO.

4.2 Introduction:

Panc is a highly aggressive and lethal cancer(Goodwin et al., 2023; Rahib et al., 2014) . Panc is the fourth most common cause of cancer-related mortality, exhibiting a 5-year survival rate of less than 5%. These numbers have remained stagnant for nearly half a century (Biankin et al., 2012; Jemal et al., 2010). It is estimated that approximately 62,210 individuals will receive a diagnosis of PanC in the United States this year (Siegel et al., 2022). Over 70% of the individuals in question will exhibit advanced pancreatic ductal adenocarcinoma (PDAC)2 that is non-resectable, rendering it incurable. PanC's poor prognosis is linked to advanced presentation and late diagnosis, where there aren't many curative treatment options or reliable biomarkers. The sole therapeutic recourse available for these patients is chemotherapy, specifically nab-paclitaxel, gemcitabine, and folfirinox. sadly, chemotherapeutic agents exhibit significant toxicities, and patients frequently acquire chemoresistance, resulting in mortality within a year of commencing treatment (Goldstein et al., 2015a; Sadot et al., 2015a).

Considerable actions have been made towards the advancement of immunotherapeutic interventions for PanC . Immunotherapy is a cancer treatment approach that aims to counteract tumor-mediated immunosuppression by targeting immune response checkpoints. The ultimate objective is to enable the patient's immune system to eliminate cancer. Immunotherapy has demonstrated

efficacy against several types of cancer, such as melanoma, non-small-cell lung cancer, renal-cell cancer, and ovarian cancer(Brahmer et al., 2012; Postow et al., 2018). Nevertheless, despite checkpoint inhibitors' efficacy in treating a variety of malignancies, they have not proven as successful in treating Panc. PanC develops resistance not only to chemotherapy, but also to resistance but also inhibit anti-tumor immune activity, leading to significant resistance against immunotherapies such as Checkpoint blockers. This dual effect further restricts the available treatment options for patients. Immunotherapeutic agents have exhibited remarkable and long-lasting advantages in various types of cancer, although their efficacy has not been observed in PanC (Trials#NCT00112580 and NCT025274340). There presents a critical need for agents that can improve the sensitivity of PanC to chemotherapeutic and immunotherapeutic treatments, thereby leading to sustained clinical advantages for patients.

In this study, we demonstrated that CRC2631, a strain of Salmonella typhimurium that has been genetically attenuated, is capable of safely infiltrating and eradicating pancreatic tumors that KRAS drives in xenograft, and an establish Panc mouse model, KPC. Furthermore, our findings demonstrate that CRC2631 elicits activation of effector T-cells that are associated with tumors, resulting in a significant reduction in tumor burden relative to the control group. The study emphasizes the potential of bacterial cancer therapy (BCT) in achieving immune re-potentiation and re-sensitization of panC to IO.

4.3 Methods:

Cell Lines, Medium, and Culture - Murine Panc02-H7 Panc cells are an invasive cell line derived from Panc02(B. Wang et al., 2001). Panc02-H7 cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM) with 2 mmol/L L-glutamine, 10 mmol/L HEPES (ThermoFisher), supplemented with 100 U/mL penicillin (ThermoFisher), 100 mg/mL streptomycin (ThermoFisher), and 10% fetal bovine serum (Sigma-Aldrich) at 37°C with 5% CO_2 in a humidified atmosphere.

Mice – KPC (LSL-$Kras^{G12D/+}$; LSL-$Trp53^{R172H/+}$; Pdx-1-Cre) Mice were housed under standard conditions with a 12-hour light/12-hour dark cycle at the University of Missouri. Mice were maintained in individually ventilated cages (IVC) with access to water and a standard mouse diet (PicoLab Rodent Diet 20, 21% protein, 11.3% fat) *ab libitum*. All experiments with mice were performed under a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Missouri. All mice received humane care according to the criteria outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. To create the B6Panc02-H7 pancreatic orthotopic xenograft model, mice were anesthetized, and a left flank incision of 1.5cm was made to open the peritoneal cavity. The spleen was manipulated to visualize the pancreas, and $2.5x10^5$ Panc02-H7 cells in 20µl PBS were injected into the pancreatic tail. All organs were returned to the peritoneal cavity, the incision sutured, and skin clipped. Mice were allowed to recover for at least seven days before further manipulation.

In vivo fluorescent imaging - All mice were fed 2019 Teklad global 19% protein extruded rodent diets for 3 days before fluorescent imaging. This defined

diet excludes alfalfa chlorophyll, minimizing feed-related autofluorescence in the gastrointestinal system(Inoue et al., 2008). Up to $2.5x10^7$ $CRC2631_{iRFP720\text{-}cat}$ in 100 µl phosphate-buffered saline (PBS) (Rocky Mountain Biologicals) was introduced into mice by tail IV injection using 29G syringes (Monoject) on day eight (female B6Panc02-H7 group) or day ten (male B6Panc02-H7 group) post-xenograft implantation. In vivo, imaging was performed using a Xenogen IVIS 200 fluorescence system (Perkin-Elmer) 96 h after $CRC2631_{iRFP720\text{-}cat}$ introduction. To perform imaging, animals were handled in a biosafety cabinet where induction anesthesia and maintenance was delivered at 2.5-3% isoflurane in oxygen at approximately 1 L/min via a non-rebreathing inhalation anesthesia system). When anesthesia level was appropriate (verified via toe pinch), animals were transferred from the induction box to the nose cones on the imaging platform. Images were acquired using the predefined filters for the fluorescent agent being used in the study. Animals were removed and immediately sacrificed to collect pancreas, Panc02-H7 xenograft tumors, Liver, spleen, kidneys, lungs, and heart for ex vivo imaging, cryosection, and isolation of lymphocytes. Ex vivo fluorescent images of tissue samples were immediately taken after sacrifice. The fluorescent signal was analyzed using Living Image software (Perkin-Elmer).

Cyrosection and fluorescent microscopy - Cyrosection was performed by using a cryostat (Thermo Scientific HM525 NX Cryostat). Harvested tumors or organ tissues were embedded in OCT compound (Thermofisher Scientific) and put on dry ice as soon as possible. A 16um thick section of tissue was cut and placed onto slides using a cryostat. Tissues were stained with DAPI (Thermofisher).

Formalin (4%) was applied to fix these tissues for fluorescence imaging using a fluorescence microscope (Keyence, Itasca IL).

Isolation of mixed lymphocytes from pancreatic cancer and pancreas - Harvested tumors or tissues were cut into small pieces and incubated with enzymes, including 0.04% Collagenase IV (Sigma) at 37°C for 0.5 hours. Cell suspension was passed through a filter (mesh size 70um) and washed with GBSS (Sigma).

Isolation of spleen lymphocytes - Spleen was harvested from mouse and minced in DMEM (Thermo fisher scientific), filtered with 40um filter, and centrifuged at low speed to pellet cells. Cell pellets were incubated with RBC lysate buffer (Thermofisher Scientific), then centrifuged at low speed to collect pellet spleen lymphocytes.

Flow Cytometry - Immediately after harvesting, tumors were minced, followed by digestion in 0.04% collagenase IV (9001–12–1, Gibco) and 0.02 mg/mL DNase I (D5025, Sigma) in GBSS with shaking at 240 rpm for 45 minutes at 37°C. After digestion, samples were filtered using a 40μm strainer, then centrifuged. To lysis RBC, cells were placed in RBC lysis Buffer (555,899, BD at 37°C. Pharm Lyse) for 5 mins at 37°C, then centrifuged and resuspended 0.04% BSA in PBS with fluorochrome-labeled antibodies. Antibody-stained cells were analyzed by FACS using a flow cytometer (BD Biosciences). FlowJo software (Tree Star; https://www.flowjo.com/). The entire cell population was gated with cell debris, then gated for live cells(7AAD). Next, CD4 and CD8 markers distinguish CD4 from CD8 T cells and are gated for CD8 T cell activation or exhaustion.

Biodistribution analysis - B6 or B6Panc02-H7orthotopic xenograft mice were injected intravenously (tail vein injections) with up to 200 µl sterile PBS containing $2.5x10^7$ CRC2631$_{iRFP720\text{-}cat}$. Mice were euthanized 96 hours post-injection. Whole blood, liver, primary pancreatic tumors, and any discrete metastatic tumor masses were collected, weighed, and kept on ice. Whole blood samples were immediately diluted 1/10 in 25% glycerol and PBS and stored at −80°C. Tissue samples were homogenized in 3 mL sterile PBS for 20 seconds on ice using a TissueRuptor homogenizer (Qiagen) with sterile tips, mixed with 3 mL of sterile 50% glycerol, and stored at −80°C. All tissue samples were later thawed, passed through 40 µm sterile filters (BD Biosciences), 20 µl dilutions spotted in triplicate on selective LB +200 µg/ml thymine +20 µg/mL chloramphenicol plates, incubated at 37°C and enumerated after 24 h following the Miles and Misra method (Hedges, 2002).

Statistical analyses - All statistical analyses (Log-rank Mantel-Cox analysis of Kaplan-Meier curves, Student t-test test analyses of CRC2631 biodistribution and tumor weight differences) were performed using GraphPad Prism software (v6.0 h).

4.4 Results:

CRC2631 safely targets pancreatic cancer tissues - We recently showed that CRC2631, a genetically attenuated and stable PC cancer cells(Kazmierczak et al., 2020). We sought to assess to what extent CRC2631 tumor targeting capabilities translates to metastatic PanC , a disease that has limited therapeutic options. We first asked whether CRC2631 targets PanC tissues using an

orthotopic xenograft mouse model. In this model, intrapancreatic delivery of murine PanC cells (Panc02H7) into immune competent mice (BL6 strain) gives rise to pancreatic lesions and distant metastases that kill the animal within 3 weeks following implantation(X. Liu et al., 2019). To enable in vivo visualization of CRC2631 via live fluorescence imaging we introduced a fluorescence reporter (iRFP720). Additionally, a chloramphenicol resistance cassette was inserted downstream of the iRFP720 reporter, enabling us selectively isolate and quantify CRC2631 tissue load from harvested organs(Kazmierczak et al., 2020) The resulting strain CRC2631$^{iRFP720\text{-}cat}$ was used for tumor targeting studies. Tumor-bearing (B6Panc02-H7) or wildtype B6 mice were treated intravenously with saline (Phosphate-buffered saline/PBS) or $2.5x10^7$ colony forming units (CFU) of CRC2631 (*N*=20 animals/group; 10 males and 10 females). iRFP720 fluorescence imaging was performed at 4days following treatment to determine the tumor targeting capability of CRC2631$^{iRFP720\text{-}cat}$. CRC2631$^{iRFP720\text{-}cat}$ fluorescence was enriched in tumor bearing mice compared to non-tumor bearing wild-type controls (Fig. 1a). Further, immunohistochemistry assays showed that CRC2631$^{iRFP720\text{-}cat}$ was enriched in pancreatic tissue sections obtained from tumor-bearing animals compared to tumor-free animals (Figure 1b and 1C versus 1d and 1e). Furthermore, tumor tissues exhibited a higher CRC2631 burden compared to non-targeted tissues. Enumeration of CRC2631 from harvested organs under chloramphenicol selection showed significant enrichment of CRC2631$^{iRFP720\text{-}cat}$ per gram of pancreatic or metastatic tumor tissues compared to CRC2631$^{iRFP720\text{-}cat}$ counts in the Liver, a clearing organ (Fid. 1f). Compared to PBS controls, CRC2631

treatment did not cause animal lethality (Fig.1g), demonstrating that tumor targeted CRC2631 is well tolerated. Consistent with its safety and PanC cells versus non-cancerous pancreatic cells in MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) in vitro assays (Fig. 1h). Taken together, the above data indicate that CRC2631 safely targets pancreatic tumor tissues.

Tumor targeted CRC2631 stimulates tumor CD8 effector T-cells - CRC2631 tumor tropism suggested that tumor localized CRC2631 may convert PanC into an immunologically active milieu, resulting in a therapeutic effect. Thus, we used fluorescence-activated cell sorting (FACS) approaches to determine the extent to which CRC2631 stimulates effector CD8 T-cells in spleen and tumor tissues. During the study, we noted that male mice lived longer than their female counterpart in response to CRC2631 (see below). Therefore, male, and female mice were examined separately to detect any possible gender effect. We found that CRC2631 increased the frequency of activated ($CD69^+$) CD8 T-cells in the spleen of both male and female mice (Fig. 2a, 2e). However, we detected a gender effect in the exhaustion status of tumor-infiltrating lymphocytes: CRC2631 had no effect on the proportion of exhausted ($PD1^+$) tumor-infiltrating CD8 T-cells in females compared to PBS control animals (Fig. 2b), but it specifically reduced the proportion of $PD1^+/CD8^+$ T-cells in male pancreatic tumor tissues (Fig. 2f).

We further explored these effects in a syngeneic mouse model of PanC KPC [LSL-KrasG12D/+, LSL-Trp53R172H/+, Pdx-1-Cre)]. The KPC model recapitulates the genetic basis (oncogenic KRAS and p53 mutations), key immune aspects, clinical course, and drug response of PanC in human patients (Beatty et

al., 2011; Royal et al., 2010; Song et al., 2016). As such, it is widely considered as a predictive mouse model of PanC . We first sought to establish a safe CRC2631 dosing regimen in this model. KPC animals received a total of 4 doses of CRC2631 ($5x10^7$ or 10^8 or 2 $x10^8$ CFU) every 3 days via intraperitoneal (ip) or subcutaneous (sc) routes. Toxicity indicators such as treatment-induced animal distress, weight loss, necropsy macrohistological observations, and lethality determined $5x10^7$ CFU (c) to be the best tolerated dose (Fig. 3). Compared to 10^8 and 2 $x10^8$ CFU, $5x10^7$ CFU cause less weight loss. 10^8 and 2 $x10^8$ CFU had the highest weight loss of 10% among the groups (Fig. 3 a, c, e, f). Congruent to this, group treated with $5x10^7$ had a better survival compared to the other groups (Fig. 3 b, d, h).

Tumor targeted CRC2631 eliminates pancreatic cancer - The observations that CRC2631 targets and kills pancreatic tumor cells and stimulate effector CD8+ T-cells suggested a therapeutic potential for CRC2631. To directly test this, mice harboring PanC02H7 xenograft or KPC-induced pancreatic tumors were treated with saline (PBS) control or CRC2631 ($2.5x10^7$ or $5x10^7$ CFU). Whole pancreases were harvested and weighed 96hrs post-treatment termination to determine the effect of the treatment on tumor size. Consistent with the bias toward male specific potentiation of tumor-infiltrating CD8 T-cells in PanC02H7 xenografts, CRC2631 reduced the size of PanC02H7 pancreas by ~30% and extended animal survivorship in males but not in female mice (Fig. 3c, 3d females versus 3g, 3h males). As expected, anti-PD1 checkpoint blockade (invivomab) failed to reduce tumor weight (Fig. 4p). However, CRC2631 reduced the weights of these IO-resistant tumors (Fig.4p). In contrast to the sex effect of CRC2631 on

tumor weight response in PanC02H7 xenografts, CRC2631 reduced disease burden in both, male and female KPC mice to a similar extent. Thus, although CRC2631-mediated activation of CD8 T-cells is less pronounced in females compared to males, this response is sufficient to induce immune-mediated tumor elimination.

4.5 Discussion:

PanC remains one of the most lethal cancers in the world. Current standard of care chemotherapy is considerably toxic and inefficacious (Goldstein et al., 2015b; Sadot et al., 2015b). Although checkpoint blockade immunotherapies generate durable benefits in other cancers (Rizvi et al., 2015; Wolchok et al., 2013), it has been difficult to realize these much-needed benefits in PanC r. Only for ~1% of PanC patients with well-defined tumor genetic features (Trials# NCT00112580, NCT02527434) respond, further limiting treatment options for most Panc patients.

The limited efficacy of immunotherapies is due to the fibrous (hard to penetrate) and immunosuppressive nature of the PanC milieu. Breaking PanC's physical and immune barriers remain the most direct and likely path to generating durable clinical benefits for patients. Here we show that CRC2631 safely targets pancreatic tumor tissues. Tumor-targeted CRC2631 recruits and re-potentiates effector T-cells, resulting in elimination of pancreatic tumors. Thus, CRC2631 overcomes PanC physical and immune barriers.

CRC2631 tumor tropism is likely aided by cell surface bacteria/tumor cognate molecular interactions. It is well known that Salmonella targets tumor

tissues. The underlying molecular mechanisms are not fully understood but involves chemotaxis of salmonella toward chemo-attractants enriched in tumors (Kasinskas & Forbes, 2006). Auxotrophy for aromatic amino acids and thymine was introduced in *crc2631* (*aroA* transposon insertion and *thyA* deletion, respectively (Hoiseth & Stocker, 1981; Kok et al., 2001; Mir et al., 2015)). This auxotrophy contributes to crc2631 tumor targeting capability by favoring colonization of cancer cells where aromatic amino acids and thymine are abundantly present (Juhász et al., 2012; Platten et al., 2019; Puccetti et al., 2015). Additionally, we found that *crc2631* binds to mannose-linked terminal disaccharides surface glycoproteins 10- to > 400-fold more efficiently than to glycoproteins lacking mannose-linked terminal disaccharides (**data not shown**). These high-affinity glycoproteins are enriched on cancer cells (Chandrasekaran et al., 2006). Thus, cancer-specific surface molecules likely promote the selective entry of CRC263*1* into cancer cells.

In addition to direct killing of PanC cells, tumor localized CRC2631 likely eliminates pancreatic tumors by converting pancreatic tumors into an immunologically active milieu. It is known that Salmonella-infected tumor cells release chemokine and cytokines that enhance the recruitment and activity of tumor infiltrating CD8+ T-cells (Grille et al., 2014; Xu et al., 2022). Congruent with both direct and indirect cancer cell killing mechanisms, CRC2631 preferentially kills PanC cells in vitro in the absence of T-cells. In vivo, CRC2631 induces the release of immune chemokines [1] and increases the proportion of tumor-associated CD8+ T-cells in pancreatic tumors. Interestingly, CRC2631 immune re-potentiation

and tumor-size reduction capabilities are more robust in males than they are in females, highlighting gender-based difference(s) in how the host interacts with this biologic. One possibility is that the immune system of female mice is more efficient at mounting a defense against and clearing CRC2631 from the circulation following repeated challenges compared to males. As such, elevated clearance would limit the pool of CRC2631 available to stably colonize tumor tissues in females. Consistent with this, female mice are more efficient at clearing bacteria than males (Scotland et al., 2011) and we found that, rather than localizing to the tumor-bearing pancreas, CRC2631 is more enriched in the Liver of female mice compared to males following the fourth treatment (Fig. 4k, 4l versus 4g, 4h). The Liver plays a central role in the clearance of bacteria from the blood circulation (Broadley et al., 2016; Jenne & Kubes, 2013). Additionally, unknown female-specific factors in the tumor microenvironment may dampen the stimulatory effect of CRC2631 on effector T-cells in the PanC02H7 xenograft model, but not in the syngeneic and autochthonous KPC model, highlighting a fundamental difference between these two models.

The therapeutic effects of CRC2631 can be maximized by exploiting its tumor penetrating- and tumor-targeting capabilities to deliver immune potentiating agents (checkpoint inhibitors) specifically to the PanC milieu. Ideally, such agents would synergize with CRC2631 to generate tumor-localized maximum anti-cancer immune activities. Also, tumor-targeted delivery of these immune stimulating agents will potentially limit toxicities associated with systemic administration, contributing to the improvement of clinical outcome. A genetically attenuated strain

of Salmonella typhimurium expressing Interlukin-2 (Saltikva) recently received FDA fast-track designation for the treatment of metastatic PanCs following phase 2 clinical studies (NCT01486329), highlighting new opportunities ahead for salmonella-based cancer therapeutics.

Finally, recent achievements in KRAS-targeted therapy have been highly celebrated, as they should, especially in the PanC space. Over 90% of PanC (all stages) are driven by oncogenic genetic alterations in the KRAS gene. Because oncogenic KRAS mutations are associated with resistance to checkpoint blockade immunotherapy, KRAS-targeted therapies offered an opportunity to sensitize PanCs to immunotherapies. However, existing KRAS-targeted therapies are allele specific and benefit only a fraction of PanC patients, leaving most patients with no treatment option. Other pan-KRAS inhibitors are currently under development (NCT04111458, NCT04117087, NCT03592888), suggesting that more patients may benefit from this approach. However, emerging data indicate that cancers quickly acquire resistance to KRAS-targeted agents (Awad et al., 2021; Tanaka et al., 2021; Y. Zhao et al., 2021). Further, combination of KRAS-targeted therapy with checkpoint blockade caused severe toxicities in lung cancer(Brahmer et al., 2012), limiting the therapeutic prospect of this approach. There is a critical need for alternative strategies. CRC2631 also targets tumors in a mouse model of PC (Transgenic Adenocarcinoma of Mouse Prostate/TRAMP) caused by *p53* and *Rb* inhibition (Kazmierczak et al., 2020), independent of oncogenic KRAS. Together with our finding that KRAS targets and eliminates KRAS-driven PanCs, this

suggests that CRC2631 target tumors more broadly, not in a gene/allele-specific manner.

4.6 Figures:

Figure1

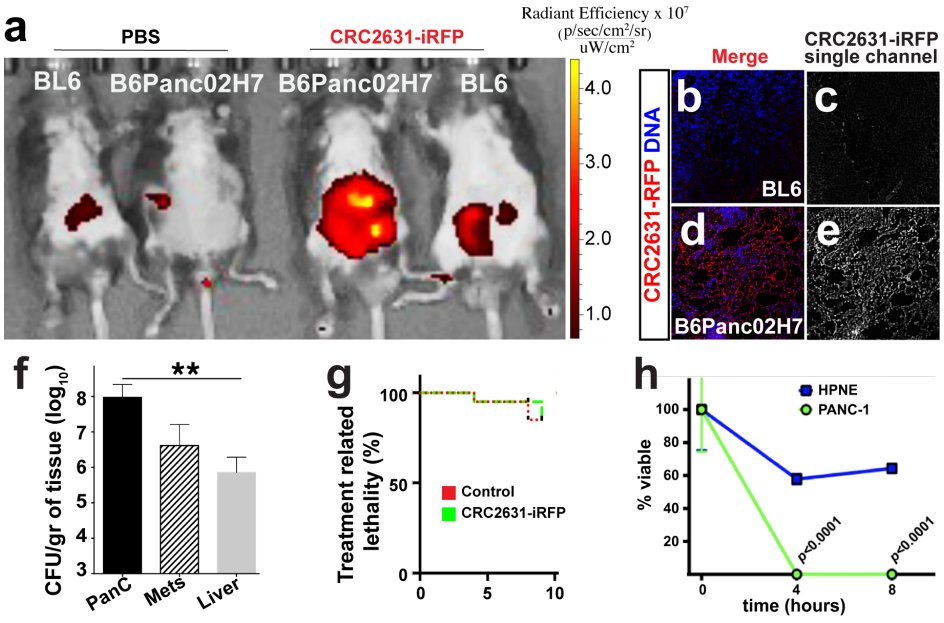

Figure 1. CRC2631 targets and eliminates PanC and extend survivorship in a Xenograft PanC Mouse Model. All C57BL/6J mice received orthotopic xenograft implantation of 2.5x105 Panc02H7 cells 10 days before treatment initiation (a) Groups (N=15) of tumor free (BL6) or PanC bearing animals (B6Panc02H7) were treated intravenously with saline control (PBS) or RFP-labeled CRC2631 (CRC2631-RFP, emit red fluorescence readily detectable in live animals). In vivo fluorescence imaging was performed 4days following treatment to assess the CRC2631 biodistribution. CRC2631-RFP signals were enriched in tumor-bearing B6Panc02H7 mice (red fluorescence. The yellow signal represents signal saturation, indicative of high CRC2631 load). Much less CRC2631 was detected in tumors-free BL6 animals (forth animal) where signals were slightly

above background (compared to 1st and second animal from the left). Bright to dark red signal = decreasing CRC2631-RFP load. (b-e) CRC2631-RFP is enriched in pancreatic cryo tissue sections obtained from tumor- bearing animals (b, d) compared to tumor-free controls (b, c). (f) Quantitative biodistribution of CRC2631-RFP (count per gram of tissue) from the indicated harvested tissues shows that CRC2631 is enriched in B6Panc02H7 tumor tissues compared to the non-specific clearance organ the liver (**=$p<0.0077$) eight days after treatment. CRC2631 shows a similar trend in liver and lung metastases. (g) Lifespan of male B6Panc02H7 mice significantly increased (N=10, $p<0.0375$) as a function of CRC2631 (green) treatment (4 doses of 5x107 CRC2631, three days apart, administered (tail IV) at 0, 3, 6, 9 days post treatment initiation) versus untreated (red) PBS carrier volume control. (h) CRC2631 preferentially kill PanC cells. MTT ([3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] assays rely on the reduction of MTT by mitochondrial dehydrogenases to Formazan, a reliable measure of cellular metabolic function and viability. The abundance of formazan is determined spectrophotometrically and is indicative of relative cell viability between treatment conditions. Normal pre- cancerous (HPNE) versus cancerous pancreatic (PANC-1) cells were treated with CRC2631 using a 4:1 bacterial to human cell ratio or multiplicity of infection/MOI. CRC2631 selectively kills all hTERT-HPNE cells 4hours after treatment and this effect perdured 8hours after treatment at the indicated MOI. In contrast, CRC2631 killed ~40% of the control cells and the proportion of viable cells began to increase by 8hours post- treatment, suggesting recovery.

Figure 2

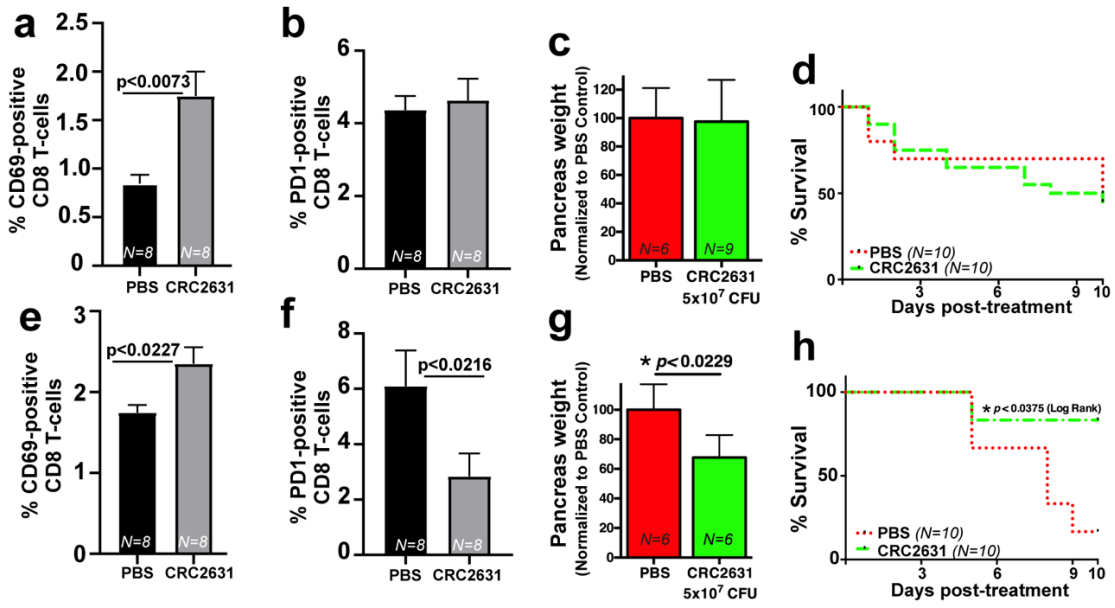

Figure 2. CRC2631 Immune response in the B6Panc02H7 orthotopic xenograft pancreatic cancer model. B6 mice received orthotopic xenograft implantation of 5x105 Panc02H7 cells 10 days before treatment initiation. (c) CRC2631 treatment of Panc02H7 significantly reduced (N=6, p<0.0229) primary pancreatic tumor weight burden in male B6Panc02H7 mice 10 days after start of treatment versus untreated control. (d) Lifespan of male B6Panc02H7 mice significantly increased (N=10, p<0.0375) as a function of CRC2631 (green) treatment (4 doses of $5x10^7$ CRC2631, three days apart, administered (tail IV) at 0, 3, 6, 9 days post treatment initiation) versus untreated (red) PBS carrier volume control. (g) tumor burden reduction (N=9, $5x10^7$ CRC2631 treatment; N=5, no treatment) was not observed in female B6Panc02H7 mice under the same treatment conditions. (h) Life extension {N=20, $5x10^7$ CRC2631 treatment (green); N=10, untreated (red)

Figure 3

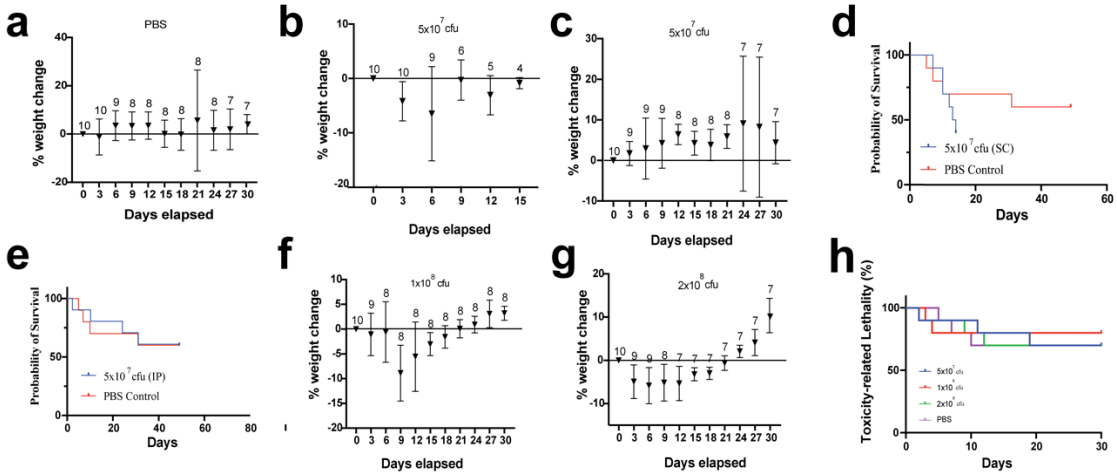

Figure 3. Comparative CRC2631 toxicological assessment

(a-h) 19 to 21 weeks old KPC mice were treated with PBS (controls) or escalating doses of CRC2631 administered subcutaneously (sc) or intraperitoneally (ip). Baseline animal weights were recorded before the study and daily thereafter. Surviving animals were recorded daily (numbers shown above standard deviation bars every 3 days). Mean percentage weight change of KPC mice treated with PBS (a) or 5 doses of CRC2631 5×10^7 CFU CRC2631 administered sc (b) or ip (c). The corresponding Kaplan Meier survival analysis graphs are shown in d and e. (e-f) Mean percentage weight change of KPC mice treated ip with increasing doses of CRC2631 [10^8 (e) or $2x10^8$ (f)] administered ip every 3 days for a total or 5 doses. (h) Graph showing Kaplan Meier survival analysis from animals in e-g above.

Figure 4

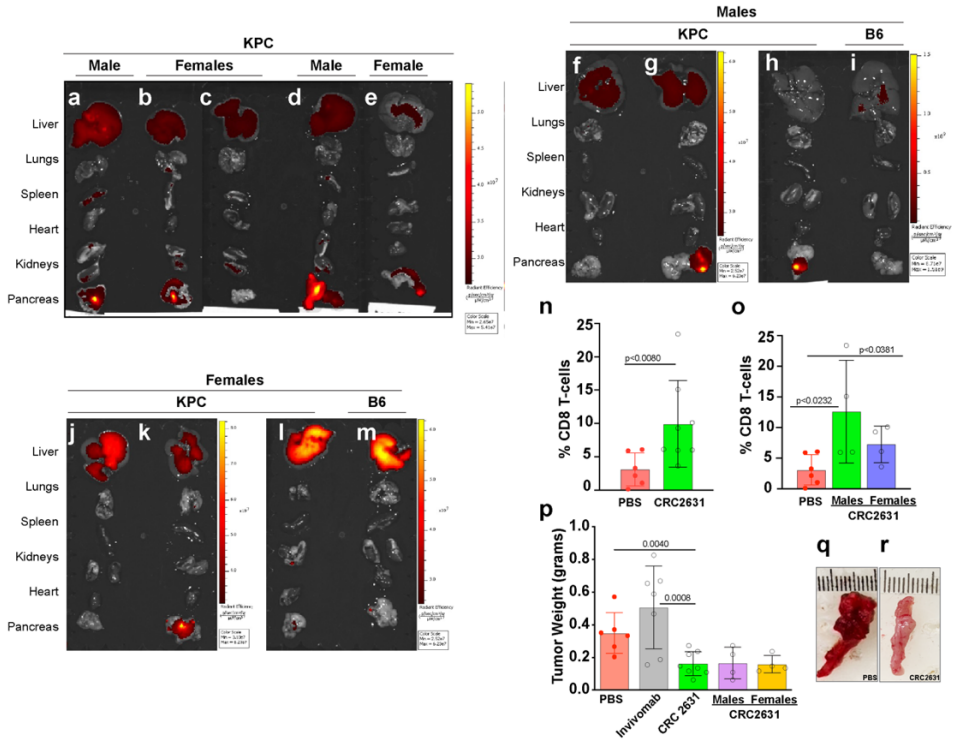

Figure 4. CRC2631 targets pancreatic tumor tissues, stimulates effector T-cells, and reduces tumor burden.

(a-m) Images showing the biodistribution of CFC2631-RFP in harvested organs 24hours after treatment with a single bolus (a-e) or after a total of 4 doses ($5x10^7$ CFU) administered every 3 days (j-i). (n, o) Graphs from flow cytometry analyses showing the frequency of CD8-positive T-cells in pancreases harvested from PBS controls (N=6 animals) or KPC mice treated with CRC2631 ($5x10^7$ CFU administered intraperitoneally every 3 days for a total of 5 doses, N=8). Pancreases were collected when half of the animal in the group experienced lethality (LD50). Error bars represent standard deviation, and the p-values were derived from one-way ANOVA. (p-r) Evaluation of the therapeutic effect of CRC2631treatment. Pancreases were harvested from the animals above and weighed. Additional pancreases were derived from KPC mice treated with antibodies against murine PD-1 (500µg/animal, administered intraperitoneally every 3 days for a total of 4 doses). Representative images of the pancreases from PBS control or CRC2631-treated mice are shown in q and r, respectively.

CHAPTER 5- CONCLUSION

Cancer drug resistance represent a significant challenge in the management of cancer patients. The underlying molecular mechanisms are complex and include non-genetic drivers. In this dissertation I provided new insights into genetic and non-genetic mechanisms of drug resistance in the context of targeted therapy: androgen or epidermal growth factor receptor directed agents in prostate or lung cancer, respectively.

In Chapter two, I delineated a dual role for PSMA2 in prostate cancer resistance to androgen deprivation therapy: on the one hand, treatment dependent transcriptional upregulation of PSMA2 sensitizes prostate cancer cells to residual androgen. On the other hand, PSMA2 promotes neuroendocrine transcriptional state in prostate cancer, contributing to the emergence of a drug resistance histological subtype. Consistent with my working model, Pharmacological or RNAi-mediated inhibition of PSMA2 suppresses the transcriptional activities of neuroendocrine genes and correspondingly sensitize neuroendocrine prostate cancer cells to androgen therapy. PSMA2 plays a central role in the neuroendocrine fate acquisition: ectopic expression of PSMA2 in prostate adenocarcinoma cells was sufficient to trigger the emergence of neuroendocrine features. I proposed that treatment-induced PSMA2 lowers AR signaling threshold required for cancer progression. HSP90 normally inhibits AR activity by sequestering AR in the cytoplasm. My data suggest that PSMA2 antagonizes the formation of HSP90/AR protein complex, resulting in accelerated AR nuclear translocation and activity in the presence of residual androgen.

The role of PSMA2 in the modulation of AR signaling and neuroendocrine state transformation in prostate cancer cells can be mediated via the known function of PSMA2 in proteosome function or via a novel proteosome-independent mechanism. To begin to distinguish between these two possibilities, I will first determine to what extent targeting of the proteosome via independent pharmacological inhibitors (MG132 or Carfilzomib) mimic the ADT sensitization effect of PSMA2 RNAi knockdown or Bortezomib treatment. Second, subcellular protein localization studies will test a proteosome-independent role for PSMA2 in controlling HSP90 subcellular distribution in prostate cancer cells. Finally, it is possible that PSAM2 role in ADT resistance is mediated via enhanced degradation of HSP90, which can readily be tested in western blotting assays.

Consistent with PSMA2 playing an important role in ADT resistance in neuroendocrine prostate cancer, Bortezomib treatment significantly prolonged animal life in a mouse model of neuroendocrine prostate cancer (TRAMP). These findings suggest the potential to enhance PC response to ADT and increase survival outcomes for patients.

In Chapter 3, through a combination of EV transcriptomics, signaling pathway analyses, we revealed that Hsa-miR-22-3p, Hsa-miR-184, and Let-7b-5p functionally converged on the WNT/β-catenin and mTOR/AKT signaling pathways, both of which are known drivers of cancer drug resistance. Utilizing pharmacological approaches in a cell culture environment, I demonstrated that targeting Hsa-miR-22-3p and Hsa-miR-184 molecules effectively desensitized NSCLC cells harboring EGFR mutations (T790M, L578R) to Osimertinib. These

findings highlight that drug resistance in lung cancer cells stems from complex non-mutational mechanisms involving emergent intercellular communication facilitated by EV microRNAs (miRNAs). I cannot rule the possibility that distinct mutations may also cooperate to elevate these EV-mediated drug resistance processes.

In Chapter 4, I discussed our ongoing efforts to develop a strategy to achieve broader and more durable benefits for patients irrespective of the tumor mutational landscape. I turned to a KRAS-driven pancreatic cancer mouse model and evaluated the tumor targeting and therapeutic capabilities of genetically engineered salmonella bacteria (CRC2631) that has been shown to target and kill cancer cells harboring RB and P53 dysfunction in prostate cancer setting. I demonstrated that CRC2631 safely infiltrate and eradicate KRAS-driven pancreatic tumors. Notably, CRC2631 activates cancer-killing effector T-cells, resulting in a significant reduction in tumor burden compared to the control group. These findings highlight the potential of bacterial cancer therapy (BCT) in achieving immune re-potentiation and re-sensitization of PanC to potent immunotherapy. Disarming cancer drug resistance mechanisms will undoubtedly require a shift in research paradigm.

In summary, these findings have significant implications in the field of cancer and drug development, as they provide critical insights into potential therapeutic targets for overcoming drug resistance in cancer.

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VITA

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