

**Hedgehog Pathway in Prostate Cancer Progression: A Novel Therapeutic
Approach to Ongoing Adaptive Responses**

by

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Abstract

Prostate cancer is the most commonly diagnosed cancer in North American men. While ever-improved androgen ablation therapies prolong life in men with advanced disease, remissions are temporary. Identifying targetable pathways underlying castration-resistant progression is essential for improving survival of patients with advanced disease. Hedgehog signalling may be one such pathway. The central hypothesis of this thesis examines the importance of adaptive responses during progression of prostate cancer to castration resistance and subsequent metastasis by reactivating developmental cues. We demonstrate that key Hedgehog pathway regulatory proteins are elevated in advanced prostate cancer compared to benign and untreated. This over-expression of Hedgehog signalling may be an adaptive response, which leads to a transition from a paracrine to autocrine Hedgehog signalling modality. Inhibition of this pathway disrupts progression of androgen sensitive, LNCaP tumours to castration-resistant prostate cancer. This inhibition has no effect on viability or key Hedgehog pathway regulators *in vitro* and no change in tumour specific Hedgehog genes *in vivo*, yet by examining host specific murine GLI1, 2 and PTCH1, key regulators of the Hedgehog pathway, we observe significant inhibition. These results suggest that Hedgehog expression promotes castration-resistant prostate cancer progression through reciprocal paracrine signalling within the tumour microenvironment. However, inhibiting the Hh pathway in our castration-resistant prostate cancer models induced down-regulation of the downstream Hh targets, *in vitro*. This inhibition hinders the migration and invasion of these cell lines *in vitro* and abrogates their invasive potential *in vivo*. These results taken with data from our prostate cancer patient cohort that show a significantly higher recurrence rate for patients with elevated SHH and GLI2 levels suggests a higher metastatic capacity driven by ligand-dependant autocrine Hedgehog

signalling. Finally, we examine the role of Hedgehog signalling inhibition in sensitizing advanced prostate cancer to palliative therapeutic modalities. Smoothed antagonist in combination with docetaxel demonstrated significant reduction in tumour growth of both LNCaP recurrent and PC3 models, compared to single therapy. These results demonstrate the integral role of Hedgehog signalling as prostate cancer tumours adapt to castration and subsequent therapeutic modalities. Targeting these adaptive responses becomes an integral part of novel cancer therapy in prostate cancer.

Preface

The body of work presented in this thesis is based on one of my PhD projects relating to the hedgehog pathway.

The publications presented in this thesis are based on the work that I carried out towards the completion of my PhD program. Manuscripts listed in this thesis either have already been published or will be submitted for publication as co-authored works.

Chapter Two: The design of all the experiments were carried out by Dr. Cox and myself. The preliminary work and the *in vitro* experiments were performed by Dr. Naokazu Ibuki and myself. The *in vivo* xenograft models were performed by Dr. Naokazu Ibuki and the analysis were performed by Dr. Naokazu Ibuki and myself. The immunohistochemistry of patient samples were performed with the help of Dr. Ladan Fazli. A version of Chapter 2 has been published in the International Journal of Cancer.

TAK-441, a novel investigational SMO antagonist, delays castration-resistant progression in prostate cancer by disrupting paracrine hedgehog signalling. Naokazu Ibuki*, Mazyar Ghaffari*, Mitali Pandey, Irene Iu, Ladan Fazli, Masahide Kashiwagi, Hideaki Tojo, Osamu Nakanishi, Martin E. Gleave and Michael E. Cox. Int. J. Cancer: 133, 1955-1966 (2013).

Chapter Three: The design of the all the experiments were carried out by Dr. Cox and myself. The preliminary work as well as all the *in vitro* experiments were carried out by myself with the help of Irene Iu. Dr. Naokazu Ibuki performed the LNCaP CRPC xenograft study and I performed the PC3 CRPC xenograft *in vivo* experiment. With the help of Dr. Ladan Fazli, prostate cancer patient specimens were immunohistochemically stained for Hedgehog pathway proteins.

The CAM assay experiments were performed by Dr. Hon Leong. All the results in this chapter were analyzed by myself. A version of Chapter 3 will be submitted for publication.

I have also included a list of my other major projects during my PhD, with associated publications:

Insulin-like growth factor pathway modulation through insulin receptor substrate and insulin-like growth factor binding protein inhibition as well as growth hormone disruption:

Ibuki N*, Ghaffari M*, Reuveni H, Pandey M, Fazli L, Azuma H, Gleave M.E, Levitzki A, Cox M.E. The tyrosine kinase inhibitor, NT157, suppresses insulin receptor substrates and augments therapeutic response of prostate cancer. *Molecular Cancer Therapeutics*. 2014 Sep 29.

Takahara K*, Ibuki N*, Ghaffari M, Tearle H, Gleave ME, Pollak M, Cox ME. The Influence of Growth Hormone/Insulin Like Growth Factor Deficiency in Prostatic Dysplasia in pARR2-CRE, PTEN Knockout Mice. *Prostate Cancer and Prostatic Disease*. 2013 May 21

Takahara K, Tearle H, Ghaffari M, Gleave ME, Pollak M, Cox ME. (2011) Human prostate cancer xenografts in lit/lit mice exhibit reduced growth and androgen-independent progression. *Prostate* 71(5):525-37.

The role of ERG translocation in carcinogenesis and epithelial to mesenchymal transition:

Becker-Santos D*, Guo Y*, Ghaffari M, Vickers ED, Oloumi A, Furukawa J, Wang Y, Nelson PS, Knudsen B, Dedhar S, Cox ME. ERG expression in prostatic epithelial cells induces ILK-dependent EMT and anchorage-independent growth. *Carcinogenesis* vol.33 no.12 pp.2558–2567, 2012

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List of Abbreviations

AD	androgen dependent
ADT	androgen deprivation therapy
AR	androgen receptors
ARE	androgen response elements
BPH	benign prostatic hyperplasia
BRT	brachytherapy
BW	body weights
CAM	chorioallantoic membrane
CIM	cell Invasion and Migration
CRPC	castration-resistant prostate cancer
DHH	desert hedgehog
DHT	dihydrotestosterone
DMSO	dimethyl sulfoxide
DRE	digital rectal exam
EBRT	external beam radiation therapy
EGF	epidermal Growth Factor
EMT	epithelial-mesenchymal transition
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GLI	glioma-associated oncogenes
GS	Gleason scores
H&E	haematoxylin and eosin
Hh	hedgehog
HIP	hedgehog-interacting protein
HIF	Hypoxia-inducible factor
IHH	Indian hedgehog
IL	Interleukin
LH	luteinizing hormone

LHRH	luteinizing hormone-releasing hormone
MAPK	mitogen-activated protein kinase
NE	neuroendocrine
NHT	neoadjuvant hormone therapy-treated
PAP	prostatic acid phosphatase
PCa	prostate Cancer
PCA3	Prostate cancer antigen 3
PIN	prostatic intra-epithelial neoplasia
PSA	prostate specific antigen
PTCH1	Patched 1
ROI	region of interest
RP	radical Prostatectomy
RT	radio-Therapy
RTCA	real-time cell analysis
RTKs	receptor tyrosine kinases
SHH	sonic hedgehog
SMO	smoothened
SUFU	suppressor of fused
TMA _s	tissue microarrays
TURP	transurethral resection of Prostate
UGM	urogenital sinus mesenchyme
UGS	urogenital sinus
VEGF	vascular endothelial growth factors

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Chapter 1: Introduction

1.1 The Prostate

1.1.1 Location and Function

The prostate is a fibromuscular exocrine gland specific to male genitalia. It surrounds the urethra, located inferior to the bladder and anterior to the rectum, between the bladder and the genitourinary membrane (Figure 1-1). At birth, the size of the prostate in humans is approximately 1-2 grams that increase to approximately 20 by 25-30 years of age [1, 2]. The main function of the adult prostate is as an accessory sex gland that plays a critical role in the mammalian male reproductive system by contributing about 25-30% of the volume of seminal fluid. Secretions from the seminal vesicle comprise the majority of seminal plasma and provide amino acids and sugars necessary for the survival of the sperm as well as phosphorylcholine and prostaglandins that protect the sperm from the vaginal immune response [1, 3]. As an exocrine gland, the prostate produces and secretes many of the components involved in coagulation, gelation and liquefaction to facilitate the female egg fertility. This secretion adds proteolytic enzymes, including prostate specific antigen (PSA), fibrinolysin, acid phosphatases and zinc to the seminal plasma. The addition of these alkaline fluids protects the spermatozoa from denaturation in the acidic environment of the vaginal canal and liquefies semen to enhance motility [1, 4, 5]. In addition, the prostate has a highly developed smooth muscle stromal compartment that functions as a secondary urinary sphincter which controls urine output and the transmission of seminal fluid during ejaculation [1].

1.1.2 Prostate Development

An adult human prostate is composed of three zones: transitional, central and peripheral [6-8]. The transitional zone is the innermost and smallest component of the prostate, immediately surrounding the urethra, which is the main site of the development of benign prostatic hyperplasia (BPH) and less commonly, adenocarcinoma. The central zone is the next region, making up approximately 25% of the gland, and the peripheral zone is located closest to the rectum, making up approximately 70% of the prostatic volume and comprises all the prostatic glandular tissue at the apex of the gland as well as all of the tissue located posteriorly near the capsule. It has been reported that the incidence of carcinoma, chronic prostatitis and post-inflammatory atrophy are relatively higher in the peripheral zone compared with the other zones (Figure 1-2) [9-11].

Development of the prostate gland is a result of epithelial invaginations of the posterior urogenital sinus (UGS) [12, 13]. This process happens under the direct influence of the underlying mesenchyme during the third fetal month [4, 12, 13]. UGS develops into the prostate, prostatic urethra and bulbourethral glands in males, the lower vagina and urethra in females, and the bladder in both [14].

The prostate gland is recognizable at week 10 of the embryonic stage [9] and its development initiates when androgens, testosterone and dihydrotestosterone (DHT), secreted from testis act on androgen receptors (AR) found in the urogenital sinus mesenchyme (UGM) stimulate epithelial budding as well as proliferation and differentiation of the prostatic bud to form ductal structures. [15-17]. The development of prostate gland is therefore, determined by androgen exposure rather than by genetically defined sex of the fetus. Therefore, the UGS of either sex fetus could develop into functional prostatic tissue if stimulated by androgen [15, 16]. In normal prostate, AR signalling within prostate stromal cells induces secretion of paracrine factors, termed

andromedins, which stimulate growth of the epithelial cells. In response to a variety of andromedins produced by the developing prostatic mesenchyme, the epithelial cells differentiate into the various cell types of the prostate, while proliferation and differentiation of the UGM forms prostatic smooth muscle and fibroblasts [9, 18].

Cunha et al. have shown that the interaction between the stroma and epithelial compartments of the prostate gland plays a major role in the normal prostate development [19]. Their experiments demonstrated that testosterone acts through the AR in the UGM to induce the development and differentiation of the epithelial compartment of the prostate [19, 20]. Conversely, the developing epithelium induces the differentiation of the primitive mesenchymal cells into the smooth muscle cells [19, 21]. In the absence of epithelial compartment the UGM will not form smooth muscles [19].

1.1.3 Histological and Cellular Makeup of the Prostate

Prostate epithelium is composed of three types of cells: columnar secretory luminal, basal, and the infrequent neuroendocrine (NE) [12, 22]. Secretory luminal epithelium are differentiated cells forming the exocrine component of the gland responsible for the production of enzymes such as PSA, prostatic acid phosphatase (PAP) and human kallikrein-2 that are secreted into the glandular lumen [7, 22]. Secretory cells express AR at high levels and low molecular weight cytokeratins 8 and 18 and require testosterone for maintaining their secretory activity [7, 23, 24]. The basal cells are non-secretory cells that constitute the proliferative component of the prostate epithelium and are responsible for the self-renewing of the gland. These cells are androgen independent and are characterized by the expression of cytokeratins 5 and 14. They play a role in modulating the endocrine and paracrine functions of the gland [24, 25]. NE cells are characterized

as non-proliferative and androgen-independent [26, 27]. They can be characterized by the expression of the neuropeptides such as chromogranin A and calcitonin and have been attributed with regulating glandular homeostasis [28].

Prostate stroma consists of fibroblast, smooth muscle and endothelial cells. Fibroblasts produce extracellular matrix and collagen as part of the connective tissues of the stroma, smooth muscle cells which are composed of non-striated muscle fibers allow for glandular flexibility, and endothelial cells help supply vascularization to the entire gland. Through the interaction with the epithelial compartments, these cells create a nutrient-rich androgen-sensitive microenvironment [29].

1.1.4 Androgens

Androgens are gonadal steroid hormones involved in development and maintenance of primary and secondary sex characteristics in males. Testosterone is the major circulating androgen in males, secreted by the testis, under the control of the hypothalamo-pituitary-gonadal axis. It is synthesized from cholesterol through a series of enzymatic steps by the testicular Leydig cells [30-32]. The majority of this secreted testosterone is bound to albumin and sex-hormone-binding globulin, leaving only about 2% in the active unbound form [2]. It is only this unbound form that can be taken up by target cells and converted into its active intracellular form, DHT by the enzyme 5α -reductase, in the cells of the reproductive tract, testes and ovaries, skin, and prostate [33].

Androgens play a crucial role in the control of prostate gland growth at the time of puberty and in maintaining it in maturity. Growth of the prostate gland during puberty includes both the stromal and epithelial elements of the prostate [34]. The binding of DHT to AR induces a conformational change in the receptor resulting in disassociation from AR-interacting heat shock

proteins and subsequent dimerization and translocation of the receptor from the cytoplasm to the nucleus. Binding of AR homodimers to androgen response elements (ARE) in conjunction with co-activator and co-repressor proteins, alter transcription of AR target genes such as PSA [35, 36]. Many of the genes regulated by the AR are critical for the development and maintenance of the prostate. However, of the components of the prostatic epithelium, the secretory luminal cells are the only androgen responsive component [15].

1.1.5 Prostatic Diseases

The main pathologies associated with prostate are prostatitis, benign prostatic hyperplasia (BPH) and prostatic intra-epithelial neoplasia (PIN) leading to prostate cancer (PCa). Prostatitis is an inflammatory disease of the prostate that can become a chronic condition with symptoms such as pelvic pain and urinary problems, mainly in aging men [37]. There has also shown to be a correlation between prostatitis and sexually transmitted disease with an increased risk of PCa [38].

BPH is the most common prostatic disease and is caused by an increase in the size of the prostate, due to prostatic stromal hypertrophy alone or in conjunction with the epithelial compartment. BPH is androgen dependent (AD) and results in the formation of expanding nodules in the transitional zone of the prostate, which can compress the urethra causing urinary problems. BPH is not believed to be associated with the development of malignant of PCa [39, 40].

PIN, is considered the precursor lesion that can progress to malignant PCa. PIN results from increased proliferation and anaplasia prostatic ducts and acini in the peripheral zones of the prostate and is classified into low and high grade. High grade PIN possesses many of the morphologic and phenotypical traits of cancer and hence considered the earliest stage of PCa. As

it progresses, there is an increased disruption of the basal cell layer of the prostatic ducts and stromal invasion that results in invasive carcinoma development [41].

1.2 Prostate Cancer

1.2.1 Epidemiology and Risk Factors

PCa is the most common cancer and the third leading cause of cancer-related mortality in men worldwide. Based on the 2015 Canadian Cancer Statistic annual report, 1 in 8 males will be diagnosed with PCa with 24,000 new cases and 4,100 deaths from this disease in 2015, which equates to 24% of all new male cases and 10% of all cancer deaths respectively [42]. There are several predictive risk factors associated with PCa onset and progression including age, ethnicity, family history, genetic predisposition, environment, lifestyle, diet and obesity [9, 43]. Genetics based research evidences have demonstrated that male relatives of PCa and breast cancer patients have a higher chance of developing the disease [44-47] this risk was also increased two- to three-fold for first degree relatives of PCa patients [48].

Age is the number one risk factor associated with PCa. Studies on autopsies have shown that even by the age of 20, around 10% of men have PCa cells in their prostate gland [49, 50]. The risk increases to about 30% by the age of 50 and 80% by the age of 80 [49-52]. In Canada, most deaths from PCa occur in the 80 plus age group, while it is diagnosed most frequently in ages 60-69 years [42]. This strong correlation suggests that accumulated genetic damage, such as oxidative stresses and environmental risk factors over the course of one's life, has a critical role in the development of the PCa [53].

Family history of cancer, specifically PCa, has been considered a significant risk factor. Genetics based researchers have revealed that male relatives of PCa and breast cancer patients

have a higher chance of developing the disease. This risk was also increased 2.41 fold for first-degree relatives of PCa patients [54, 55]. This risk is increased if the cancer is diagnosed in family members at a younger, less than 55 years, age or if it affected three or more family members.

The incidence of PCa varies greatly geographically and based on ethnicity. United States has the highest rate of PCa, specifically in the African-American population (137 per 100,000 per year) followed by Canada and Western Europe, with the lowest incidence seen in Asia (1.9 per 100,000 per year in China) [56, 57]. These differences, although not fully understood, could be attributed to ethnic genetic variations that are compounded by environmentally induced epigenetic changes that may be brought upon through change in factors such as diet and life style. It is also critical to account for differential use of screening and detection methods, in addition to discrepancies in reporting and documentation. Interestingly, PCa incidence in immigrants tends to shift towards their host country and enhances with length of stay. This strengthens the role of diet, as well as environmental exposures in the incidence of this disease [53].

Several studies emphasize the role of diet and weight in PCa initiation and the role of obesity in modulating PCa progression [58, 59]. Obesity affects the level of insulin and insulin-like growth factor 1(IGF-1). There are many studies linking high serum IGF-1 levels to development of high-grade PIN and risk of PCa [60]. Total dietary fat consumption seems to have an impact in PCa development, progression and mortality [50, 61].

1.2.2 Prostate Cancer Development

Tumourigenesis is a multistep process that results in the transformation of normal cells to highly malignant derivatives via a series of premalignant steps to invasive cancer [62, 63]. A combination of events has to occur that directs the cell to evolve from normalcy to malignancy.

These cells must become self-sufficient in growth signals, insensitive to anti-growth signals, able to evade apoptosis, able to invade surrounding tissues, gain sustained vasculature and acquire limitless replication potential [62, 63].

More than 90% of prostate cancers are adenocarcinoma and arise from the epithelial compartment [40]. Although the causes of PCa are not yet fully understood, PCa along with other kinds of malignancies may arise as a consequence of genetic alterations in stem cell populations. It is hypothesized that cancer stem-like cells are responsible for onset, progression and relapse of malignancy. An epithelial stem cell destined for differentiation along a basal, luminal, or neuroendocrine lineage can be affected by the stromal microenvironment to undergo differentiation into a malignant cell. Disruption in the epithelial-mesenchymal equilibrium appears to be a critical step in the development of PCa [64, 65]. This, along with the classical view of altered growth factors and proteases, which triggers a reduction in smooth muscle content followed by induction of fibroblasts in the stroma and upon AR activation paracrine stimulation of epithelial stem cells results in the onset of a malignant phenotype [65, 66]. This aberrant signalling leads to changes in tissue architecture, boundaries, angiogenesis, and metastases that is characteristic of a progression to malignancy [63]. PCa is multifocal and heterogeneous cancer in nature with as many as 5 or 6 tumours occurring in a single prostate [67, 68]. One single epithelial stem cell can differentiate and metastasize into multiple heterogeneous cancer cells that combine to create a PCa tumour [22].

1.2.3 Clinical Presentation and Diagnosis

PCa initiates as a single focus, or several foci, of cancerous cells that are asymptomatic and in most cases latent [69]. This dormant stage may lead to an active disease and further growth and

progression of the cancer. Local symptoms such as increased frequency and difficulty in urination are due to the pressure of the enlarged prostate onto the bladder neck or urethra. If PCa is undiagnosed, rectal invasion, renal failure secondary to ureteral obstruction, and impotence as well as metastasis to local lymph nodes and subsequently fractures from bone metastasis will be rare and late manifestations of highly advanced local disease [70, 71]. In most cases, the initial symptoms are due to PCa metastasis. Pelvic lymph nodes and bones are the most common metastatic sites. In fact, over 84% of patients with advanced disease show skeletal metastases [72]. Involvement of bone consistently produces significant symptoms such as pain or pathologic fracture at metastatic sites. Incidental discovery during prostate biopsies and autopsies have shown that the majority of aging men will develop microscopic foci of prostatic disease, yet only a small subset will develop into an invasive disease [69, 70].

PCa diagnosis continues to be an area of widespread interest and study, debate, and controversy. In spite of extensive research on PCa, the natural history of this disease is poorly understood. This disease is remarkably heterogeneous and varies from clinically silent, indolent and non-metastatic to an aggressive and metastatic form that causes morbidity and mortality. Early diagnosis, as well as the ability to distinguish between the indolent and aggressive forms of PCa is integral in our ability to tailor treatment options and improve prognosis of this disease.

Currently PCa is screened through abnormal digital rectal exam, elevated serum PSA, and is diagnosed by prostate biopsy. In order to determine whether the prostate is enlarged or contains any abnormal cancerous nodules, a physician performs DRE by inserting their finger into the anus of the patient and feeling the general morphology of the prostate. Abnormal lumps are often indicative of PCa nodules [73]. A DRE could miss a substantial proportion of PCAs, is poorly reproducible and subjective, with high inter-examiner variability [74], and may not be ideal as a

screening tool for detection of low-grade PCa [75]. No controlled studies have shown decreased morbidity or mortality of PCa when detected by DRE [76]. Nevertheless, it is a clinically important part of physical examination that is useful in PCa detection and staging [71]. One of the limitations of this test is that many cancer foci are located in regions that evade digital palpation [40]. PSA combined with DRE appears to be the most effective screening tool for early detection of PCa, however, in the case of PSA testing, the important adverse effect of screening has been shown to be the over diagnosis and overtreatment of the patients [40, 77].

PSA is a member of the kallikrein-related family and is produced in the prostatic luminal secretory epithelial cells and secreted into seminal fluid, with normal concentrations of around 1 to 5 mg/ml. These values are approximately 1 million times greater than normal serum level of less than 4 ng/mL [78]. Increased PSA is often indicative of prostatic disease, including PCa and BPH, or inflammation. PSA serum levels can vary quite a bit between healthy individuals and those with prostatic disease, so it is a combination of serum concentration and the change over time that is diagnostically important. Historically, PSA measurements of over 4 ng/ml and a PSA increase of 0.75 ng/ml/yr have generally been considered ideal indicators for requesting prostate biopsies to be performed [79, 80].

Many non-prostatic tumours also express detectable PSA including breast and ovarian cancers [81, 82], as well as, colon and liver carcinomas [83]. This alongside the fact that PSA can't differentiate between PCa and BPH and could be elevated in response to factors and activities unrelated to PCa such as sex, ejaculation, and bike riding, leads to a non-specific screening tool [84]. This has led to an extensive effort to discover novel PCa screening tools, which could be easily implemented instead of or alongside PSA testing. One such candidate is prostate cancer antigen 3 (PCA3), a novel urine based assay utilized as a predicative tool for prostate cancer biopsy

results and treatment outcome. PCA3 or DD3 gene was found to be overexpressed in PCa tissue [85], which led to clinical trials in men undergoing repeat biopsies, where PCA3 outperformed PSA for PCa detection [86]. The utility of this test is best seen in cases with slightly elevated PSA and an abnormal DRE; wherein, PCA3 outperforms PSA testing for the prediction of positive initial and subsequent biopsy results [87, 88]. However, Serum PSA is still the most commonly used predictor of a histologic diagnostic adenocarcinoma in needle biopsy [89]. The positive predictive value of a PSA level > 4 ng/mL for a diagnosis of carcinoma in needle biopsy is 31% to 51%. This value is increased to 42% to 72% when the DRE is suspicious for PCa [89, 90]. A biopsy will be carried out to confirm and categorize the clinical grade of the cancer. Unlike the PSA test or DRE, a biopsy is capable of differentially diagnosing PCa from other conditions such as BPH. Biopsies consist of 6-12 core samples of the prostate tissue that undergo pathological evaluation to assess the patients cancer using the Gleason grading system [91].

1.2.4 Staging and Grading

Subsequent to a clinical diagnosis of PCa, the tumours are staged and graded. PCa stage is defined as anatomic extension of cancer and it aids in rationally selecting treatment approaches and to predict prognosis. The typical system used is the TNM classification, based on the size of a primary tumour (T), regional lymph node involvement (N), and distant metastasis (M) (Table 1.1) [92, 93]. The extension of the cancer is determined by multiple criteria. DRE, serum PSA, radiologic imaging, histologic grading, and surgical lymphadenectomy are most commonly used to determine PCa extension. However, pathologic T staging usually requires histologic examination of radical prostatectomy (RP) tissues, including the prostate gland and seminal vesicles. Pathologic stage greatly impacts therapeutic approach and outcome for men with localized PCa [71].

Grading describes the carcinoma in terms of gland differentiation and stromal invasion by scoring the two most prevalent histological components of the tumour section. The most prevalent grading system used worldwide is the Gleason system [91]. This grading system is based entirely on the histologic arrangement of carcinoma cells in haematoxylin and eosin (H&E) stained tissue sections [94]. Scoring for regions of each of these sections is from 1 to 5. The sum of the most predominant staining pattern and the second most common pattern is referred to as the Gleason score (or Gleason sum) and can range from 2 to 10. Lower scores in the 2 to 4 range signify a well-differentiated tumour. Scores in the 5 to 7 range signify moderately differentiated tumours, while higher scores from 8 to 10 represent poorly differentiated tumours. Patients with higher Gleason scores (GS) have a poorer clinical outcome and a higher risk of progression of cancer to metastatic disease.

1.2.5 Role of Androgen in Prostate Cancer Progression to Castration-Resistant Prostate Cancer (CRPC)

In 1940, Drs. Huggins and Hodges demonstrated the pivotal role of androgens and AR in the pathogenesis of PCa [95]. Dr. Huggins was awarded the 1966 Nobel Prize for Physiology or Medicine for this discovery. Huggins observed that estrogen treatment led to significant shrinkage of the prostate gland, which was later discovered to be due to the inhibition of androgen production by the hypothalamus-pituitary-gonadal axis [2]. After successful removal of tumours via castration in dogs, Huggins and Hodges conducted bilateral orchiectomies on advanced PCa patients and observed a remarkable response and regression of late stage tumours [95]. Later it became clear that inhibition of androgen production could also be achieved by shutting down the hypothalamus-

pituitary-gonadal axis using chemical luteinizing hormone-releasing hormone (LHRH) agonists [96].

In the absence of androgens, AR protein is not transcriptionally active in the cytosol, resulting in the reduction of AR transcribed genes. Androgen mediated AR activation initiates survival, growth, and proliferation pathways and when inhibited by castration will induce these androgen-sensitive cells to undergo rapid apoptosis [97]. The early findings by Huggins and Hodges on the dependency of PCa on androgens and a regression in tumour size upon castration setup the building blocks of our current understanding of PCa development and progression.

Based on the role of androgens, we can categorize PCa progression. This initial stage of the disease is androgen dependent where the majority of malignant cells require androgens for growth and survival [98]. This is the stage that is usually detected by initial PSA, DRE, and biopsy screening. However, over time, the selective pressure of androgen deprivation therapy (ADT) inevitably leads to physiologic adaptation of the tumour in almost all patients and the progression to CRPC. CRPC is marked by an increase in the levels of PSA and continued cell growth despite the low levels of androgen in circulation (Figure 1-3) [99]. Castration induces adaptive stress responses in PCa cells, insulates against apoptosis, while steady accumulation of further genomic and epigenomic aberration [100], facilitates progression to an aggressive tumour capable of growing in castrate levels of testosterone and thus termed CRPC. The majority of CRPC re-initiate cell division by reactivation of the AR, providing a critical therapeutic approach by targeting of the AR signalling axis, at least for a short duration, in a large proportion of CRPC patients [101].

Mechanisms involved in PCa progression to CRPC are complex and fraught with controversies. Several mechanisms have been proposed to explain the progression from an AD disease to CRPC [2]. One such mechanism is the hypersensitivity pathway, whereby the cancer

cells gain an increased sensitivity to very low level of androgen. This can occur through over-expression of the AR, as seen in approximately 30% of tumours; an increased sensitivity of AR; or by elevated intracrine androgen metabolism [2]. This is enhanced by an increase in the activity of 5 α -reductase as well as activation of steroid biosynthesis machinery [2, 102]. Recent studies also suggest that androgens and other steroid precursors secreted from the adrenal glands, which account for 10–30% of circulating androgens, are an important source of continued AR activation [103]. Another mechanism for progression to CRPC is the promiscuous pathway. Mutations in the ligand binding domain of the AR gene leads to non-specific binding to non-androgen steroids such as progestin and estrogen as well as anti-androgens such as flutamide and hydroxyflutamide [104, 105]. Over-expression of AR co-activators or a decreased expression of AR co-repressors can have similar effects [2]. The outlaw pathway describes the mechanisms through which AR, as well as other steroid hormone receptors, becomes activated through ligand independent mechanisms. Growth Factors and Cytokines such as IGF-1, Epidermal Growth Factor (EGF), and Interleukin (IL)-4 and IL-6 are overexpressed in CRPC. These growth factors and cytokines activate receptor tyrosine kinases (RTKs) and lead to AR phosphorylation via either AKT or mitogen-activated protein kinase (MAPK), resulting in activation of AR in the absence of androgens [104-108]. The bypass pathway describes alternate signalling pathways that can circumvent AR signalling to promote the growth and survival of the cell [2]. In this pathway, activation of oncogenes or inactivation of tumour suppressors in CRPC results in up-regulation of growth and survival signals via pathways other than AR pathway. Up-regulation of growth factors (IGF-1, EGF), Bcl-2 protein and PI3K pathway are examples of some of the alterations that result in growth and survival of PCa cells in a castrate environment [104, 109]. PCa may also circumvent the effects of ADT via the lurker pathway. Prostate tumours inherently vary in their level of dependence upon androgens.

Upon ADT, androgen responsive cells are eliminated but androgen independent cells, such as the epithelial stem cells, continue to proliferate resulting in clonal expansion and progression to CRPC [2, 110]. Finally, the discovery of truncated AR isoforms that lack the ligand binding domain and hence function as constitutively active, ligand-independent transcription factors, allow PCa cell progression to CRPC in the presence of androgen depletion therapies. This AR variant was initially discovered in CWR22 by Tepper et al., which created an isoform that was constitutively nuclear and could bind DNA independent of androgens and functioned independently of the full length AR [111]. The increased transcriptional pressure on the AR gene, fast tracks a Darwinian selection for these variants, which have been recently associated with resistance to abiraterone and enzalutamide [112]. There are a variety of AR splice variants lacking the ligand binding domain, with the most abundant and research focused in PCa being AR splice variant-], with a 20-fold increase in expression in CRPC compared to hormone-sensitive tissue [113]. These AR splice variants as well as the burden of ligand binding domain mutations has led to the discovery of novel inhibitors, targeting the AR N-terminal and DNA binding domains which have shown early promise [101].

1.2.6 Treatment

The key factor in deciding on the optimal treatment modality for PCa is the stage of disease. When the cancer is confined to the prostate gland, patients with localized PCa (stages I and II), the most prevalent treatments include active surveillance, Radiotherapy (RT), and RP [114, 115]. However, for patient diagnosed with advanced PCa in which the disease is not confined to prostate, ADT is the cornerstone of their treatment plan [104, 116].

Localized PCa can be itself classified into three groups of low risk, intermediate risk, and high risk. Low risk PCa are patients with a TNM staging of cT1c–cT2a and a PSA <10 ng/ml and GS of 6. Intermediate risk PCa are patients with TNM staging of cT2b–c and a PSA between 10.1 and 20 ng/ml and GS of 7. High risk PCa is a non-metastatic disease that manifests with local tissue invasion and a combined GS of at least 8 with a PSA level greater than 20 ng/ml [117-119].

Active surveillance is a strategy used for low risk PCa or early stage disease where patient's age and health status predicts a low risk of symptomatic disease progression [120, 121]. These patients are closely monitored by prostate biopsies, PSA level measurements and DRE at regular intervals, and the decisions are made based on patient condition and their disease progression [120-122]. Based on evidence that suggests that PCa patients with low risk localized and well-differentiated PCa have a 20-year PCa specific survival rate of 80-90% [123, 124], active surveillance has been adopted by many physicians to overcome the overtreatment of patients with clinically insignificant disease [122, 125].

RT is an established treatment modality for many cancers. In PCa, RT is delivered by external beam radiation therapy (EBRT) or brachytherapy (BRT) and in conjunction with other treatment options for low to high risk localized PCa [126, 127]. EBRT is the most common form of RT in which an external source of radiation is directed to the prostate in short bursts and this is repeated daily for an extensive time period. In contrast, during BRT the radiation is delivered internally via implanted radioactive seeds into the prostate in low or high doses for up to 6 month [128]. Treatment response rate for the two delivery techniques are similar. In many cases, patients who prefer to avoid the risks of invasive surgery choose radiation therapy [129].

RP is a surgical procedure to remove the prostate gland and the attached seminal vesicles [115]. It is most commonly used for healthy men younger than 70 with localized, low to

intermediate risk PCa. For these patients, RP is one of the most effective treatment approaches to maximize the quality of life [130, 131]. Due to its invasive nature complications such as urinary incontinence and erectile dysfunction may occur [119, 132].

In advance PCa, where the disease has spread beyond the prostate capsule, ADT or as it is also known, hormone ablation, is the only line of treatment. ADT is used to prolong survival, to slow down the spread of cancer cells that have escaped (secondary or adjuvant therapy), to ease pain caused by the spread of the cancer (palliative therapy), to enhance the effect of radiotherapy, and to shrink the prostate and the tumour before a procedure, in order to reduce the likelihood of escape (neoadjuvant hormone therapy (NHT)). Approximately 80% of metastatic PCa patients will respond to ADT with a median progression-free survival of 1-2 years and overall survival of 2-4 years, making this treatment the “gold standard” [133]. ADT can be performed either through the surgical removal of testicles, bilateral orchiectomy, or chemical castration [104, 116]. By exploiting the luteinizing hormone (LH) secretion pathway, LHRH agonists such as Leuprolide and Goserelin can competitively bind LHRH receptor in the pituitary gland and suppress the secretion of LH in the blood and therefore inhibit testosterone synthesis by the testis [134, 135]. The only serious side effect is the surge in the serum testosterone level at the time of first administration, which may result in the exacerbation of clinical symptoms, such as urinary obstruction, bone pain, and spinal cord compression [136]. Initially, LHRH antagonists such as Degarelix were developed to avoid the flare response. Another prevalent solution to this problem is the use of antiandrogens, such as flutamide or bicalutamide, in combination with LH-RH agonists for the first three weeks to suppress androgen flare up [135, 137]. Due to side effects associated with ADT including anemia, metabolic changes, gynecomastia and bone density loss and more importantly recurrence of PCa after approximately 18 months, intermittent ADT was

investigated as a possible treatment regime [138, 139]. By alternating cycles of ADT, surviving tumour cells can follow a path of normal differentiation, which may restore the ability to undergo apoptosis, prolong androgen dependence, and delay progression to CRPC [140]. Although the survival benefits of ADT are well established for non-localized PCa, sadly they are not curative and other treatment modalities in conjunction with ADT are needed.

Antiandrogens are another class of hormone therapy that compete with DHT to bind and activate AR [141]. Anti-androgen drugs are classified in two groups of steroidal anti-androgens such as cyproterone acetate and non-steroidal anti-androgens such as flutamide, bicalutamide, nilutamide, and the most recent addition, enzalutamide. [142-144]. Enzalutamide has shown great promise by having an eight-fold greater affinity for the AR in comparison with bicalutamide and reduces the efficiency of AR nuclear localization [142, 145]. Despite the availability and use of a combination of treatments to block androgens, progression of advanced disease to CRPC is inevitable.

In cases where the cancer is not responsive to antiandrogens or has progressed to a metastatic CRPC, another treatment modality includes the use of steroidogenesis enzyme inhibitors such as ketoconazole and abiraterone acetate. Ketoconazole inhibits the broad-spectrum production of steroids whereas abiraterone acetate inhibits the testicular, adrenal, and intratumoural androgen synthesis by inhibiting the enzyme CYP17. Abiraterone acetate has been shown to prolong survival of late stage metastatic PCa, in individuals whose disease has progressed after receiving chemotherapy [142, 146].

A number of chemotherapeutic agents have been employed for the treatment of CRPC, yet very few act both to prolong survival and improve quality of life. Docetaxel was the only one with

both palliative and life-prolonging results in CRPC [147-149]. Docetaxel is an anti-mitotic agent that works through stabilization of microtubules and in turn inhibition of mitotic cell division [150]. As with many other cancer therapies, patients treated with docetaxel either developed resistance or were not able to tolerate its side effects over the long term [149]. More recently, cabazitaxel received FDA approval in 2010 as a second-line treatment option for patients who failed docetaxel therapy [142, 151]. Due to the fact that patients with metastatic CRPC develop resistance to these therapeutics approaches, it is apparent that other drugs and novel targets are needed.

The immunogenic aspects of PCa have been given great attention and this prolific area of research has led to the development of novel therapeutics, including the first immunotherapeutic treatment of CRPC, sipuleucel-T, which was approved by the FDA in 2010 [152, 153]. Sipuleucel-T is an immunotherapy antibody against immune checkpoint programmed death-1 protein and is used in treatment of asymptomatic or minimally symptomatic metastatic CRPC [142, 153, 154]. Only through examining the underlying molecular biology of PCa progression have we been able to develop the aforementioned drugs and by further probing these and other novel mechanisms can we develop more effective drugs.

1.3 Hedgehog Signalling

1.3.1 Hedgehog Signalling Pathway Overview

The Hedgehog (Hh) pathway is a highly conserved signalling pathway that has been shown to play a vital role in embryonic development. The first member of the Hh pathway was identified in 1980 by Christiane Nüsslein-Volhard and Eric Wieschaus, who identified mutations that were linked to phenotypic changes through performing a series of genetic screens on the fruit fly

Drosophila melanogaster [155]. This work alongside a multitude of discoveries in the field of developmental biology led to a Nobel Prize in Physiology or Medicine in 1995.

The mutation in the Hh gene resulted in a distinct phenotype of short larva where the ventral denticles formed a dense lawn instead of the distinct segmented bands in the wild type *Drosophila*. The gene harbouring the loss-of-function mutation was named Hedgehog, due to the close resemblance between this stubby and “hairy” phenotype and a hedgehog. This gene is highly conserved in vertebrates, as homologues were identified in a variety of species including *Homo sapiens* [156-158]. In mammals, there are three homologs of the gene encoding the *Drosophila* Hh ligand: Sonic hedgehog (SHH), Indian hedgehog (IHH) and Desert hedgehog (DHH) [156].

The expression of Hh isoforms are tightly controlled and the mechanisms involved in Hh processing and secretion are evolutionarily conserved [159]. Hh ligands are produced as 45 kDa full-length proteins, which undergo posttranslational modifications including autocleavage and cholesterol modification to the N-terminal fragment [160, 161]. The cholesterol modification results in the association with the plasma membrane, facilitating the final processing step in which a palmitoyl moiety is added to the N-terminus by transmembrane acyltransferase. All signal activities are mediated by the N-terminal, but the C-terminal region is required to catalyze the autoproteolytic cleavage [162].

Hh ligands initiate signalling by binding the receptor Patched 1 (PTCH1), a twelve-pass trans-membrane, which in turn removes the inhibition of a seven-pass G-protein-coupled membrane protein called smoothened (SMO) [163]. In addition to binding Hh to initiate signalling, PTCH1 also sequesters Hh to modulate the extracellular gradient of the Hh ligand [164]. Activation of SMO involves a conformational switch, where in the absence of the Hh ligand, SMO

is found as a dimer in a closed inactive conformation. Pathway activation leads to a sequential phosphorylation of the C-terminal domain of SMO, leading to an open conformation. This switch is postulated to be a critical step in cell surface accumulation and signal transduction. SMO activation leads to a change in the balance of the repressor and activator forms of the three glioma-associated oncogenes (GLI) transcription factors, GLI1, GLI2, and GLI3 [165, 166]. This occurs by regulating their post-translational proteolytic processing. In the absence of the Hh ligand, PTCH1 inhibits SMO, GLI2 and GLI3 undergo sequential phosphorylation by three protein kinases, PKA, CK1 and GSK3 β and are proteolytically processed, leading to the conversion of GLI3, and to a lesser extent GLI2 into transcriptional repressors [167-171]. In the presence of the Hh ligand, PTCH1 inhibition of SMO is removed; GLI2 and GLI3 proteolysis is inhibited by the activated SMO and leads to the formation of GLI2, and to lesser extent GLI3 activators.

In an active state, GLI2 and GLI3 increase the expression of target genes, including GLI1 (Figure 1-4) [166, 172]. This response is mediated by the binding of the GLI1-3 transcription factors to a GLI-consensus binding sequence, 'TGGGTGGTC', in promoter and enhancer regions of target genes [173, 174]. As discussed, GLIs act as both transcription activators and repressors of a number of genes that vary between organisms and tissues. These targets include components of the pathway, PTCH, GLI1 and, hedgehog-interacting protein (HIP), which binds and sequesters Hh ligands, limiting the range and strength of Hh signals. In addition, the GLI transcription factors target genes of various protein families such as FGF, BMP, Myc, Cyclin, VEGF, and other proteins including, BCL-2, WNT, and SPOP, to name a few [175-179]. There are also targets that act in a feedback mechanism, such as GLI1, which is a transcriptional target of Hh signalling and is a constitutive activator that initiates a positive feedback on the pathway [180]. The relative levels of

GLI activator and repressor activities and their ratios determine the effect of the Hh pathway activation. GLI1 and PTCH1 are conserved target genes in a variety of tissues, and are indicative of activation of the Hh signalling pathway [181].

Canonical Hh signalling in vertebrates requires the key components of the pathway to organize along a microtubule-based organelle that projects from the surface of most vertebrate cells, called the primary cilia [182]. In the absence of Hh, PTCH1 is enriched in primary cilia, while in response to Hh, relocates out of the cilia and in turn enriches through activation, mobilizes SMO along the primary cilia base and activates the pathway by inhibiting the suppressor of fused (SUFU), an critical negative regulator of Hh signalling. Activation of the pathway increases the levels of GLI1, GLI2, and SUFU in the tip of the primary cilia. Removal of the suppression of SUFU, which is due to the dissociation of the GLI-SUFU complex. The exact nature of the regulation of this complex is not clear, but recent studies have shown the presence of an intrinsically disordered region that is an inherent feature of human SUFU. This domain is rearranged upon GLI binding and acts as a central signal organizer and plays a key role in modulation of regulated auto inhibition, properties that are found to be common among proteins with intrinsic disorder regions and therefore exist in different structural states [183, 184]. This complex dissociation allows GLI2 and GLI3 to become activators and translocate into the nucleus and bypass proteolytic processing, where they promote expression of Hh target genes [185, 186]. However, the role of the primary cilia in Hh signalling seems to be specific to mammalian cells since the loss of their orthologs does not seem to affect Hh signalling in *Drosophila* or Zebrafish [187].

1.3.2 Hedgehog Signalling in Development

The Hh pathway controls a variety of developmental processes including cell proliferation, cell differentiation, tissue patterning, and organogenesis, depending on the specific tissue and developmental timing [188]. The first insights into the role of the Hh in development were its involvement in the fruit fly larva segmentation [155]. In *Drosophila*, Hh is required for the development of the wings and legs as well as the patterning of the embryonic segments and the formation of posterior/anterior and dorsal/ventral axis in the early embryo [181]. Of importance, is the ability of Hh ligands to both act on short distance, and act over a long range, controlling cell fate as a function of Hh concentration.

In mammals, SHH and IHH play critical roles in embryonic development and DHH regulates spermatogenesis [189, 190]. However, among the three ligands, SHH has emerged as the most expressed and the most extensively studied. SHH is involved in the patterning of several parts of the embryo, including the neural tube and midline structures in the brain, limbs, teeth, internal organs, gut, and bone growth [191]. During early embryogenesis, SHH plays a key role in left/right axis and dorsal/ventral patterning of the embryo through expression in midline tissues including the node, notochord, and floor plate [192, 193]. SHH is also expressed in the zone of polarizing activity of the limb bud and controls anterior/posterior limb patterning [194]. These examples of anterior/posterior and dorsal/ventral axis formation all build on the principle of generating a signalling gradient by Hh ligands that act as a morphogen. Later in development, SHH is involved in the development of most epithelial tissues, including Hair, lung, foregut and glands [195, 196].

While playing a vital role in development, Hh signalling is also implicated in the maintenance of tissue homeostasis in the adult. SHH is required for hair follicle bulb and hair production during the postnatal hair cycle [197]. Hh signalling has also been identified as an important factor in wound healing, which may be due to its effect in promoting angiogenesis as well as inducing motility to close the wound [198-200]. Several studies suggest Hh signalling is involved in maintenance of stem or stem-like progenitor cells of various epithelia [201]. SHH has also been reported to be critical in tissue regeneration following injury, including regeneration of CNS, retina, lung, gastric, pancreas and prostate tissue [202-205]. Many of these same tissues are the site for aberrant Hh signalling that leads to the development of cancers.

1.3.3 Hedgehog Signalling in Prostate Development

During prostate ductal morphogenesis, SHH is expressed in the epithelium of nascent UGS buds and localizes to active growth sites. During ductal budding, SHH expression in the epithelium is up regulated and concentrates at sites of epithelial invagination [196]. The pattern of SHH expression is mirrored by the expression of GLI1 and PTCH1 in the adjacent UGS mesenchyme [206]. Paracrine signalling directly affects mesenchymal proliferation, but also influences epithelial proliferation and differentiation through feedback mechanisms [207, 208]. Interestingly, in the nascent buds and at the tips of the growing ducts there is a concentrated epithelial expression of PTCH1 and GLI1, which may be an indication of autocrine signalling [209]. SHH expression in the UGS is not dependent upon testosterone, however testosterone slightly increases the expression and redistribution of SHH during budding, which is tied to an androgen-induced morphogenetic event [206].

Studies utilizing antibody blockade or chemical inhibition of Hh signalling in E15 UGS grafts implanted under renal capsule or prostate organ cultures, demonstrated disruption of ductal budding and glandular morphogenesis [196, 206]. In the same line of thought, SHH^{-/-} embryos at E18.5 show a complete absence of prostatic ductal budding [210]. Yet, subsequent work demonstrated that normal glandular structures can be derived from SHH null mice when the UGS is implanted under the renal capsule of adult male mice or cultured in the presence of androgen [210].

The effects of Hh activation on ductal morphogenesis are stage dependent. Using cultured UGS from rats, Wang et al. demonstrated that inhibition of Hh signalling by cyclopamine resulted in enlarged ductal tips and increased ductal branching, while introducing recombinant SHH protein increased the number of differentiated epithelial cells [208]. Yu and Bushman, in an effort to resolve the discrepancies in previous studies, examined the effects of Hh activation on prostate ductal growth using organ cultures and transgenic mouse models. They found that co-culture of embryonic or postnatal prostates with a mesenchymal cell line overexpressing SMO results in increased ductal growth in embryonic prostates but decreased ductal growth in postnatal prostates [211]. These stage dependent effects of paracrine Hh signalling are likely due to differences in the response of the stroma at different developmental stages.

In situ hybridization experiments using a highly specific radiolabeled probe localized SHH to the prostatic epithelium and GLII expression almost exclusively to the periglandular stroma. PTCHI, was expressed at a basal level, due to absence of Hh, in both compartments [212]. Another group performed the same technique with a digoxigenin-labeled probe and immunostaining to demonstrate relatively weak co-expression of SHH, PTCH and GLII in the prostatic epithelium

[213]. These studies suggest that Hh signalling exerts multiple effects and in the normal adult prostate may involve a combination of autocrine and paracrine signalling.

1.3.4 Hedgehog Signalling in Disease

Given the critical role of Hh signalling in embryonic development, its aberrant activity during embryogenesis causes severe birth defects. A loss-of-function mutation in SHH, leads to a loss of ventral midline that results in a disorder called holoprosencephaly [214]. The phenotypic characteristic of this disorder affects the forebrain and mid-face which may result in impaired separation of the two halves of the forebrain and cyclopia. Mutations in PTCH and GLI2 have also been linked to similar phenotypes [215, 216]. Mutations in GLI3 are linked to disorders of head, face, and mainly limbs, where varying deformities in the digits are observed [217]. Mutations disrupting cholesterol biosynthesis, such as Smith-Lemli-Optiz syndrome and also defects in microtubule dynamics and primary cilia functionality, such as in Joubert syndrome and polycystic kidney disease, include phenotypes reminiscent of Hh pathway mutations [218-220]. In addition to the developmental disorders, aberrant Hh signalling is also involved in human disease in young and adult individuals such as modulation in hair growth and erectile dysfunction [197, 221]. Yet the most prominent human disease involving the Hh pathway is cancer.

1.3.5 Hedgehog Signalling in Cancer

Due to the critical role of Hh signalling during the developmental processes of patterning, morphogenesis, and proliferation, it is understandable that cancer recapitulates the same developmental cues, which leads to aberrant Hh signalling in a variety of tumours. The first study linking the Hh pathway to cancer was identification of a mutation of PTCH in nevoid basal cell carcinoma syndrome, also called Gorlin syndrome [222]. Patients with this disorder suffer from

birth defects such as skeletal malformations and frequently develop neoplasia and cancer [223]. This identified PTCH as a tumour suppressor and predisposes patients with the Gorlin syndrome to various cancers, including multiple basal cell carcinomas, medulloblastomas, ovarian fibromas, and with a lower frequency of occurrence rhabdomyosarcomas, and cardiac fibromas [224]. In recent years, the role of Hh signalling in cancer initiation and progression has been established and shown to be aberrant in over 30% of human cancer [225].

Three main modalities of Hh pathway activation have been proposed in cancers [226]. Type I are ligand-independent cancers harbouring a pathway activating mutation. Type II are ligand-dependent, autocrine cancers that both secrete and respond to the Hh ligand. Type III are also ligand dependent, but are paracrine cancers that secrete the Hh ligand and target stromal cells. A variation of Type III cancers is a reverse paracrine model. In these tumours, the tumour stroma produces the Hh ligand, targeting the tumour cells (Figure 1-5).

The case of Gorlin syndrome is an example of type I ligand independent activation of the Hh pathway, wherein a loss of function mutation of PTCH leads to constitutively activated Hh signalling in the absence of the ligand [227]. In addition to the inhibiting mutations in PTCH1, gain of function mutations in SMO, reduce its ability to be inhibited by PTCH and have been shown to be associated with medulloblastoma and sporadic basal cell carcinoma. Inactivating mutations in the SUFU gene have also been identified in basal cell carcinoma, and loss of heterozygosity of both PTCH and SUFU have been associated with rhabdomyosarcomas as examples of type I cancers [228, 229]. In other tumours, aberrant Hh signalling is mainly caused by ligand-dependent Hh activation, yet whether this is due to autocrine or paracrine signalling or due to a combination of both is to be fully studied.

There is a large subset of tumours that have been shown to respond to autocrine Hh signalling and present with overexpression of Hh ligands. These cancers including prostate [205], lung [202], pancreatic [230], breast [231], melanoma [232], bladder [233], and gastrointestinal [234] fall into the category of type II, ligand-dependant autocrine, cancers . These tumour cells demonstrate a cell-autonomous Hh pathway activation and inhibition of Hh signalling, in cell lines derived from these tumours, through ligand-neutralizing 5E1 antibody, SMO or GLI1 knockdown, or a natural inhibitor of SMO activity, cyclopamine, demonstrates an inhibition of growth in a mono-culture setting [213, 230, 235].

A variation of Type III Hh activation modality is a paracrine signalling model in which the Hh ligand is produced by the stroma and acts on the tumour cells to activate the Hh pathway. Evidence for the existence of this type of signalling in cancer comes mainly from hematological tumours, particularly B-cell lymphoma, multiple myeloma, and glioma [236, 237]. In these studies, Hh ligand produced by bone marrow stromal cells or endothelial cells enhance tumour growth and proliferation.

Due to the fact that Hh signalling functions in a paracrine fashion during development, it is understandable to expect to find cancers that recapitulate the same dynamic. The first observation of a type III, ligand-dependant paracrine, cancer was made by Bushman and colleagues in PCa [212]. In this study, human LNCaP cells transfected with SHH grew at the same rate *in vitro* but faster *in vivo* compared to untransfected, suggesting that the mouse stroma influenced the xenograft growth rate. They further confirmed their results by in situ hybridization and species-specific polymerase chain reaction (PCR) experiments that showed Hh levels in the tumour correlated with murine GLI1, GLI2 and PTCH1 expression in the stroma, but not in the

human tumour cells [212]. These results have also been demonstrated in pancreatic, ovarian, and metastatic colorectal tumour tissue samples [238].

1.3.6 Hedgehog Signalling in Prostate Cancer

There are increasing findings implicating a role for an aberrant activation of Hh signalling in the development and progression of PCa. However, the exact role of Hh signalling and its mode of action are still to be discerned. Studies have shown evidence of all three modalities of Hh activation, as discussed above, in PCa. There is only one study that demonstrates the presence of a loss of function mutation of SUFU in prostatic tumour tissue, which may suggest a role for ligand-independent Hh activation [239]. The rest of the findings demonstrate a clear role for ligand dependent activation of Hh signalling in PCa, yet controversy remains on whether the mode of action is paracrine, autocrine, a combination of both, or a progression from one to the other. During prostate development, Hh activation occurs through epithelial-stromal signalling, in a paracrine fashion. This would suggest that PCa might recapitulate the same mechanism. This is consistent with reports that Hh ligands secreted by tumour cells signal to the stroma, thereby inducing the production of growth factors leading to tumour growth and survival [212]. However, there are also studies demonstrating an autocrine requirement for Hh signalling in which the tumour cells both produce and respond to the ligand [205, 213]. Regardless of the differences in mode of action, these studies underline a clear role for Hh signalling in PCa. Sanchez et al. found increased SHH expression in tumours compared to normal adjacent tissue, correlating with increased proliferation [213]. In addition, there seems to be a positive correlation with high levels of Hh signalling and tumour grade and stage [205, 239, 240]. Tzepeli et al. confirmed this by demonstrating that expression of PTCH in the tumour tissue correlated with tumour grade and stage and add that epithelial PTCH expression was also found to be higher in metastatic tissue compared to primary

PCa tissue [241]. In contrast, other studies have shown that expression of SHH is localized to tumour epithelium and GLI1 is restricted to the stroma, suggesting a paracrine mechanism [212, 242]. In another study of prostate tumour samples, a significant correlation between SHH expression and Gleason score as well as metastasis in the lymph nodes was found. Interestingly, DHH expression in the epithelium was associated with Gleason score and tumour stage [243]. There have also been associations made between elevated Hh signalling and poor prognosis, including correlation between SHH, PTCH, SMO, and GLIs and Gleason score. In the same study elevated SHH expression was found to be a prognostic factor for PSA recurrence [240, 244].

Other studies attempted to examine the tumourigenic potential of aberrant Hh signalling. In one study, using *Pb-Cre4/+ R26-SMOM2/+* mice, which expresses a constitutively active form of SMO in the prostate epithelium, no hyperplastic or neoplastic lesions were detected after 12 months [245], suggesting that epithelial cells are not a permissive cell type for tumour initiation and development. Yet, another study showed that overexpression of SHH ligand by electroporation of the adult mouse prostate was sufficient to cause PCa in 90 days [246]. Since this technique can lead to overexpression of SHH in both the epithelium and the stroma, it will be difficult to reach a conclusive result, yet it seems activation of the stromal compartment may be required early in the development of PCa.

Although aberrant Hh signalling is associated with advanced PCa and metastatic potential, due to the complexity of this pathway, its mechanism is not fully understood. Examining Hh pathway targets that play a pivotal role in the progression of PCa may shed a light. SHH induces expression of vascular endothelial growth factors (VEGF) and angiopoietins, two key pro-angiogenic molecules. Inhibitory components of the Hh pathway, such as HIP, are expressed in

resting endothelial cells and are down regulated in prostate xenografts undergoing angiogenesis [247]. This angiogenic response may be triggered indirectly by the Hh pathway through regulation of other factors such as hypoxia induced factor (HIF)-1 α . This has been demonstrated in high Gleason grade PCa, where destabilizing HIF-1 α represses transcription of VEGF and in doing so inhibits nuclear localization of Snail1 and epithelial-mesenchymal transition (EMT)[248]. Hypoxia has been shown to induce Hh signalling activation, mediated through accumulation of HIF-1 α [249]. This accumulation triggers non-canonical Hh signalling to facilitate hypoxia induced EMT and invasive processes [250]. Recent studies in breast cancer have established a clear link between HIF-1 α and GLI1 and demonstrate that knockdown of GLI1 mitigated the hypoxia enhanced EMT and invasion of breast cancer [251]. These studies pave the way for further examination of aberrant Hh signalling in PCa to better understand its role in the progression of the disease.

Hh signalling also plays a role in cell cycle regulation mainly via targeting cyclins. Increased expression of GLI2 led to an accelerated cell cycle progression, consequently resulting in an increased cell growth and knockout of GLI2 suppressed PCa tumour growth both *in vivo* and *in vitro* [252]. Hh signalling has been shown to mediate EMT, which facilitates cell motility and aids in the invasive properties of metastatic PCa. Overexpression of GLI1 in a non-metastatic PCa cell line stimulated expression of Snail, a marker of EMT, to levels seen in metastatic lines and increased cell invasion *in vitro*. In addition, Hh inhibition after treatment with the SMO inhibitor NVP-LDE-225 resulted in suppression of EMT, as illustrated by decreased cell motility, invasion and migration of PCa cells [253]. In addition, Hh signalling may contribute to PCa metastasis to

bone, as bone marrow stromal cells are responsive to Hh ligands, and both SHH and IHH stimulate bone remodelling [254].

In several recent studies, long-term androgen deprivation has been shown to upregulate Hh signalling, which may elicit a role for this pathway in the progression to CRPC [242, 243, 255]. It has been demonstrated that levels of SHH, SMO, GLI1, and GLI2 were elevated after ADT both in human and mouse xenograft samples. In addition, inhibition of Hh pathway led to down-regulation of AR signalling activity, which may be due to direct binding of GLI1 and GLI2 to the AR [256, 257]. This provides an ideal opportunity for the development and implementation of combinational therapies of ADT followed by Hh pathway inhibition. Moreover, targeting Hh pathway could represent an integral part of PCa treatment, especially in more aggressive and drug resistant stages of this disease.

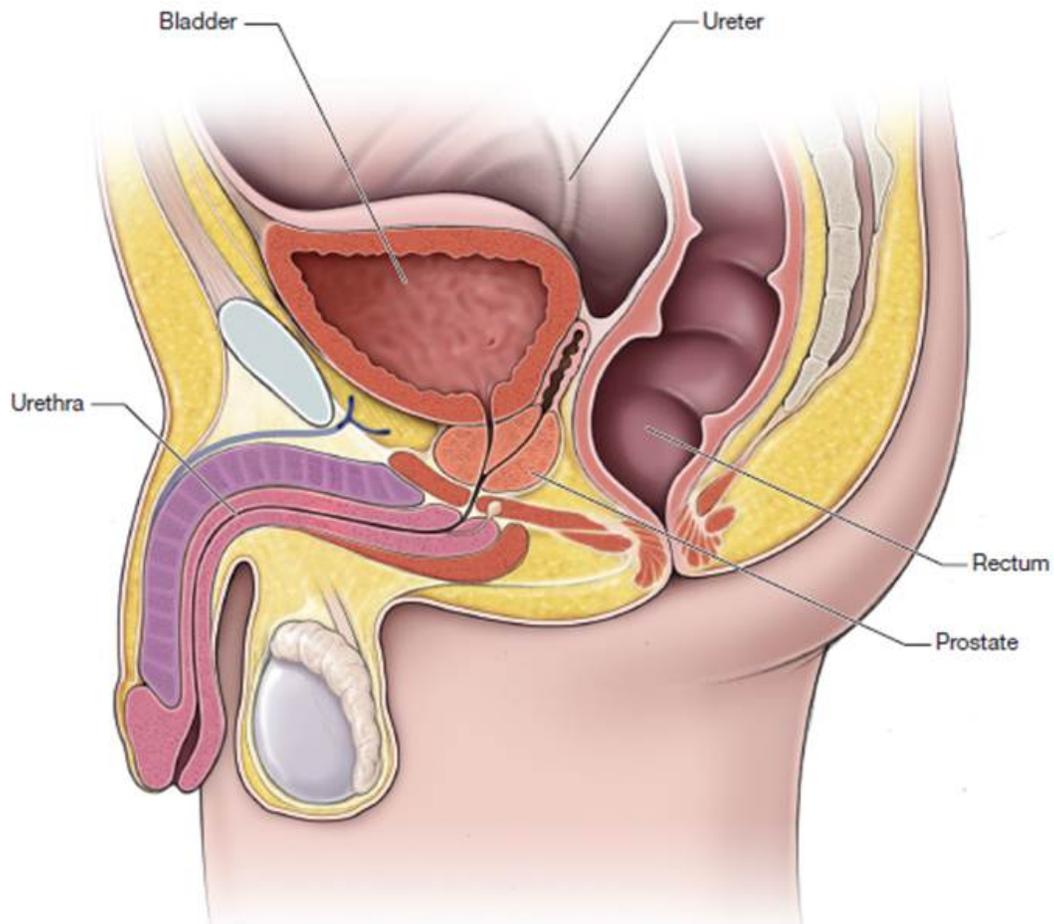


Figure 1-1 Location of the prostate gland.

The prostate gland is located anterior to the rectum and inferior to the urinary bladder in the pelvic body cavity.

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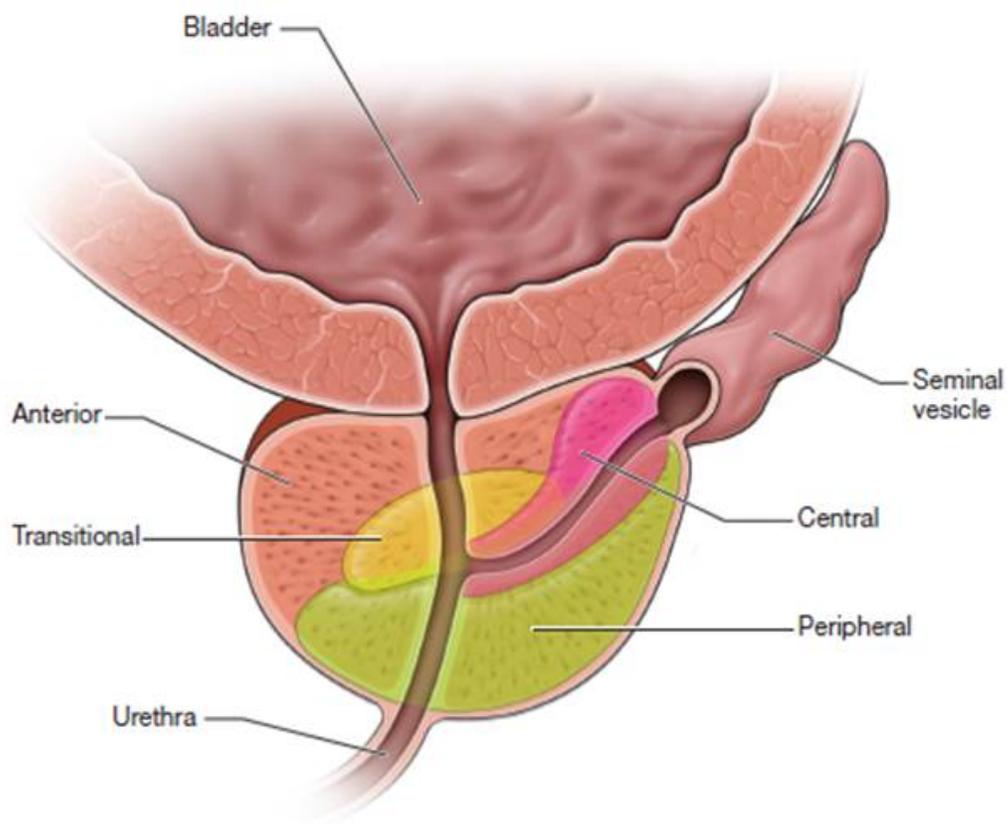


Figure 1-2 Location of the prostate gland.

An adult prostate is composed of three zones: peripheral zone, central zone and transition zone, enclosed by a capsule composed of collagen elastin and abundant smooth muscle.

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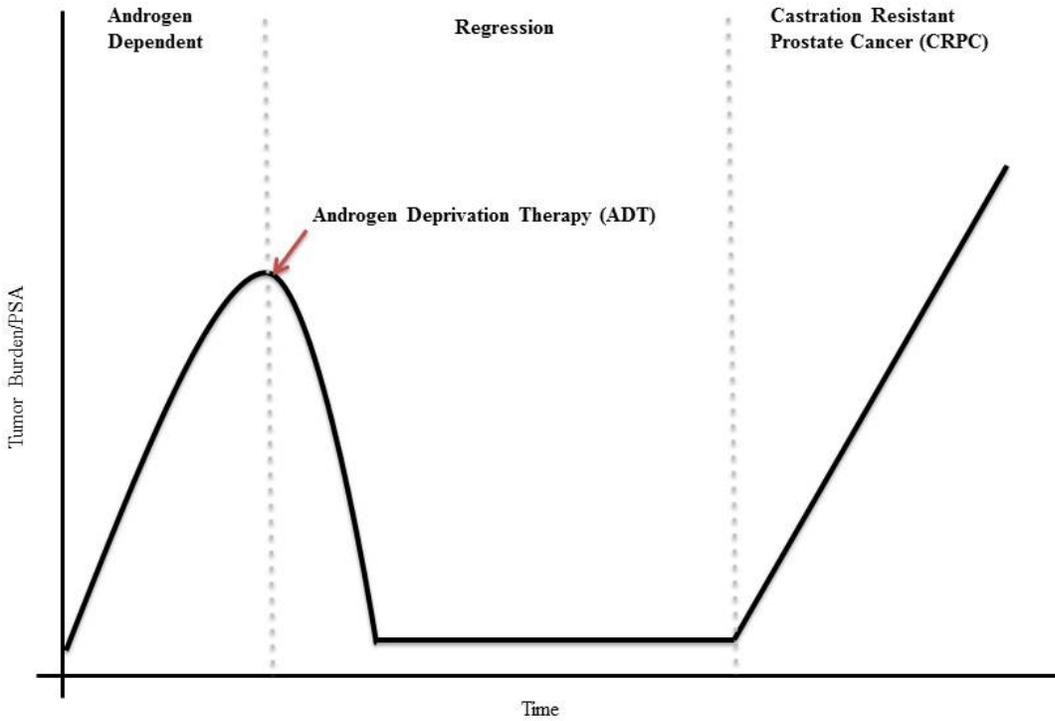
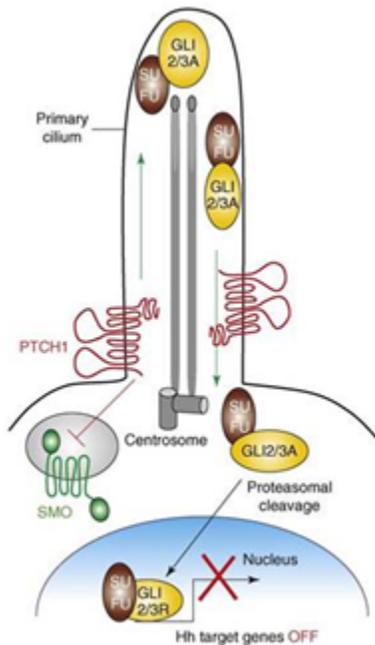


Figure 1-3 Schematic of clinical PCa progression.

PCa progression is classified into three different phases. The initial PCa growth phase which elevates the PSA level is the androgen dependent stage (AD); following androgen deprivation therapy, which initiates the regression phase of the disease, the androgen dependent tumour PSA levels and tumour burden are decreased and the tumour will be dormant. The Final stage initiates with the increase of patient PSA levels, signifying that the tumour is in the Castration Resistant Prostate Cancer (CRPC) stage. At this stage, all treatments are palliative.

A. Absence of Hh ligand



B. Presence of Hh ligand

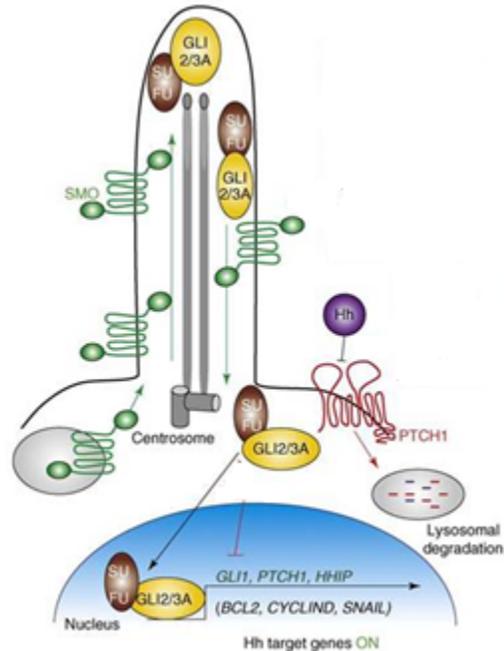
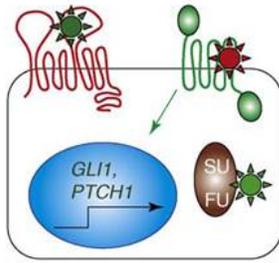


Figure 1-4 Hedgehog Signalling Pathway.

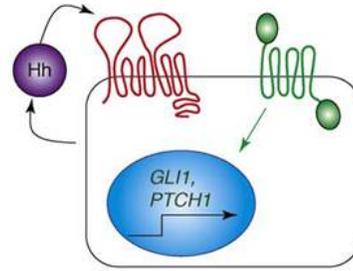
Components of the Hedgehog pathway. (a) In the absence of Hh ligands, Patched (PTCH1) inhibits Smoothed (SMO) from entering the cilium, keeping the pathway off. GLI2 and GLI3 activators move up and down the cilia by intraflagellar transport, and are cleaved by the centrosome-proximal proteasome into repressor forms (GLI2/3R) that cannot activate target gene transcription upon binding to DNA in the nucleus. (b) Upon Hh binding, PTCH1 translocates out of the primary cilium, losing its ability to inhibit SMO, which moves into the cilium, thus stimulating the pathway and preventing GLI2 and GLI3 cleavage. PTCH1 and Hh are internalized and degraded in lysosomes. (Modified from Scales et al., 2009)[226].

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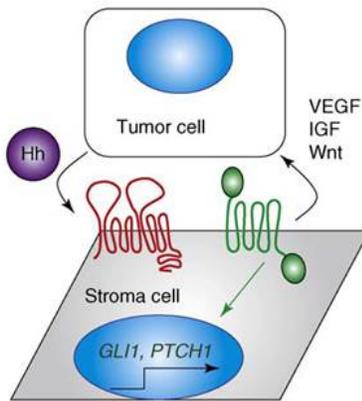
A. Type I ligand independent



B. Type II autocrine ligand dependent



C. Type III paracrine ligand dependent



D. Type IIIb “reverse” paracrine ligand dependent

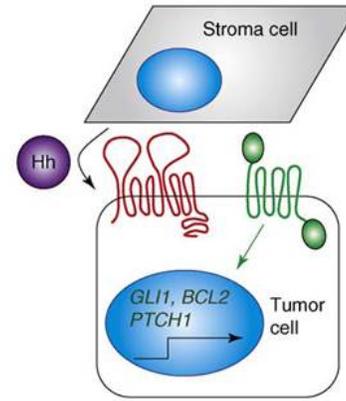


Figure 1-5 Models of Hedgehog Activation in cancer.

Illustrations of the different models of Hh pathway activation in cancer. (a) Type I ligand-independent cancers harbour inactivating mutations in PTCH1 or SUFU, or activating mutations in SMO, leading to pathway activation in the absence of ligand. (b) Type II ligand-dependent autocrine cancers both secrete Hh and respond to it, leading to cell-autonomous pathway activation. (c) Type III ligand-dependent paracrine cancers secrete Hh, which binds to PTCH1 on stromal cells, leading to pathway activation in that cell type and feedback of other growth or survival signals to the tumour. (d) Type IIIb ‘reverse paracrine’ tumours receive Hh secreted from stromal cells, which leads to pathway activation in the tumour. (Modified from Scales et al., 2009)[226].

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Table 1-1 TNM Staging of Prostate Cancer.

TNM Staging of Prostate Cancer				
Stage	Description			
T Stage				
TX	Primary tumor can be assessed			
T0	No evidence of primary tumor			
T1	Clinically unapparent tumor neither palpable nor visible imaging			
T1a	Tumor incidental histologic findings in ≤ 5% of tissue resected			
T1b	Tumor incidental histologic findings in > 5% of tissue resected			
T1c	Tumor identified by needle biopsy (eg, because of elevated PSA level)			
T2	Tumor confined within prostate			
T2a	Tumor involves no more than half of one lobe			
T2b	Tumor involves more than half of one lobe but not both lobes			
T2c	Tumor involves both lobes			
T3	Tumor extends through the prostatic capsule**			
T3a	Extracapsular extension (unilateral or bilateral)			
T3b	Tumor invades the seminal vesicle(s)			
T4	Tumor is fixed to or invades adjacent structures other than seminal vesicles (eg, bladder, levator ani muscle, pelvic wall)			
N Stage				
NX	Regional lymph nodes not assessed			
N0	No regional lymph node metastasis			
N1	Metastasis in regional lymph node(s)			
M Stage***				
M0	No distant metastasis			
M1	Distant metastasis			
M1a	Nonregional lymph node(s)			
M1b	Bone(s)			
M1c	Other site(s) with or without bone disease			
Stage Grouping				
Stage I	T1a	N0	M0	G1 (GS 2-4)
Stage II	T1a	N0	M0	G2,3-4 (GS 5-10)
	T1b	N0	M0	Any G
	T1c	N0	M0	Any G
	T1	N0	M0	Any G
	T2	N0	M0	Any G
Stage III	T3	N0	M0	Any G
Stage IV	T4	N0	M0	Any G
	Any T	N1	M0	Any G

* Tumor found in one or both lobes with needle biopsy but not palpable or reliably visible at imaging is classified as T1c disease

** Invasion into the prostatic apex or into (but not beyond) the prostatic capsule is classified as T2 disease, not T3.

*** When more than one site of metastasis is present, the most advanced category is used

Chapter 2: Inhibiting Smoothed, Delays Castration Resistant Prostate Cancer by Disrupting Hedgehog Signalling

2.1 Introduction

While ever-improved androgen ablation therapies prolong life in men with advanced PCa, remissions are temporary because surviving tumour cells progress to establish castration-resistant disease [258, 259]. Castration-resistant progression is a complex process by which cells acquire the ability to both survive and proliferate in the absence of androgens and involves variable combinations of clonal selection, adaptive up-regulation of anti-apoptotic genes, and alternative growth factor pathways [99, 260]. Identifying targetable pathways underlying castration-resistant progression is essential for improving survival of patients with advanced disease.

Hh signalling is one such pathway. It is a highly conserved inter- and intracellular regulator that governs processes including cell proliferation, differentiation, patterning and organogenesis of numerous tissues, including the prostate, during embryogenesis and is involved in stem cell maintenance, tissue repair and regeneration in adults [261].

Aberrant Hh signalling has been implicated in carcinogenesis of a variety of human tumours such as medulloblastoma, melanoma, basal cell carcinoma and cancers of the colon, lung, breast, pancreas, and prostate [171, 202, 205, 213, 234, 238, 239, 262], yet how it impacts cancer development and/or progression remains to be fully resolved. Canonical paracrine Hh signalling is strongly implicated in ductal morphogenesis of the prostate through production of Hh ligands by the developing prostatic epithelium and transcriptional activation of GLIs in the adjacent mesenchyme (reviewed in [263]). Evidence for aberrant Hh signalling occurring in PCa in regions of EMT, and during metastatic and CRPC progression [205, 213, 239, 243] suggest that Hh-regulated developmental processes may be recapitulated in PCa. Up-regulated Hh ligand

expression in androgen-deprived and castration-resistant PCa (CRPC), and elevated Hh ligand expression and Hh signalling activity in a variety of PCa cell lines is indicative of activation of canonical Hh signalling in PCa.

Whereas Hh ligand expression is suggestive of canonical signalling activation, the relative contribution of autocrine and paracrine signalling remains controversial. Although targeting canonical Hh pathway activation with the Smo antagonist or a GLI1-targeted siRNA inhibits proliferation of numerous PCa cell models *in vitro* [205, 213, 239, 264], this effect is not always correlated with inhibition of Hh signalling activity [265]. Additionally, while the androgen-responsive PCa model, LNCaP, exhibits elevated SHH expression and modest cyclopamine sensitivity under castrate conditions [266], correlation of their accelerated growth *in vivo* with GLI-1 expression in the adjacent stroma implicated a paracrine model for SHH-mediated xenograft development [212]. Furthermore, the LNCaP-derived castration-resistant cell line, C4-2 can drive SHH-dependent paracrine signalling and growth of normal prostate stromal cells [267] and LNCaP cells engineered to over express SHH drive tumour cell proliferation by paracrine signalling with rodent urogenital mesenchyme *in vitro* and with xenograft-associated stroma [268, 269].

Several studies reveal potential therapeutic efficacy of targeting SMO using PCa xenograft models. Initial xenograft studies demonstrated that SHH-expressing LNCaP cells exhibited accelerated xenograft growth in intact mice [212] and that cyclopamine treatment suppressed tumour growth of CWR22Rv1 and PC3 in female hosts [205]. Recently the SMO antagonist, GDC-0449 (vismodegib), was shown to suppress PC3 xenograft growth [264]. This drug has also shown to suppress GLI1 and Ptch1 expression in the stromal compartment, and decrease mitotic index of SHH-producing MDA PCa 118b xenografts, but this effect was not shown to suppress total tumour growth in hormonally intact hosts [270].

These studies strongly implicate aberrant Hh signalling as a potentially viable therapeutic target for management of advanced PCa. Thus we hypothesize that Hh signalling is a critical adaptive response during progression to castration resistance, which recapitulates paracrine developmental cues. In this study we confirm up-regulation of DHH in androgen ablated PCa specimens and elevated SHH expression in CRPC. Hypothesizing that castration-induced Hh expression promotes CRPC progression through reciprocal canonical paracrine signalling within the tumour microenvironment, we assessed the inhibitory efficacy of a novel investigational SMO antagonist, TAK-441 in CRPC progression.

TAK-441 was discovered as part of a high throughput screening for SMO inhibitors and was modified for enhanced inhibitory activity and metabolic stability by Takeda Pharmaceutical. As part of the high throughput screen, thieno[3,2-c]quinoline-4-one derivative 9a was identified as a SMO inhibitor with potent *in vitro* activity but poor metabolic stability. Enhancement of the inhibitory activity and metabolic stability was achieved through core ring conversion and side chain optimization. This led to the discovery of pyrrolo[3,2-c]quinoline-4-one derivative 11d, which has a structure distinct from previously reported Hh signaling inhibitors. Compound 11d, named TAK-441, suppressed stromal Gli1 mRNA expression in tumour associated stromal tissue and inhibited tumour growth in a mouse medulloblastoma allograft model [271, 272].

We observed that TAK-441 does not impact SHH expression, or viability of LNCaP cells cultured under androgen-deprived conditions *in vitro*, but that TAK-441 effectively suppressed CRPC progression of LNCaP xenografts as assessed by tumour size and serum PSA levels. The decreased tumour growth under castrate conditions correlated with decreased Ki67 staining of tumour cells and decreased expression of murine GLI1, GLI2 and Ptch1, but not their human orthologues, in xenograft specimens. These studies suggest that castration-induced SHH

expression in PCa tumours promotes CRPC by driving paracrine SMO signalling in host stroma. Thus, paracrine Hh signalling may offer unique opportunities for prognostic biomarker development, drug targeting and therapeutic response monitoring of PCa progression.

2.2 Materials and Methods

2.2.1 Prostate Cancer Cell Lines

The human PCa cell lines, LNCaP (ATCC) and C4-2 (Dr. Martine Gleave, Vancouver Prostate Centre, Vancouver, Canada) were maintained in RPMI 1640 (Invitrogen, Grand Island, NY) supplemented with 5% FBS and 2 mM L-glutamine in a humidified 5% CO₂/air atmosphere at 37 °C. PC3 (Dr. Paul Rennie, Vancouver Prostate Centre, Vancouver, Canada) and DU145 (ATCC) cells were maintained in DMEM (Invitrogen) supplemented with 5% FBS. For androgen deprivation studies, LNCaP cells were cultured in phenol red-free RPMI 1640 supplemented with 5% charcoal-stripped FBS (CSS; Invitrogen). Cells lines were verified to be mycoplasma free and authenticated by STR profiling by the Genetic Resources Core Facility, Fragment Analysis Facility (Johns Hopkins School of Medicine, Baltimore, MD) and comparison to known ATCC fingerprints (ATCC.org).

2.2.2 Prostate Cancer Immunohistochemistry

A total of 210 needle biopsy cores from 92 PCa patients were obtained from Vancouver Prostate Centre Tissue Bank (Table 2-1). All the specimens were from RP except for 48 cores from 12 CRPC specimens obtained from transurethral resection of prostate (TURP). 59 patients received preoperative androgen deprivation therapy (neoadjuvant hormone therapy, NHT). 44 cores from 21 patients were defined as short-term (≤ 6 months) and 76 cores from 38 patients were defined as

long-term (> 6 months). Tissue microarrays (TMAs) were prepared as 1 mm cores from each specimen. TMAs were stained with rabbit monoclonal primary antibodies targeting SHH (Abcam, Cambridge, MA), rabbit polyclonal DHH and IHH (Santa Cruz Biotechnology, Santa Cruz, CA), using a Ventana autostainer model Discover XT (Ventana Medical Systems, Tucson, AZ) with enzyme labeled biotin streptavidin system and solvent resistant diaminobenzidine immunohistochemistry kit (Invitrogen). Immunostaining intensity of each core was scored by an independent pathologist on a four-point scale. Descriptively, 0 represents no staining by any tumour cells, 1 represents a faint or focal, questionably present stain, 2 represents a stain of convincing intensity in a minority of cells and 3 represents a stain of convincing intensity in a majority of cells.

2.2.3 Smoothened Antagonists

Cyclopamine (Sigma Chemical Co, St. Louis, MO) was dissolved in dimethyl sulfoxide (DMSO) as a 10 mM stock. The SMO antagonist, TAK-441 (6-Ethyl-N-[1-(hydroxyacetyl)piperidin-4-yl]-1-methyl-4-oxo-5-(2-oxo-2-phenylethyl)-3-(2,2,2-trifluoroethoxy)-4,5-dihydro-1H-pyrrolo[3,2-c]pyridine-2-carboxamide) was provided by Takeda Pharmaceuticals Inc. (Fujisawa Japan). TAK-441 is a highly potent and orally bioavailable synthetic small molecule SMO antagonist in clinical development. It has an IC₅₀ of 4.6 nM evaluated using luciferase reporter activities in NIH3T3 cells carrying a stably-transfected GLI-reporter construct [272]. For *in vitro* studies, TAK-441 was added to media at indicated final concentrations from a 10 μM stock solution in DMSO. For *in vivo* studies, TAK-441 was administered by oral gavage once daily at 10 or 25 mg/kg from a 0.5% methylcellulose aqueous stock.

2.2.4 Cell Growth Assays

Cell growth was assessed using crystal violet assay as described previously [273]. Briefly, cells were plated in 24-well plates, treated with escalating doses of cyclopamine or TAK-441 for 48-72 hrs, and stained with crystal violet. The absorbance was determined by optical density at 562 nm and impact of treatment was calculated as the percent optical density of time-matched vehicle-treated cells.

2.2.5 Western Blot Analysis

After the treatments indicated in the legend, detached and attached cells were lysed in RIPA buffer (50 mM Tris, pH 7.2, 1% NP-40, 0.1% deoxycholate, 0.1% SDS, 100 mM NaCl, 1X Roche complete protease inhibitor cocktail). Whole cell lysate protein (30 µg/lane) were subjected to SDS-PAGE, transferred to nitrocellulose filters, blocked in PBS containing 5% non-fat milk powder for 1 hr, and incubated at 4°C overnight with primary SHH and DHH antibodies described above, anti-vinculin mouse monoclonal Ab (Sigma Chemical Co.), anti-GLI1 and anti-GLI2 rabbit polyclonal Ab (Cell Signalling Technology, Danvers, MA). Immunoblots were incubated with horseradish peroxidase-conjugated anti-mouse or rabbit IgG antibody (Santa Cruz Biotechnology) and visualized with enhanced chemiluminescence Western blotting analysis system (Amersham Life Science, Arlington Heights, IL) using a DYVERSITY imaging system with GeneSnap Ver. 7.04 software (SynGene, Cambridge, England).

2.2.6 Assessment of *In Vivo* Tumour Growth

For *in vivo* xenograft studies, 2×10^6 LNCaP cells were suspended in 0.1 ml Matrigel (BD Biosciences, San Jose, CA) and inoculated subcutaneously in the flank of 6- to 8-week-old male athymic nude mice (Harlan Sprague-Dawley, Inc., Indianapolis, IN) via a 27-gauge needle under

isoflurane anesthesia. Gonadally intact animals harboring xenografts that grow to $>150 \text{ mm}^3$ with circulating PSA levels of $>100 \text{ ng/ml}$ between 4 and 6 weeks of inoculation were castrated and randomized to 10 mg/kg TAK-441 or 25 mg/kg TAK-441 or vehicle orally treated once daily for 7 days. Each experimental group consisted of 8 mice. Tumour volumes and body weights (BW) were measured once weekly. Serum PSA levels were determined weekly using a total PSA immunoassay on a Cobas e 411 immunoassay analyzer (Roche Diagnostics PSA, Indianapolis, IN), according to the manufacturer's instruction. Data points were expressed as average tumour volume \pm SEM or average PSA concentration \pm SEM. All animal procedures were performed according to the guidelines of the Canadian Council on Animal Care and with appropriate institutional certification.

2.2.7 Quantitative RT-PCR

Total RNA was extracted from cultured cells at times and conditions indicated in respective figure legends using TRIzol reagent (Invitrogen-Life Technologies, Inc.). Two micrograms of total RNA was reverse transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Brandfort, CT). Real-time monitoring of PCR amplification of cDNA was performed using the following primer pairs and probes: SHH (Hs00179843_m1), DHH (Hs00368306_m1), human GLI1 (Hs00171790_m1), mouse GLI1 (Mm00494645_m1), human GLI2 (Hs01119974_m1), mouse GLI2 (Mm01293111_m1), human Ptch1 (Hs00181117_m1), mouse Ptch1 (Mm00436026_m1) on the ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems) with TaqMan PCR Master Mix (Applied Biosystems, Foster City, CA). Target gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels in respective samples as an internal standard, and the comparative cycle threshold method

was used to calculate relative quantification of target mRNAs. Each assay was carried out in triplicate.

2.2.8 Statistical Analysis

All *in vitro* data were assessed by ANOVA and Mann-Whitney post hoc test. Tumour volume and serum PSA levels were compared using the Kruskal–Wallis test. Overall survival was analyzed using Kaplan–Meier curves, and statistical significance between the groups was assessed with the log-rank test (GraphPad Prism). Levels of statistical significance were set at $p < 0.05$.

2.3 Results

2.3.1 SHH and DHH Expression are Associated with Hormone Responsiveness and CRPC Progression

Expression of the three Hh ligands in the PCa microenvironment of untreated, short term NHT, long term NHT, and CRPC disease was examined by IHC. As previously described, IHH was predominantly expressed in prostatic stroma [243]. In our PCa specimen series, we observed increased IHH expression in stroma in response to castration; however, overall IHH IHC intensity was indistinguishable between short and long-term NHT and CRPC specimens. Conversely, SHH and DHH were expressed predominantly in the adenocarcinoma cells (Figure 2-1). In general agreement with previous reports [241, 243], expression of SHH and DHH was relatively weak in untreated PCa and tend to be increased with disease progression, however, in our cohort, we observed a marginal increase in expression in a minority of the short-term NHT specimens, but the average expression level was not distinguishable from that of the untreated cohort. While SHH and DHH expression levels trended higher in long-term NHT-treated specimens, only DHH expression was found to be significantly up-regulated. In the CRPC cohort, SHH levels were

significantly elevated relative to naïve and NHT cohorts while DHH levels declined such that they were indistinguishable from the levels observed in the untreated and short-term NHT cohorts. These results indicate that SHH and DHH up-regulation occurs as a consequence of long-term androgen ablation but that as the cancers become CRPC, SHH expression persists while DHH levels decline suggesting that SHH may be important in sustaining CR state.

2.3.2 Smoothened Antagonism does not Affect SHH Expression and LNCaP Viability *In Vitro*

Basal expression of Hh ligands was assessed in a panel of PCa cell lines to determine if they recapitulated Hh expression of the disease states they are presumed to model. (Figure 2-2A). SHH and DHH proteins were readily detected in the androgen-responsive LNCaP and C4-2 cell lines. In the androgen receptor–negative cell lines, DU145 and PC3, SHH protein expression was higher than in the LNCaP and C4-2 lines while DHH expression was very low to undetectable. While potentially coincidental, these observations are consistent with patient data indicating that LNCaP and C4-2 cells reflect androgen responsive PCa and express both SHH and DHH while DU145 and PC3 cells reflect restricted SHH expression of CRPC.

We first assessed the impact of TAK-441 on viability of androgen-deprived LNCaP cells (Figure 2-2B). In a dose-response analysis, we observed no decrease in cell viability at up to 100 times its reported *in vitro* SMO antagonistic IC₅₀ of 5 nM [272]. Similarly, in a time-course analysis, we found that neither TAK-441 nor cyclopamine at their respective IC₅₀s affected viability of androgen-deprived LNCaP cells (Figure 2-2D). We were, however, able to recapitulate the previously observed sensitivity of LNCaP cell growth to cyclopamine with an IC₅₀ of 10 μM [274] under standard culture conditions (Figure 2-2B). We also noted that SHH protein levels were

increased in LNCaP cells cultured in CSS for 12 days ~4-fold, while GLI1 expression exhibited an apparent 2-fold increase; yet neither TAK-441 nor cyclopamine at their respective IC₅₀s affected expression of SHH or GLI1 cultured under these conditions (Figure 2-2E). The lack of impact on cell viability, SHH and GLI1 expression is consistent with the conclusion of Chen et al [266] that, androgen-deprived LNCaP cells are not sensitive to disruption of autocrine canonical Hh signalling *in vitro*. SHH expression under androgen-deprived conditions resulted in a 5- and 10-fold increase in Shh mRNA expression when cultured for 9 and 15 days in CSS, respectively (Figure 2-2F).

2.3.3 TAK-441 Treatment Inhibits LNCaP Xenograft Growth and Delays CRPC

While previous studies have indicated that Hh signalling antagonism can suppress PCa xenograft growth, none have assessed whether SMO antagonism affects castration-resistant progression. We therefore evaluated whether oral delivery of TAK-441 could affect castration-resistant progression of LNCaP xenografts established subcutaneously in male athymic mice (Figure 2-3). Once serum PSA levels exceeded 100 ng/ml, animals were castrated and randomized to 10 mg/kg or 25 mg/kg TAK-441 or vehicle treatment cohorts. While tumour growth rate was decreased for 2 weeks post-castration in the vehicle treated cohort before regaining pre-castration growth rate, in both of the TAK-441 treated cohorts, tumour volume remained essentially unchanged for 8 weeks (Figure 2-3A). Average tumour growth rate of mice treated with TAK-441 at 10 mg/kg and 25 mg/kg was 16% and 9% of vehicle treated mice (Table 2-2; vehicle: 164.3 ± 31.5 mm³/week, TAK-441 10 mg/kg: 26.1 ± 21.6 mm³/week, TAK-441 25 mg/kg: 15.0 ± 12.0 mm³/week). A waterfall plot of tumour volume change for each mouse analyzed in this study shows that all but one vehicle treated tumour grew more than all of the TAK treated tumours and that in half of the TAK-441 treated mice either no growth, or regression of the xenografts was

observed (Figure 2-3B). Mitotic activity of LNCaP tumours from TAK-441-treated mice collected at end of study, was measured by Ki67 IHC analysis of AR-positive cells (Figure 2-3 C and D), Consistent with delayed tumour growth, mitotic activity was significantly lower in the TAK-441-treated samples than in the vehicle treated tumours (~20% suppressed relative to vehicle treated samples while androgen receptor staining index was indistinguishable between cohorts).

Serum PSA levels are used as a surrogate for tumour load and for castration-resistant progression of LNCaP xenografts. We operationally define an LNCaP xenograft as being castration-resistant at the time following castration that serum PSA levels equal pre-castrate PSA level [275]. Paralleling tumour volume results above, TAK-441-treatment significantly delayed time to castration-resistance in castrated mice bearing LNCaP xenografts, (Figure 2-4A). Consistent with previous studies examining changes in PSA production by LNCaP xenografts following castration, in the vehicle-treated cohort, average PSA level reached a nadir at 1 week following castration and began rising thereafter, reaching pre-castration PSA levels 3.5 weeks after castration. In both TAK-441-treated cohorts, average post-castrate PSA levels declined dramatically after 1 week and achieved nadir at 2 weeks. While average PSA levels of the TAK-441-treated cohorts began rising slowly thereafter, they did not achieve pre-castrate PSA levels until 8 weeks after castration. The waterfall plot at 8 weeks post-castration shows that all but one vehicle treated mouse exhibited increased PSA while 6 and 5 of the 8 mice in the 10 and 25 mg/kg TAK-441-treated cohorts, respectively exhibited a decrease in serum PSA levels relative to time of castration (Figure 2-4B and Table 2-3). In the TAK-441-treated cohorts, those animals that exhibited an increased serum PSA level 8 weeks following castration, show a fold-change measured that was less than the median observed in the vehicle cohort. Consequently, progression-free survival of both the vehicle vs. TAK-441 10 mg/kg and the vehicle vs. TAK-441 25 mg/kg

groups were significantly prolonged in the TAK-441-treated group (Figure 2-4C). No significant effect on animal body weight was observed between vehicle and TAK-441 treated mice bearing LNCaP xenograft during this treatment period (Figure 2-4D). These studies indicate that TAK-441 profoundly suppressed castration-resistant progression of LNCaP xenografts.

2.3.4 TAK-441 Selectively Suppresses Murine Hh Target Gene Expression in LNCaP

Xenografts

Since TAK-441 treatment did not affect LNCaP viability or SHH production under androgen-deprived conditions *in vitro*, but profoundly suppressed xenograft growth following castration, we suspected that TAK-441 might function by affecting host response to LNCaP-derived SHH in the tumour microenvironment. We therefore performed qPCR analysis for expression of human and mouse orthologues of GLI1, GLI2 and Ptch1 in the vehicle and TAK-treated LNCaP xenograft specimens (Figure 2-5). As predicted from the *in vitro* analysis, expression of human GLI1, GLI2, and Ptch1 was unaffected in the TAK-441-treated cohorts, however expression of murine GLI1, GLI2 and Ptch1 was significantly lower in the 25 mg/kg cohort and murine GLI1 and Ptch1 were lower in the 10 mg/kg cohort when compared to their respective expression in the vehicle cohort. These results suggest that the pharmacodynamic effect of TAK-441 is exerted through inhibition of paracrine Hh signalling.

2.4 Discussion

Several recent reports have shown that Hh signalling pathway activation is an important regulator of growth, survival, and metastatic potential of PCa [213, 239, 241, 243, 264, 270]. A critical step in Hh signalling is binding of SHH to the trans-membrane receptor Ptch1, an action that relieves repression of another trans-membrane protein, SMO, and triggers transcriptional

activation of Hh target genes including *GLI1*, *Ptch1* and *Hip*. Up-regulation of *SHH* and *DHH* expression in hormone ablation-treated PCa [243] and increased expression of Hh ligands in LNCaP cells under androgen-depleted conditions [266] are consistent with a model in which Hh signalling is part of an adaptive process that facilitates PCa cell survival during androgen deprivation. In this work, we confirm that *SHH* and *DHH* expression are elevated in PCa adenocarcinomas responding to NHT and once they become castrate-resistant. Our results differ from previous reports [243] in that we observe a trend for increased *SHH* and *DHH* during NHT but only a significant increase in *DHH* expression in those men treated for >6 months. Additionally, only *SHH* levels were significantly elevated in the CRPC cohort.

These clinical observations suggest that targeting Hh signalling may be part of an effective management scheme for advanced PCa, but mechanisms for how Hh signalling might impact PCa remain to be resolved. While in tumours such as basal cell carcinomas and medulloblastoma loss-of-function *Ptch* mutations and/or gain-of-function *SMO* mutations, along with potential *Ptch*/*Smo*-independent mechanisms for *GLI* activation have been reported [276, 277], evidence to date suggests that, with the exception of the minority of cases harbouring *SuFu* mutations [239], the Hh ligands are important for either autocrine or paracrine signalling in PCa. Paracrine Hh signalling is an important growth mediator of several solid tumours [238, 278]. Furthermore, paracrine Hh signalling is important for embryonic development of the prostate from the UGS where *SHH* produced by the developing epithelium acts on adjacent mesenchymal cells to activate the Hh signal transduction pathway and elicit paracrine effects on epithelial proliferation and differentiation [196, 207, 210, 279].

A number of PCa models have been used to assess the role of Hh signalling in regulation of cell growth. We show that the androgen responsive lines, LNCaP and C4-2 produce both SHH and DHH and that the androgen receptor-negative lines, DU145 and PC3 predominantly express SHH, indicating that these lines generally recapitulate the expression profile for these Hh ligands in cancer progression. While early studies concluding that cyclopamine decreased growth of LNCaP, VCaP, PC3, DU145 and CWR22Rv1 PCa cells *in vitro* [205, 213, 280], were suggestive of an autocrine mechanism in which tumour cell-derived Hh ligands would drive SMO-dependent tumour growth, later reports demonstrated that this inhibition of PCa cell lines (LNCaP, PC3, CWR22Rv1) proliferation is not accompanied by the expected changes in expression of the endogenous target genes, GLI1 and PTCH1 [265, 281]. Because, these studies were performed in FBS-containing media, the impact of androgen withdrawal on growth of the androgen-responsive lines, LNCaP and CWR22Rv1 could not be assessed. Our results demonstrate that while cyclopamine suppressed LNCaP growth when cultured in FBS-containing media, viability was unaffected when cultured in CSS-containing media. This result is consistent with observations of Sirab et al. [280] indicating that cyclopamine-responsiveness may be regulated by androgen receptor activation.

While the specificity of cyclopamine as a SMO antagonist has been questioned [238, 282], the highly specific and selective SMO inhibitor, TAK-441 did not affect LNCaP viability under FBS or CSS culture conditions nor did it affect up-regulated expression of SHH under androgen-deprived conditions indicating that this androgen-responsive PCa model is not responsive to selective SMO antagonist treatment. We therefore postulate that, the selective impact of TAK-441 on LNCaP xenograft growth is due to up-regulated SHH expression by the tumours in response to castration and the subsequent activation of Hh signalling in tumour-associated murine stroma. The

recent report demonstrating that *in vitro* growth of LNCaP cells engineered to express SHH can be driven by activation of TGF β expression by rodent urogenital mesenchyme [283] corroborate our results indicating that LNCaP cells are not directly responsive to SMO antagonism and support a model for paracrine Hh signalling in PCa.

A number of botanical and synthetic agents that interfere with Hh signalling have been described (reviewed in [284]). Reports of *in vitro* IC₅₀s for these agents often vary considerably depending on the systems tested. At present, eight small molecule inhibitors targeting the Hh pathway are in clinical development for the management of a variety of cancers (reviewed in [285], [286]). The agent described here, TAK-441, has an *in vitro* IC₅₀ comparable to the lowest reported to date [287]. Importantly, the SMO antagonist, GDC-0449 (vismodegib), has been FDA approved for the treatment of adults with advanced basal cell carcinoma and is in clinical trials for several additional indications. While results such as these indicate that Hh inhibitors are valid therapeutic approaches for cancer management, few have been rigorously assessed in PCa models.

Hh pathway targeting in PCa remains in early development. In PCa models, a diverse array of botanical agents were reported to antagonize GLI activation and TRAMP tumorigenesis with IC₅₀s ranging from 1 to 25 μ M, however whether these effects were directly related to SMO antagonism was not clarified [288]. Two agents are in clinical trials for PCa. In preclinical testing, while GDC-0449 treatment suppressed PC3 *in vitro* and xenograft growth, it did not affect MDA PCa 118b xenograft volume increase, but was shown to decrease tumour cell proliferation rate and to down-regulate expression of the Hh-activated transcriptional target genes, GLI1 and Ptch1 by stromal cells [270]. However, both of these PCa models are androgen receptor-negative. In addition previous work with CWR22Rv1 xenografts in female hosts cannot be taken as mimicking

tumour growth under castrate conditions as these cells harbour the androgen receptor mutation H874Y that renders the receptor promiscuous to non-androgen steroids such as estrogen [289]. The other agent, itraconazole, is an antifungal ascribed with a variety of activities including Hh antagonist activity that effectively suppresses growth of several PCa models and has been reported to have modest antitumour activity in men with CRPC [290]. In this report, we demonstrate for the first time a pre-clinical therapeutic effect of the selective SMO antagonist, TAK-441 on castrate resistant progression in PCa using the LNCaP xenograft model. However, the profound impact of TAK-441 treatment on tumour volume and circulating PSA increase following castration was contrasted by a modestly suppressed mitotic index (20%) and indistinguishable apoptotic and necrotic indices. It must be noted that these physiologic indices are measured using end of study tumours. It is unknown how these indices, or other relevant causes of tumour growth suppression such as necrosis and autophagy, might change during the course of study. Nevertheless, targeting SMO by daily oral delivery of TAK-441 following castration significantly enhanced the effect of castration on growth and PSA production by the LNCaP xenografts and thus prolonged the time to castrate-resistant progression.

Consistent with results from the GDC-0449 study on MDA PCa 118b xenografts, LNCaP xenografts from TAK-441-treated mice exhibited down-regulation of murine Hh target genes, GLI1, GLI2 and Ptch1, but not of their human orthologues. These are important pharmacodynamic indicators of interruption of Hh signalling. Ptch 1 and GLI 1 are well described transcriptional targets of Hh signalling and GLI 2 is a key mediator of Hh transcriptional signalling whose expression level is regulated by Hh-mediated suppression of processing and degradation [171]. While there is evidence for expression of Hh target genes in both tumour [213] and tumour-adjacent stromal cells [212], such contradictory results might be resolved by a shift in Hh signalling

from paracrine to autocrine mechanisms during disease progression [241]. These results indicate that LNCaP cells represent a paracrine model of Hh signalling in PCa similar to that detected in a subset of other cancers in which the tumour stroma provide a Hh-mediated role in supporting tumour growth [291] that mimics embryogenic and wound healing mechanisms of Hh signalling [196, 201]. The precise nature of the stromal-mediated events that mediate tumour growth remain to be defined, induction of angiogenic and epithelial-to-mesenchymal transition mediators have been reported [177, 241, 269] as candidate factors.

In conclusion, the current study shows for the first time that progression of CRPC *in vivo* can be delayed by targeting SMO using the selective SMO antagonist, TAK-441, by disrupting paracrine Hh signalling in the context of the tumour environment. TAK-441 is currently in an ongoing clinical trial, and based on these preclinical animal model results, may also hold promise for treatment of CRPC progression.

Figure 2-1 A.

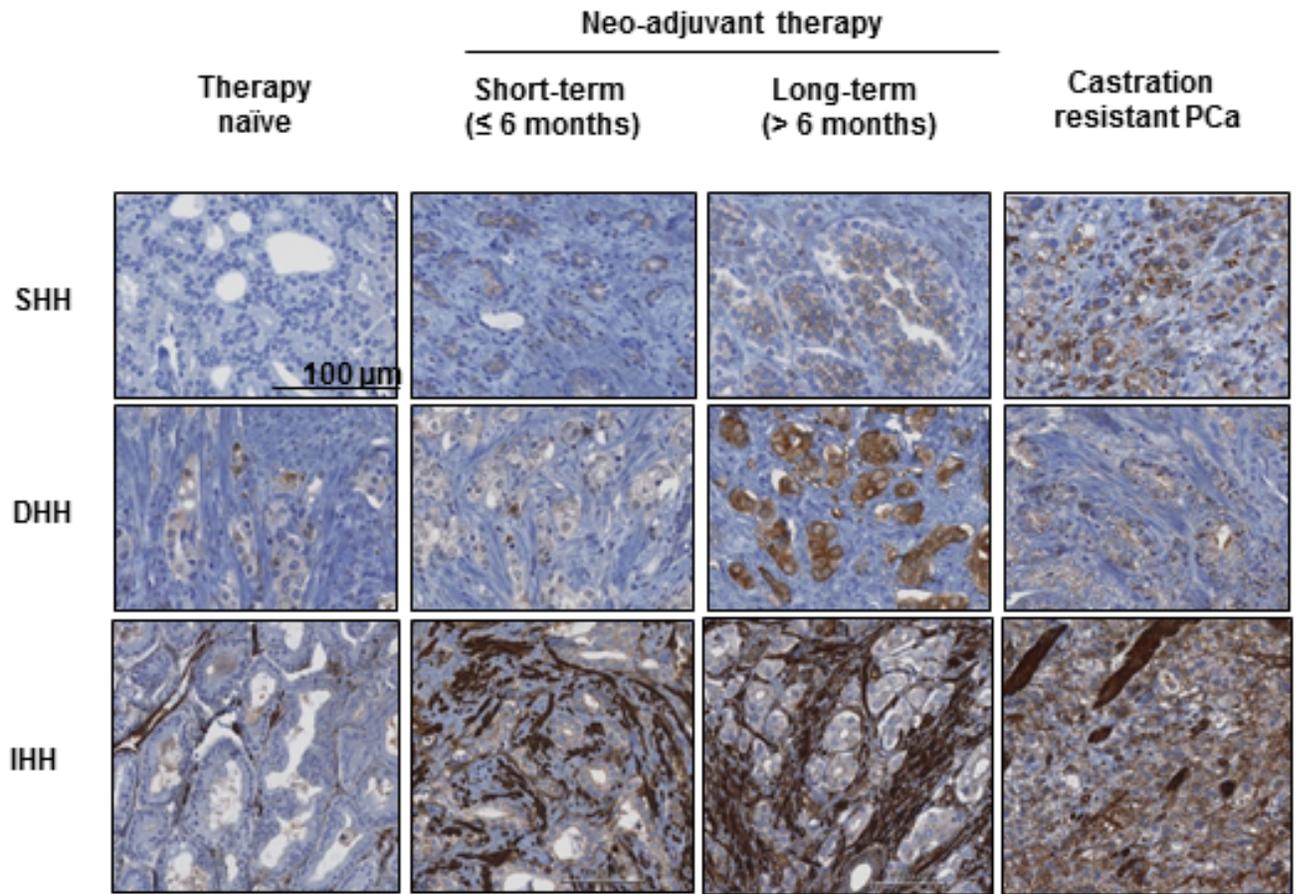


Figure 2-1 B.

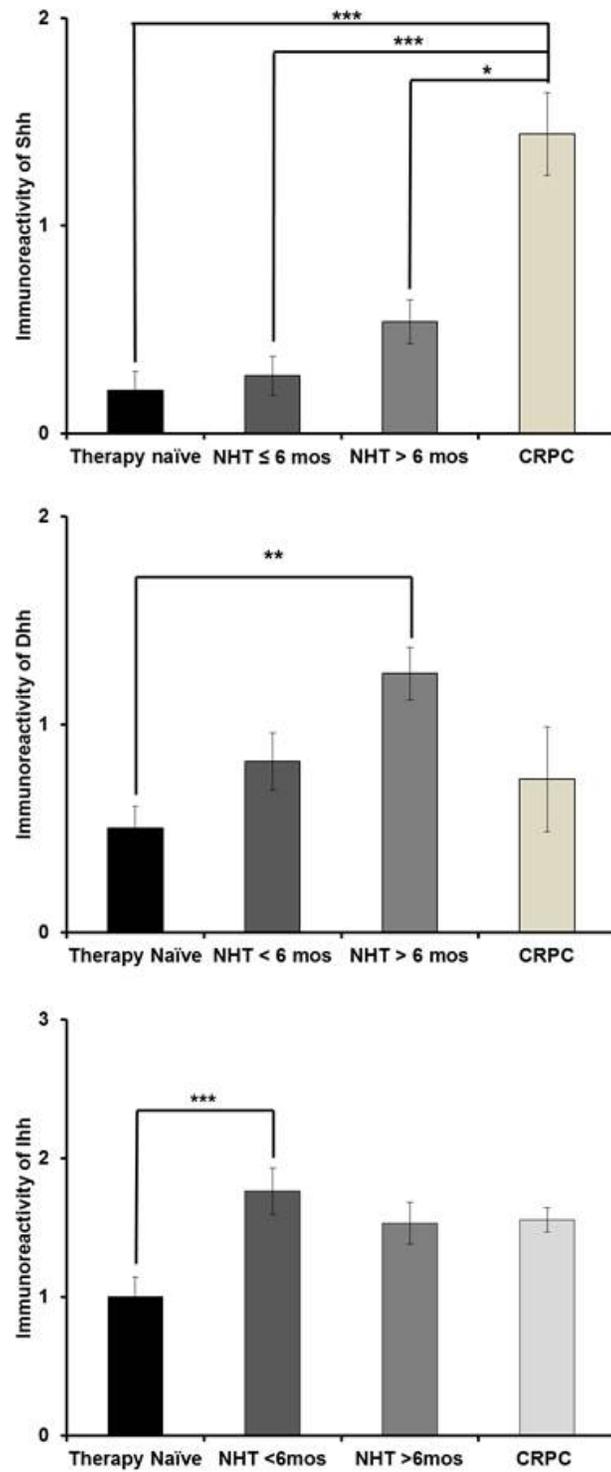


Figure 2-1. Expression of hedgehog ligands (SHH, and DHH) are elevated in long-term NHT prostatectomy and CRPC specimens.

A. Representative IHC photomicrographs are presented showing the extent and intensity of SHH, DHH, and IHH immunoreactivity in therapy naïve, post short-term (≤ 6 months) and long-term NHT (> 6 months) and CRPC tissue stained in serial sections (scaler = 100 μm). **B.** The mean intensity score of SHH-, DHH-, and IHH-positive cells expressed as the average \pm SEM for each tissue cohort as detailed in methods and materials. Expression of both proteins is not normally distributed in any cohort and Dunn's multiple comparisons test indicates that significant median variation for SHH and DHH expressions are calculated between each groups, * , $p < 0.05$, **, $p < 0.01$, *** $p < 0.001$.

Figure 2-2 A.

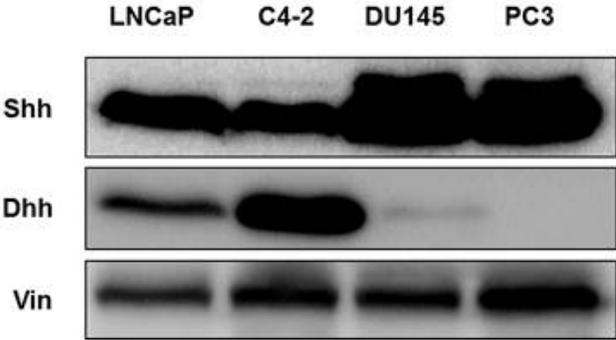


Figure 2-2 B.

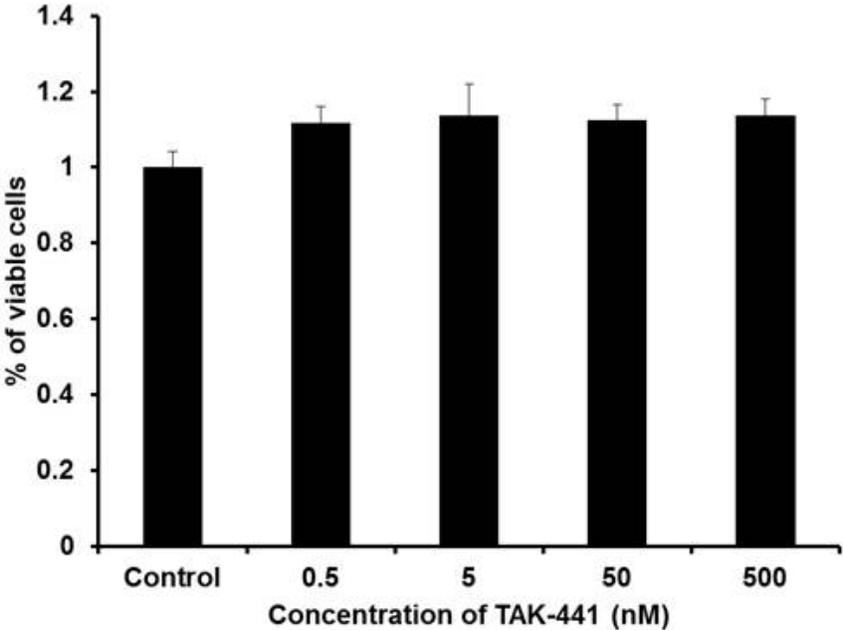


Figure 2-2 C.

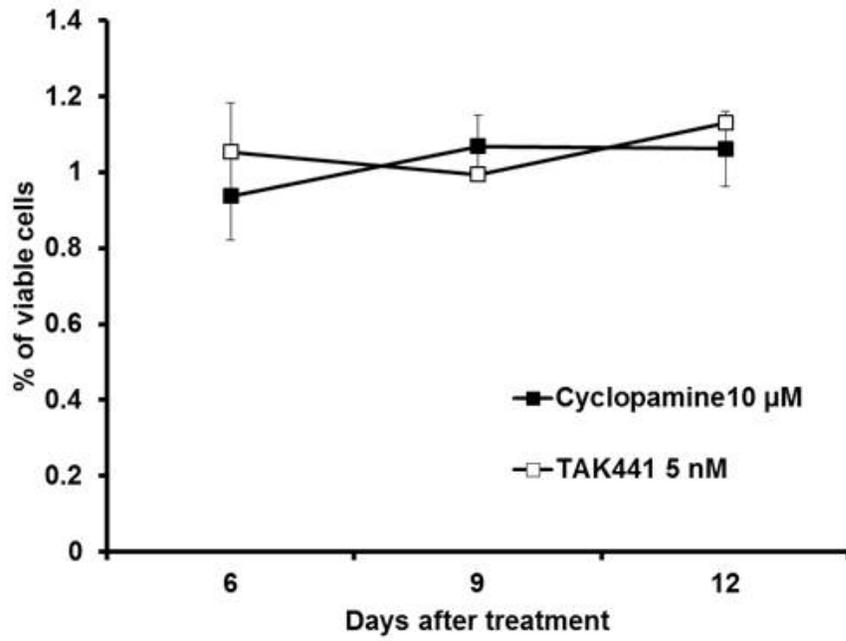


Figure 2-2 D.

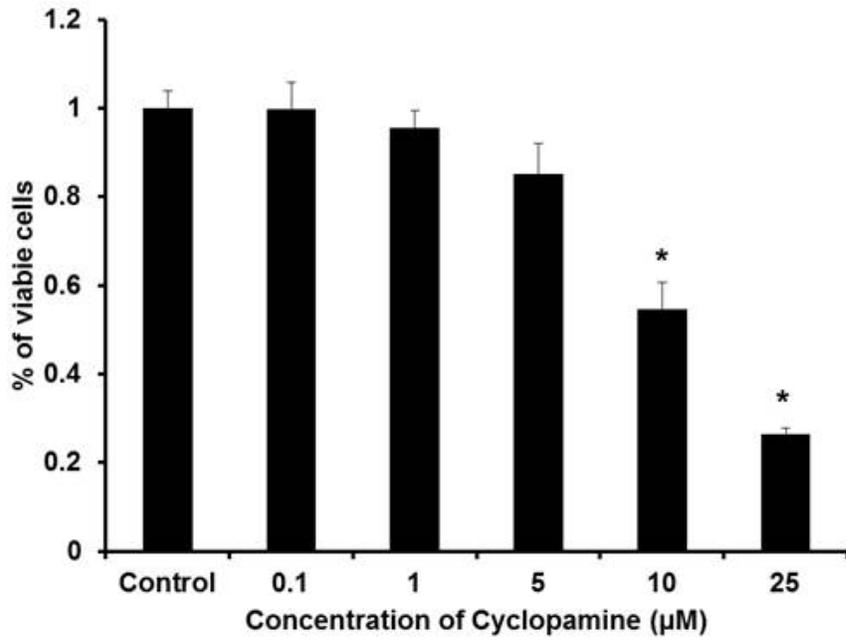


Figure 2-2 E.

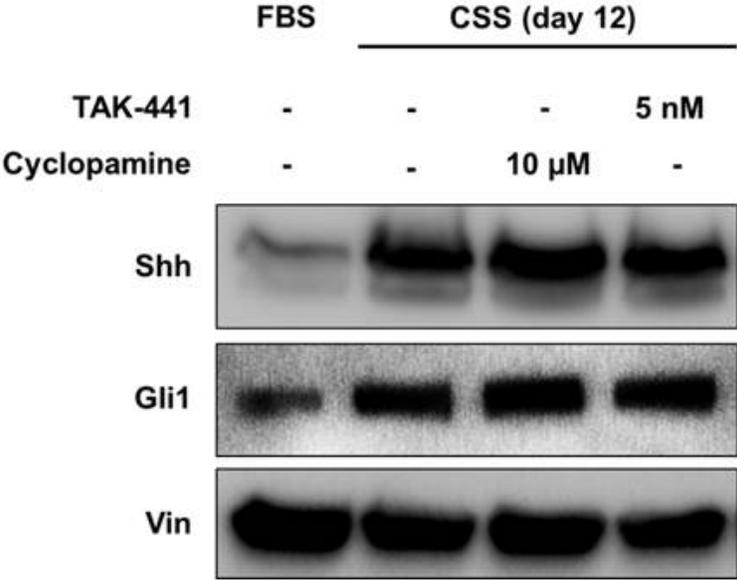


Figure 2-2 F.

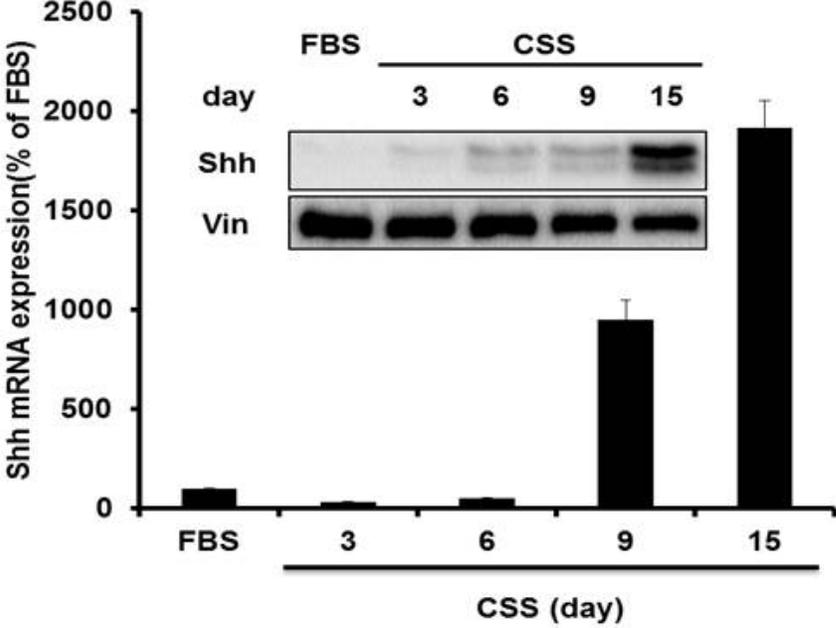


Figure 2-2 Expression of SHH and DHH in human prostate cancer cell lines and effect of TAK-441 on *in vitro* growth of LNCaP cells under androgen stimulated or depleted conditions.

A. Western blot analysis of SHH and DHH expression in LNCaP, C4-2, DU145 and PC3 human PCa cell lines under standard culture conditions. **B.** LNCaP cells were cultured in FBS media, and treated by indicated TAK-441 concentrations for 2 days. Cell viability was determined by crystal violet assay. **C.** LNCaP cells were cultured in CSS for 6, 9, 12 days, and treated by indicated concentration of cyclopamine and TAK-441 for the last 2 days of treatment. Cell viability was determined by crystal violet assay. **D.** LNCaP cells were cultured in FBS media, and treated by indicated cyclopamine concentrations for 2 days. Cell viability was determined by crystal violet assay. **E.** LNCaP cells were cultured in FBS and CSS media for 12 days and treated by cyclopamine or TAK-441. Whole cell lysates were extracted from cultured cells and SHH, GLI1, and Vinculin protein levels were analyzed by Western blotting. Histograms are densitometric scans of 3 independent western blots normalized to vinculin and. *, $P < 0.05$ vs FBS condition. **F.** Real-time RT-PCR analysis for mRNA expression of SHH gene in LNCaP cell cultured in FBS and androgen-free media is shown as the histogram. Experimental groups are as indicated under each data column, representing relative levels of expression normalized to the mRNA level of GAPDH. Calculations are expressed as mean \pm SEM. Insert is representative Western-blot analysis for SHH proteins in LNCaP cells cultured in FBS and androgen-free media.

Figure 2-3 A.

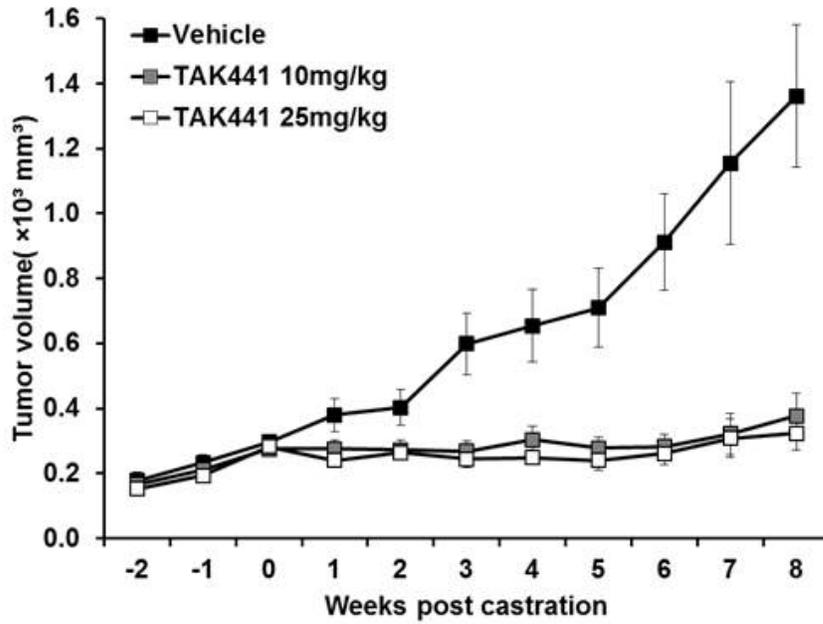


Figure 2-3 B.

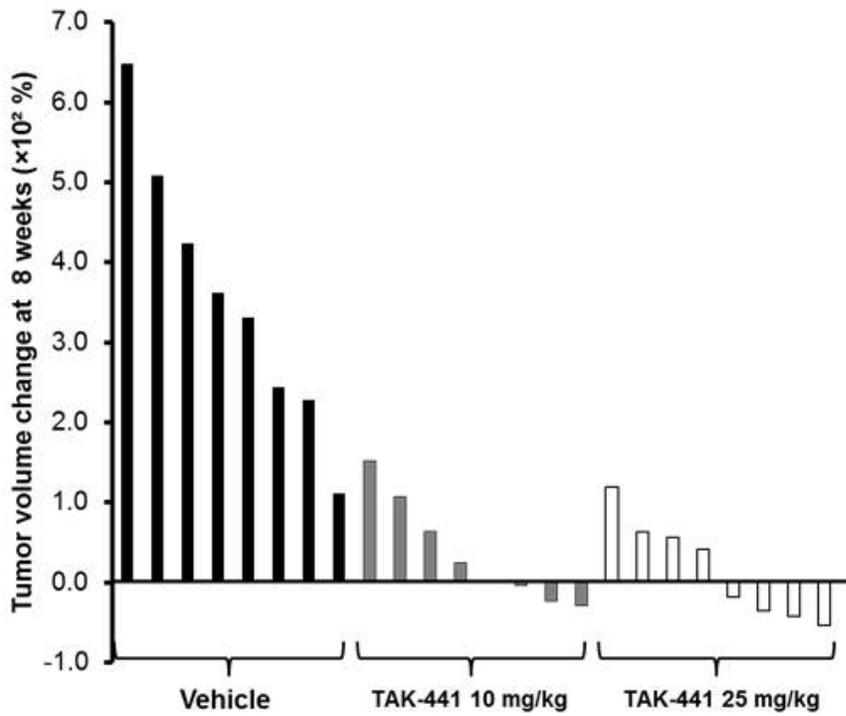


Figure 2-3 C.

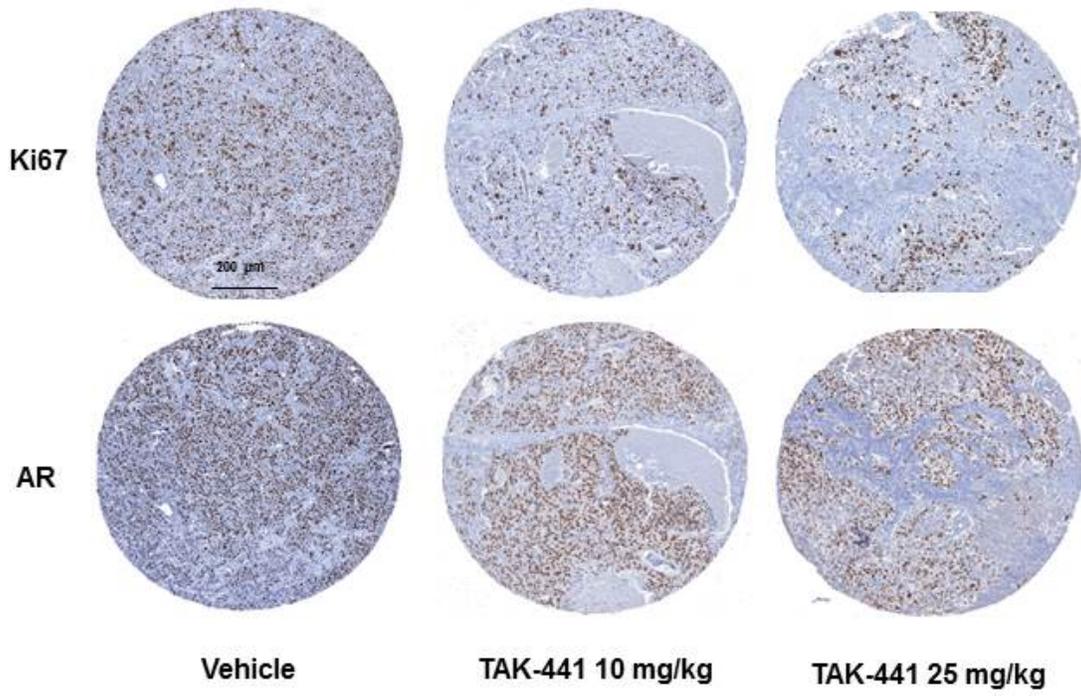


Figure 2-3 D.

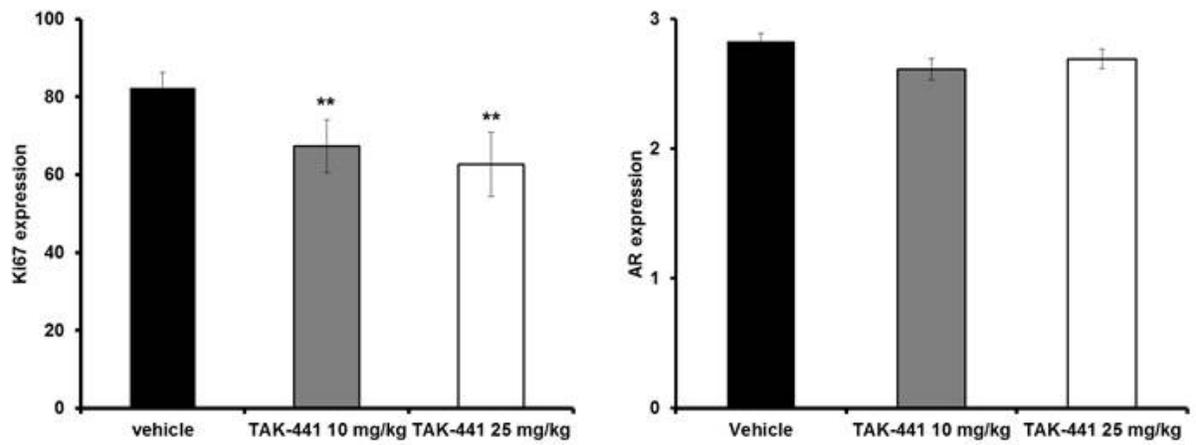


Figure 2-3 Smoothened antagonist suppresses post-castration LNCaP xenograft growth and inhibited mitotic activity.

LNCaP cells were inoculated subcutaneously and when PSA values exceeded 100 ng/ml, mice were castrated and randomly selected for treatment with 10 mg/kg or 25 mg/kg TAK-441- or vehicle (0.5% methylcellulose/H₂O) orally treated once daily. **A.** Xenograft growth curve: Data points represent mean tumour volume of each group of 8 mice \pm SEM. **B.** Waterfall plot showing the percent change in volume for each tumour after 8 weeks. Mitotic activity in the xenografts was evaluated by Ki67 immunohistochemical analysis. AR immunostaining was specific for LNCaP cells in xenograft tissue. **C.** Representative images of Ki67 and AR staining in each group. Scaler = 200 μ m

D. Quantification of Ki67, and AR IHC intensity expressed as the average % positive adenocarcinoma nuclei for each group \pm SEM. ** Ki67 expression in TAK-441 treated tumour was significantly lower than vehicle treated tumour ($p < 0.05$).

Figure 2-4 A.

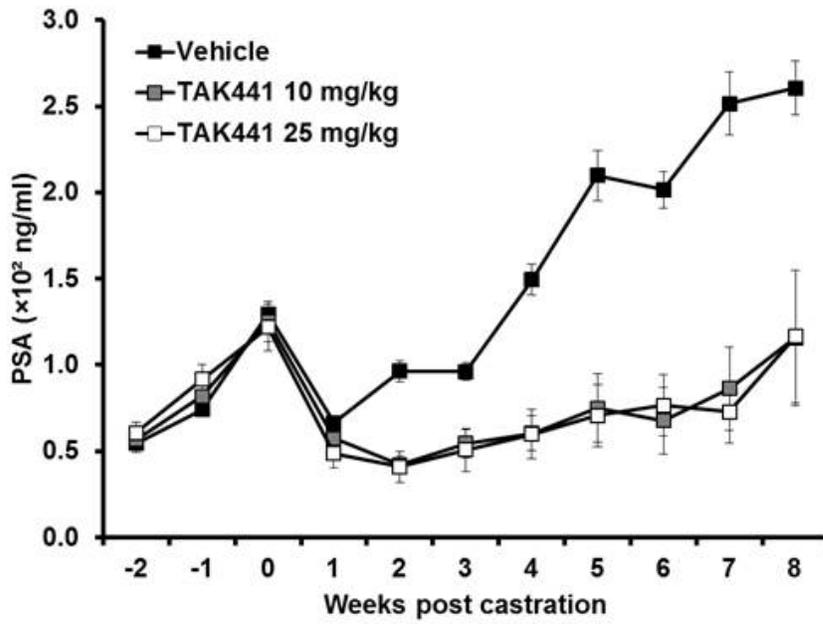


Figure 2-4 B.

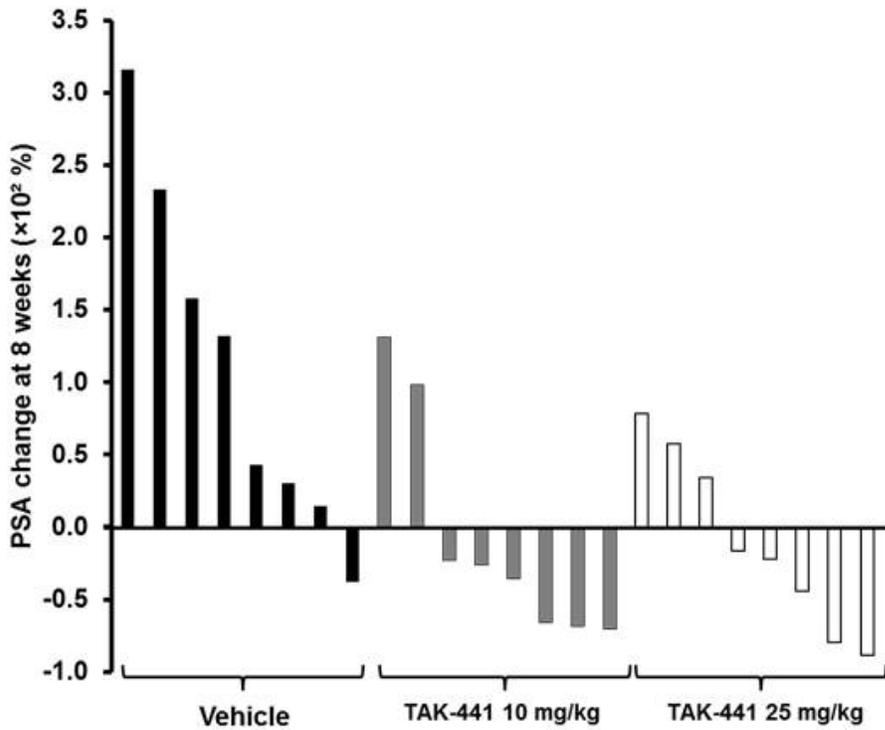
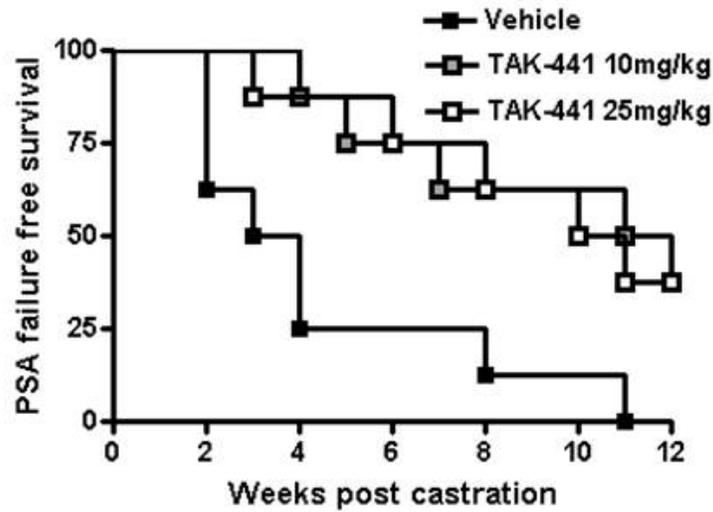


Figure 2-4 C.



Kaplan-Meier statistic analysis (vs Vehicle)

	TAK-441 10 mg/kg	TAK-441 25 mg/kg
Logrank Test		
Chi square	7.298	5.813
P value	0.0069	0.0159
Median survival		
PSA failure	Vehicle 3.500	Vehicle 3.500
PSA failure	TAK-441 10 mg/kg 11.50	TAK-441 25 mg/kg 10.50
Ratio	0.3043	0.3333
95% CI of ratio	-0.02279 to 0.6315	0.006195 to 0.6605
Hazard Ratio		
Ratio	3.632	3.254
95% CI of ratio	1.645 to 22.86	1.340 to 17.07

Figure 2-4 D.

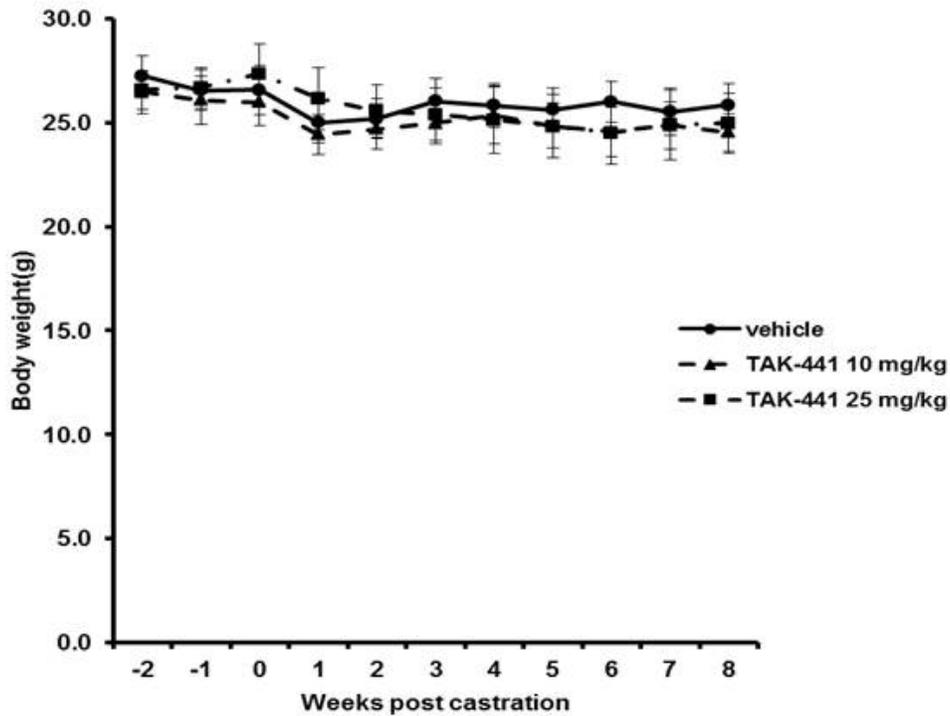


Figure 2-4 Smoothened antagonist delays CRPC progression.

TAK-441 was administered beginning at the time of castration as described in Materials and Methods. Serum samples were obtained from the tail vein of the mice once weekly to measure serum PSA. **A.** PSA curve: Data points represent mean PSA of each group of 8 mice \pm SEM. **B.** Waterfall plot showing the percent change in PSA for each tumour after 8 weeks post castration. **C.** Kaplan Meier analysis in which PSA failure is determined by the time required for serum PSA levels to equal the pre-castrate level. (Vehicle vs. TAK-441 10 mg/kg groups: $p = 0.0159$, vehicle vs. TAK-441 25 mg/kg groups: $p = 0.0069$). **D.** Body weight of the mice over the course of the xenograft studies did not vary significantly. The average weight of each mouse was included once in the calculation of mean \pm SEM for 8 mice in each group.

Figure 2-5.

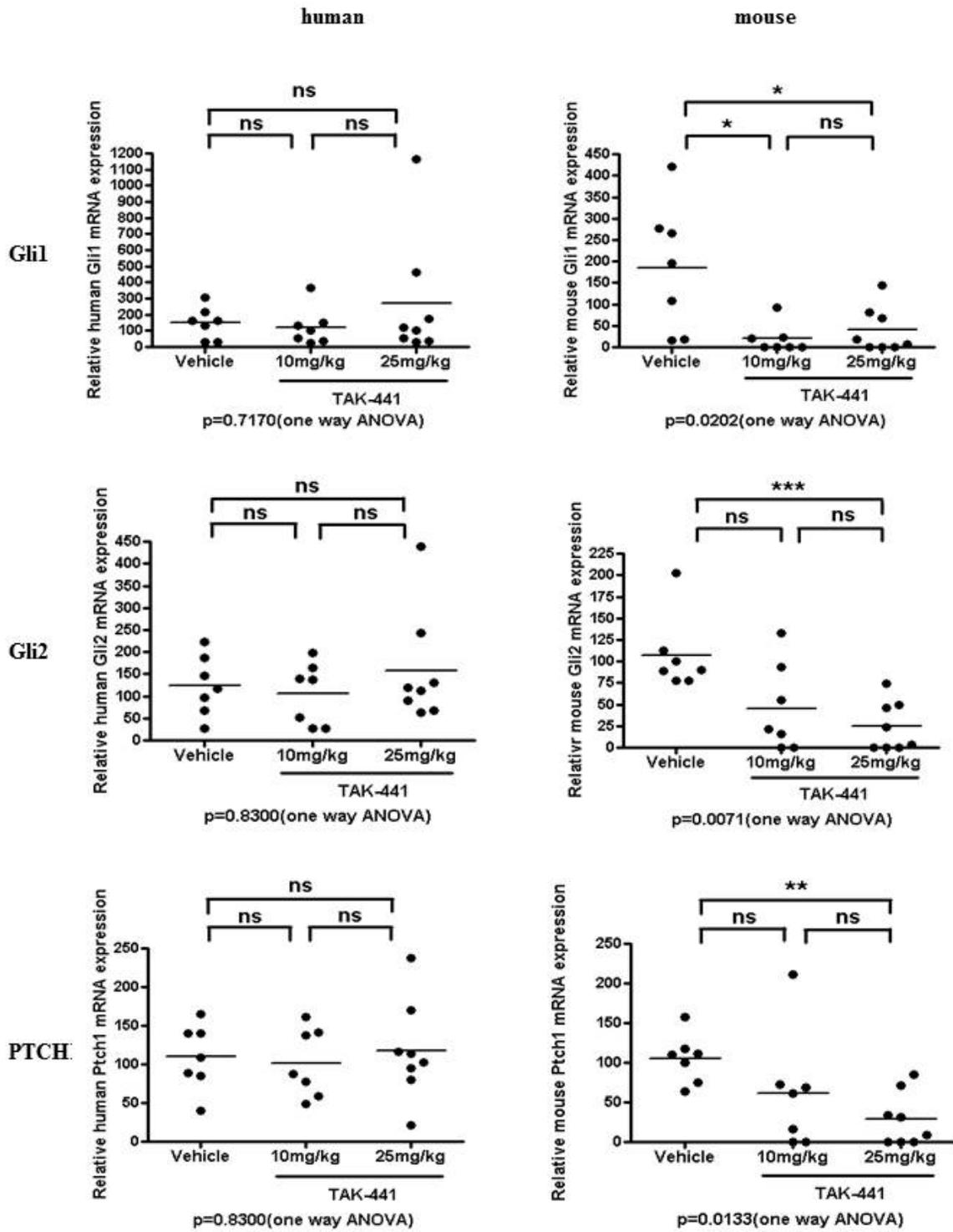


Figure 2-5 Effects of smoothened antagonist treatment on human and mouse Hh signalling in LNCaP xenografts.

Total RNA was extracted from flash frozen xenografts treated with 10 mg/kg and 25 mg/kg TAK-441 or vehicle at end point. Human and mouse GLI1, GLI2 and Ptch1 mRNA expression levels were determined by quantitative RT-PCR. Experimental groups are as indicated under each data column, representing mean \pm SEM relative levels of expression normalized to the mRNA level of GAPDH. Significant difference from vehicle treated group are indicated as *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Table 2-1 Prostate cancer tumour microarray characteristic

	Therapy naïve	NHT*		CRPC**
		Short term	Long term	
Cores (patients)	42 (21)	44 (21)	76 (38)	48 (12)
Pathological stage				
pT2a	6 (3)	8 (4)	4 (2)	2 (1)
pT2C	20 (10)	22 (10)	24 (12)	4 (1)
pT3			2 (1)	
pT3a		4 (2)	14 (7)	2 (1)
pT3b	2 (1)	2 (1)	2 (1)	2 (1)
pT3c	10 (5)	8 (4)	24 (12)	4 (1)
pT4			2 (1)	
Undefined	6 (3)	0	4 (2)	34 (7)
PSA at diagnosis				
Median PSA at D _x (ng/mL)	9.6	9.85	15.63	1.4
Median pathological Gleason score	7.63	7.38	7.06	9.07

*Short term = ≤ 6 months NHT treatment, Long term = > 6 months NHT treatment

**All cores obtained from radical prostatectomy specimens except for CRPC specimens collected by TURP

Table 2-2 Calculation of tumour growth rate for LNCaP xenografts tumour growth rate (mm³/week)

Animal	Vehicle	TAK-441 10 mg/kg	TAK-441 25 mg/kg
1	179.0	16.8	-22.8
2	111.5	0.7	-14.1
3	75.8	-16.7	-16.0
4	152.3	-0.9	17.7
5	300.8	160.2	24.4
6	88.1	-6.3	17.2
7	274.6	44.5	46.5
8	132.6	10.0	67.2
Average	164.3	26.1	15.0
SEM	31.5	21.6	12.0

One way ANOVA $p=0.0015$

Dunn's multiple comparison test

Vehicle vs TAK-441 10 mg/kg : $p < 0.001$

Vehicle vs TAK-441 25 mg/kg : $p < 0.001$

TAK-441 10 mg/kg vs TAK-441 25 mg/kg: $p > 0.05$

Table 2-3 Calculation of PSA growth rate for LNCaP xenografts tumour rate (ng/ml/week)

Animal	Vehicle	TAK-441 10 mg/kg	TAK-441 25 mg/kg
1	23.2	1.5	-2.1
2	17.9	6.8	7.2
3	23.6	3.7	-2.2
4	8.8	-0.9	4.7
5	54.2	34.2	7.7
6	26.9	-4.1	8.9
7	62.1	24.4	26.2
8	77.3	6.9	18.4
Average	36.8	9.1	8.6
SEM	9.2	5.0	3.7

One way ANOVA $p=0.0042$

Dunn's multiple comparison test

Vehicle vs TAK-441 10 mg/kg : $p < 0.05$

Vehicle vs TAK-441 25 mg/kg : $p < 0.01$

TAK-441 10 mg/kg vs TAK-441 25 mg/kg: $p > 0.05$

Chapter 3: Inhibiting Hedgehog Signalling in CRPC Hinders Metastatic Potential and Enhances Chemosensitivity

3.1 Introduction

Despite improvement in PCa diagnosis, management options for patients with advanced forms of the disease are mainly limited to androgen-ablation and palliative chemotherapeutics. However most of the patients will suffer disease progression to CRPC within 2 years of treatment initiation and subsequent lines of chemotherapeutic agents, which eventually leads to chemo-resistance and metastasis [292-297]. Tumour metastasis is responsible for about 90% of deaths of cancer patients, yet remains one of the most poorly understood facets of the pathogenesis and progression of cancer [298]. The adaptive tumour cells that metastasize from the primary tumour often activate normal developmental pathways. These pathways are believed to affect the survival, progression and finally metastasis of these tumour cells [299, 300]. Understanding these pathways will provide critical insight into the mechanisms of tumour metastasis, which provides great promise for the discovery of novel therapeutics and the treatment of advanced metastatic PCa. As described in previous chapters, Hh pathway is a developmental signalling pathway that is necessary for early embryogenesis [301]. The pathway is silenced in most adult tissues, making it a good candidate for targeted therapeutic in PCa.

Aberrant activation of Hh signalling has been implicated in human prostate carcinogenesis. However, the complexity of this pathway is still being unraveled. Its mode of action is fraught with controversy and its role in cancer progression, specifically in advanced PCa unclear. There is an abundance of preclinical data suggesting a benefit to targeting this pathway. However, clinical evidence to date that demonstrates the benefit to patients has been limited to tumours with mutations in the Hh pathway. Early preclinical studies in other malignancies, including breast,

pancreatic, and prostate cancer suggest a potential role for these agents. Yet beneficial clinical results will require further understanding of the mode of action of this pathway as well as thoughtful combination with cytotoxic agents, since the activated Hh pathway is not the primary driver of malignancy [302].

To be able to utilize Hh targeted therapies in the appropriate setting and with maximal efficacy, we need to examine the mode of action as well as the role of Hh signalling in the various stages of PCa. Although there are compelling data supporting both the autocrine and paracrine Hh signalling, as described in the previous chapter; we demonstrate a transition from a paracrine androgen responsive PCa to an autocrine CRPC with elevated SHH, GLI1 GLI2 expression and pronounced activation of Hh signalling. We demonstrate that this transition to an autocrine state, alongside increased activation of the Hh pathway, leads to a more aggressive PCa with increased metastatic potential. These results, suggest that inhibiting the Hh signalling pathway will provide a beneficial therapeutic modality for advanced and metastatic PCa. Hh inhibitors as monotherapy modalities in clinical settings have been effective in tumours with mutations in the Hh pathway, yet in other cancers including solid tumours they have not performed as well [303]. Taken together, our results demonstrate a clear role in utilizing Hh pathway inhibitors as a therapeutic modality in advance and metastatic PCa.

3.2 Material and Methods

3.2.1 Prostate Cancer Immunohistochemistry

A more comprehensive immunohistochemical evaluation of the Hh pathway was performed by examining a total of 354 needle biopsy cores from 190 PCa patient specimens obtained from Vancouver Prostate Centre Tissue Bank. 3 TMA, including a Gleason graded, NHT,

and CRPC/TURP were constructed manually (Beecher Instruments, MD, USA) by punching duplicate cores of 1 mm for each sample. All specimens were from RP except for 28 cores from 12 CRPC samples that were obtained from TURP. From these 3 TMAs, 55 cores were designated benign including BPH samples, 160 cores were untreated, 110 cores were either short or long-term NHT, 42 cores were associated with biochemical PSA recurrence including cores from therapy naïve and NHT patient specimens, and 28 cores obtained from TURP specimens designated as CRPC. TMAs were stained with rabbit monoclonal primary antibodies targeting SHH, IHH, and GLI2 (Abcam, Cambridge, UK), rabbit polyclonal GLI1 and GLI3 (Santa Cruz Biotechnology, Dallas, TX), and goat polyclonal DHH, SMO, and PTCH primary antibodies (Santa Cruz Biotechnology, Dallas, TX). The immunohistochemical staining was conducted by Ventana autostainer model Discover XT (Ventana Medical Systems) with enzyme labeled biotin streptavidin system and solvent resistant diaminobenzidine immunohistochemistry kit (Invitrogen). The staining concentration was examined by optimizing using 3 different concentrations. An independent pathologist scored the intensity of each core on a four-point scale. Descriptively, 0 represents no staining by any tumour cells, 1 represents a faint or focal, questionably present stain, 2 represents a stain of convincing intensity in a minority of cells and 3 represents a stain of convincing intensity in a majority of cells.

3.2.2 Prostate Cancer Cell Lines

The human PCa cell lines, PC3, PC3M, and DU145 cells were maintained in DMEM (Invitrogen, Carlsbad, CA) supplemented with 5% FBS. LNCaP and C4-2 were maintained in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 5% FBS and 2 mM L-glutamine in a humidified 5% CO₂/air atmosphere at 37 °C. Cells lines were verified to be mycoplasma free and

authenticated by STR profiling by the Genetic Resources Core Facility, Fragment Analysis Facility (Johns Hopkins School of Medicine, Baltimore, MD) and comparison to known ATCC fingerprints (ATCC.org).

3.2.3 Hedgehog Pathway Antagonists

The SMO antagonist, TAK-441 was provided by Takeda Pharmaceuticals Inc. (Fujisawa Japan). For *in vitro* studies, TAK-441 was added to media at indicated final concentrations from a 10 μ M stock solution in DMSO. Cyclopamine (Sigma-Aldrich, St. Louis, MO) was dissolved in DMSO as a 10 mM stock and was used for *in vitro* studies at a final concentration of 10 μ M, with its IC₅₀ ranging from 8.79-30 μ M [304, 305].

3.2.4 Western Blot Analysis

Total protein was obtained by lysing the treated cells in RIPA buffer (50 mM Tris, pH 7.2, 1% NP-40, 0.1% deoxycholate, 0.1% SDS, 100 mM NaCl, 1X Roche complete protease inhibitor cocktail). Whole cell lysate protein (30 μ g/lane) were subjected to SDS-PAGE, transferred to nitrocellulose filters, blocked in PBS containing 5% non-fat milk powder for 1 hr, and incubated at 4°C overnight with rabbit monoclonal primary antibodies targeting SHH and GLI2 (Abcam, Cambridge, UK), rabbit polyclonal GLI1 (Santa Cruz Biotechnology, Dallas, TX), and goat polyclonal SMO, and PTCH primary antibodies (Santa Cruz Biotechnology, Dallas, TX).

3.2.5 Quantitative RT-PCR

Total RNA was extracted from PC3 cells at 24 hrs post treatment as indicated using TRIzol reagent (Invitrogen-Life Technologies). Total RNA was reverse transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science). Real-time monitoring of PCR amplification of cDNA was performed using the SHH (Hs00179843_m1) primer pairs and probes

on the ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems) with TaqMan PCR Master Mix (Applied Biosystems). Target gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels in respective samples as an internal standard, and the comparative cycle threshold method was used to calculate relative quantification of target mRNAs.

3.2.6 GLI-Luciferase Reporter Assay

PC3 cells were seeded in 6 well plates and were transfected at 80% confluence with 800 ng GLI luciferase reporter pGl3-GBS promoter (provided by Takeda) and 200 ng of pRL-TK per well using TransIT-2020 Transfection Reagent (Mirus, Madison, WI). After 6 h, cells were trypsinized and replated into a 12-well plate and treated with either 10 μ M of Cyclopamine or escalating doses of TAK-441 +/- 200ng of rSHH. Luciferase activity was determined 48 h later with the Dual-Glo Luciferase Assay System (Promega, Madison, WI) using the Tekan 200 pro (Tekan, Mannedorf, Switzerland). Values were divided by Renilla luciferase activities to normalize for transfection efficiency and are shown as the mean +/- SEM.

3.2.7 Cell Viability/Proliferation Assay

PC3 cells were seeded in 12 well plates in normal conditions. After 48 hrs of treatment with vehicle (DMSO) or escalating doses of either TAK-441 or cyclopamine, viability of the cells were measured using the CellTiter AQueous Cell Proliferation Assay (Promega). This is a colorimetric method for determining number of viable cells using a tetrazolium compound, MTS, and an electron-coupling reagent. MTS is bio-reduced by cells into a formazan product that has an absorbance at 490 nm. The plates were incubated at 37°C in a humidified 5% CO₂ atmosphere for

1-4 hours after the addition of this solution followed by recording the 490 nm absorbance of each plate. The results were normalized to vehicle and graphed as mean +/- SEM.

3.2.8 Wound Healing 2-D Migration Assay

PC3 cells were seeded in 6-well plates with a density of 1.5×10^5 cells/well in normal conditions. The cells were treated 24hrs prior to scratch with vehicle (DMSO) or indicated concentrations of TAK-441 or cyclopamine +/- rSHH. When the cells reached confluence, they were treated with 30 $\mu\text{g/ml}$ of mitomycin C (Sigma) for 2 hours in order to remove the effect of cell proliferation. Each well was scratched horizontally and vertically to create a reference intersection point for imaging. Using microscopy, the cells were imaged at T=0, 12, 24, 36, 48, and 60 hrs. The images consist of a composition of a 5×5 panel of images taken at 10X magnification centering on the intersection point. This enhances the reliability and accuracy of the repeated images taken at various time-points. The wound closure rate for each treatment was normalized to T=0 and used to calculate migration rate, which was graphed as a mean +/- SEM.

3.2.9 Real-time Automated XCELLigence Trans-well Migration and Invasion Assay

Real-time trans-well migration and invasion assays used the Real-Time Cell Analysis (RTCA) system from XCELLigence (ACEA Biosystems). For trans-well migration assays, 40,000 PC3M cells in serum-free medium seeded in the upper chamber of Cell Invasion and Migration plates migrated toward 10% FBS serum in the bottom chambers or no serum as a negative control. The ideal cell number was achieved by performing cell titration experiments for PC3 and PC3M cell lines. Cells were treated with vehicle or indicated concentrations of TAK-441 or cyclopamine with 200 ng/ml of rSHH and recorded for over 48 hrs. Automated analysis of cellular density on

the underside of the trans-well membrane occurred every 30 minutes and was plotted as cell index +/- SEM per group.

3.2.10 Boyden Chamber Trans-well Invasion Assay

PC3M cells were grown to sub-confluence in normal conditions. The cells were stained with 8 μ M Calcein-AM for 45 min and 80,000 cells were seeded in the upper chamber of a 12-well trans-well membrane (Corning, Glendale, AZ) pre-coated with matrigel matrix in 1% FBS, while the bottom chamber contains 10% FBS to create a gradient. Treatments were added to the top chamber as indicated. After 24 hours, the media from the wells are removed and 5% trypsin was added to the bottom well to cover the bottom of the insert. The dissociated cells are aliquoted into a solid black 96-well plate (Corning, Glendale, AZ) and the fluorescence was read in a Tecan 200 Pro microplate reader (Tecan, Switzerland). Mean fluorescents normalized to vehicle was plotted +/- SEM.

3.2.11 Quantitation of Extravasation Efficiency Rates in Chorioallantoic Membrane (CAM) Assay

Fertilized chicken eggs were incubated in a rotary incubator at 37°C with 90% humidity for 4 days before being removed from the shell and placed in covered dishes and incubated at 37°C with 90% humidity until usage [306, 307]. On day 13 of embryonic development, 5×10^5 PC3 or PC3M PCa cells expressing GFP in 75 μ L of PBS were injected into a vein within the CAM [306, 307]. Prior to injection, GFP-expressing cancer cells were pre-treated with TAK-441 (0.5, 5.0, 50, 500) nM or vehicle (DMSO) for 1 hr on ice. Immediately after injection, four aluminum foil square markers (5 mm diameter) were placed on the surface of the CAM to form a large rectangular region of interest (ROI) for macroscopic imaging. Using an upright fluorescence stereoscope, all GFP

cells were enumerated within the rectangular ROI immediately after injection. At T=24 hrs post-injection, CAM vasculature was labeled with Rhodamine conjugated Lens Culinaris Agglutinin (LCA, Vector Laboratories, Burlingame, CA, USA) at 24 hrs post-injection time point, and cancer cells were visualized using a spinning-disk confocal microscope (Quorum Technologies, Waterloo, ON, Canada) [308]. Intravascular cells were identified as being present only within the CAM lumen as labeled by LCA. Extravascular cells were identified as being present within the underlying stroma, and not within the same Z-plane as the CAM lumen [308]. At least 200 cells for each ROI at T=0 was analyzed and enumerated.

3.2.12 PSA Recurrent LNCAP CRPC Xenograft Model

2×10^6 LNCaP cells were suspended in 0.1 ml Matrigel (BD Biosciences) and inoculated subcutaneous in the flank of 6- to 8-week-old male athymic nude mice (Harlan Sprague–Dawley). Gonadally intact animals harbouring xenografts that grow to $>200 \text{ mm}^3$ with circulating PSA levels of $>60 \text{ ng/ml}$ were castrated. When PSA increased to the same level as pre-castration (T=0) the animals were randomized to 4 groups of vehicle (0.5% methylcellulose/H₂O), 25 mg/kg TAK-441 orally administrated once daily, 15 mg/kg Docetaxel (Sanofi-Aventis Canada Inc., Laval, QC) intraperitoneal injections, once a week for 3 weeks, and combination of TAK-441 and Docetaxel. Each experimental group consisted of 10 mice. Tumour volumes and BW were measured once weekly. Serum PSA levels were determined weekly using a total PSA immunoassay on a Cobas e 411 immunoassay analyzer (Roche Diagnostics PSA), according to the manufacturer's instruction. Data points were expressed as mean % tumour volume change compared to T=0 +/- SEM or mean % PSA concentration change compared to T=0 +/- SEM. All animal procedures were performed according to the guidelines of the Canadian Council on Animal Care and with appropriate institutional certification.

3.2.13 PC3 CRPC Xenograft Model

2×10^6 PC-3 cells were suspended in 0.1 ml Matrigel (Becton Dickinson Labware, Franklin Lakes, NJ) and were inoculated subcutaneously in the flank of 6- to 8-week-old male athymic nude mice (Harlan Sprague–Dawley). Tumour size was monitored twice a week prior to treatment. When the tumours reached 100 mm^3 , the mice were randomly assigned to 4 groups of vehicle (0.5% methylcellulose/H₂O), 25 mg/kg TAK-441 orally administrated once daily, 15 mg/kg Docetaxel IP, once a week for 3 weeks, and combination of TAK-441 and Docetaxel. Vehicle and Docetaxel groups contained 10 mice each, whereas TAK-441 and the combination group contained 15 mice each. Tumour volume measurements were performed twice weekly and calculated by the formula: length \times width \times depth \times 0.5236. Mean Tumour volume \pm SEM was plotted for all groups. Tumour growth rate was calculated for each animal by linear regression analysis and plotted as a waterfall plot. All animal procedures were performed according to the guidelines of the Canadian Council on Animal Care and with appropriate institutional certification.

3.3 Results

3.3.1 SHH, GLI1, and GLI2 Expression are Associated with Biochemical PSA

Recurrence and CRPC Progression

Expression of the key components of the Hh pathway in PCa was examined using a comprehensive IHC analysis, containing 354 cores from 190 PCa patients grouped into benign, untreated, NHT, recurrent, and CRPC. In this study, SHH expression was very low in benign samples and increased in untreated, NHT, and recurrent, with a drastic increase in CRPC samples relative to all other cohorts, as previously reported [241, 242]. GLI1 and GLI2 expression gradually increases from benign to CRPC, with a significant difference observed between benign vs. CRPC in GLI1 expression and benign vs. both recurrent and CRPC in GLI2 (Figure 3-1 B). In

addition, we elicited the role of the Hh pathway as a prognostic indicator by examining time to PSA recurrence in light of previous studies, which have shown elevated SHH and SMO levels to be a prognostic factor for PSA recurrence [240]. We categorized the expression level of Hh pathway proteins into low and high levels based on pathological scores. Examining PSA-free survival, a significant decrease in time to PSA recurrence between low expression levels of SHH, GLI, and GLI2 compared to high expression of those proteins were observed (Figure 3-1 C). However, this association was not observed for SMO as previously reported, neither for PTCH, IHH, or DHH. Increased expression of these key Hh regulators in advanced PCa and their correlation with prognostic indicators such as time to PSA recurrence is indicative of a clear role for aberrant Hh signalling in PCa progression. Mainly, the multi-fold increase of SHH in CRPC may postulate a shift in the mode of activation of this pathway.

3.3.2 Modulating the expression and activity of the Hh pathway by inhibiting SMO in PC3 cell lines

Expression of the Hh ligand, SHH, as well as key downstream regulators of the pathway, GLI1 and GLI2 were assessed in a panel of PCa cell lines, from the androgen-responsive cell line, LNCaP, to its lineage-derived, AR-positive CRPC derivative, C4-2, and the independently-derived, AR-negative PC3 and DU145 cell lines (Figure 3-2 A). As previously described in chapter 2, we observe an increased expression of SHH between LNCaP and the CRPC cell lines. In addition to our previous results, we observe an increase in GLI1 levels in DU145 and GLI2 expression levels in both DU145 and PC3.

In order to determine the Hh pathway mode of action in PCa, we antagonised SMO by using TAK-441 or cyclopamine in both LNCaP and PC3 cell lines and examined expression of

key Hh pathway proteins, SMO, PTCH1, and GLI2. We observed no change in expression of these Hh pathway proteins in LNCaP cells treated with either TAK-441 at 100-fold above its IC₅₀ or cyclopamine at up to 10 μM (Figure 3-2 B). This is in line with our previous observations that LNCaP cells are not sensitive to modulation of canonical Hh signalling in an *in vitro* monoculture setting. However, when the same conditions were applied to a CRPC cell line model, we observed a reduction in both SMO and GLI2 expression when inhibited by TAK-441 at its IC₅₀ of 5 nM, with a dose-dependent reduction up to 100-fold above its IC₅₀. Cyclopamine treatment at its IC₅₀ of 10 μM also had an inhibitory effect on SMO, PTCH1, and GLI2 expression (Figure 3-2 C). We were cognisant of the inhibitory effect of cyclopamine on cell viability [274, 281, 282], and recapitulated these previous findings in PC3 cells. In a MTS viability assay, under standard culture conditions we observed a decrease in viability at 10 μM cyclopamine, with a decrease of over 50% at 20 μM (Figure 3-2 D). Same conditions were applied using TAK-441 up to 100-fold above its IC₅₀ with no effect on cell viability (Figure 3-2 E).

As in previous studies, the GLI levels were used as an indicator of Hh pathway activation, especially GLI2 in the case of PC3 cell lines [256, 260]. GLI2 mRNA expression is significantly reduced in PC3 cells when treated with 50 nM of TAK-441, in the presence of rSHH. In our experiments, cyclopamine had no effect on GLI2 mRNA expression (Figure 3-2 F). Although the change in protein and mRNA expression is a great indicator of modulating the Hh pathway with SMO inhibitors, it is critical to understand the role of these drugs on the activity of the Hh pathway by performing a luciferase GLI-reporter assay. We observe a significant decrease in the activity of the Hh pathway when treated with 500 nM of TAK-441 in the absence of exogenous rSHH, and although we see a decrease in the cyclopamine treated samples, it is not significant. In the presence of rSHH, we observe a significant reduction in Hh activity in as low a concentration of TAK-441

as 5 nM (Figure 3-2 G). This difference could be explained by low basal level of Hh activity, which may be due to the limitation of monolayer cell culture techniques.

3.3.3 Antagonizing SMO, inhibits migration and invasive potential of PC3 cell lines

To better gauge the role of Hh pathway in cell motility, migration of PC3 cells was assessed using a wound healing assay, by calculating the rate of wound closure in the presence of mitomycin C to control for proliferation (Figure 3-3 A). We observe a significant decrease in migratory rate of PC3 cells when treated with 500 nM of TAK-441 (Figure 3-3 B). In the presence of rSHH, the inhibitory effect of TAK-441 on migration is seen significantly at a concentration of 0.5 nM, with more than 50% reduction in the migratory capacity of PC3 cells at 500 nM. We also observe a decreased motility in PC3 cells treated with 10 μ M of cyclopamine, which may have been enhanced due to off-target toxicity at later time points. These results give a clear indication of the role of the Hh pathway in the migration of our CRPC cell line, yet the 2-dimensional nature of this experiment becomes a limitation in making assumptions without supporting experiments. On that note, we utilized a Boyden chamber trans-well migration assay, which creates a more realistic approximation of the migratory potential of these cells. For these experiments we obtained a more metastatic variant of PC3 cells, PC3M. We also performed the assays using the XCELLigence platform, which monitors the migration of PC3 cells across the membrane in real-time (Figure 3-4 A). Similar to our previous results, we observe a significant reduction in migration of PC3M cells treated with 5, 50, or 500 nM of TAK-441, as well as 10 μ M of cyclopamine in the presence of exogenous rSHH (Figure 3-4 B).

To further elucidate the role of the Hh pathway in metastatic potential of advanced PCa, we aimed to study the invasiveness of PC3M cells in a trans-well migration assay. Cells that

invaded through the matrigel matrix were quantified and normalized to vehicle treatment. Inhibiting SMO with 10 μ M cyclopamine or an escalating dose of TAK-441 had a significant effect in hindering invasion of PC3M cells (Figure 3-4 C). These experiments demonstrate a clear reduction in migration and invasion of advanced PCa models through inhibition of SMO and a clear insight into the role of the Hh pathway in metastatic PCa.

3.3.4 Hh Pathway Plays an Important Role in CRPC Cell Line Extravasation *Ex Vivo*

To establish a clearer link between the Hh pathway and PCa metastasis, we performed a CAM assay to ascertain the extravasation potential of PC3 and PC3M cells. TAK-441 pre-treated PC3 and PC3M cells expressing GFP were injected into a vein within the chorioallantoic membrane. 24 hrs post injection, intravascular cells were identified as being within the CAM lumen, labelled with Rhodamine using spinning-disk confocal microscopy (Figure 3-5 A). Extravascular cells were identified as moving out of the CAM lumen and within the underlying stroma (Figure 3-5 B). Extravasation efficiency was calculated as a percentage of extravascular cells at 24 hrs post injection compared to T=0. We observe a significant reduction in extravasation efficiency of PC3 cells treated with escalating doses of TAK-441, as low as 5 nM (Figure 3-5 C). However, in PC3M cells only TAK-441 AT 500 nM showed significant reduction in vascular extravasation (Figure 3-5 D). These are the first findings demonstrating the role of Hh inhibition in hindering CRPC cell extravasation, which may lead to an ideal therapeutic approach in prevention or delay of a metastatic CRPC.

3.3.5 Combinatory Treatment of SMO Antagonist and Docetaxel Delays Growth and Progression of CRPC Xenograft Models

Hh pathway modulation has been implicated in affecting PCa xenograft growth in a variety of models and has been used as a combination therapy with AR and MEK inhibitors [309, 310]. In addition, recent findings demonstrate the involvement of Hh pathway activation in acquired chemo-resistance [311, 312]. These along with our findings pertaining to CRPC progression led to utilizing two well established CRPC xenograft models, PSA recurrent LNCaP CRPC and PC3 CRPC xenografts, in ascertaining the effect of TAK-441 in combination with Docetaxel treatment in progression of CRPC.

We set out to evaluate whether oral delivery of TAK-441 in combination with Docetaxel post-castration affects the PSA recurrence and CRPC progression of subcutaneous LNCaP xenografts established in athymic mice. These gonadally intact mice were castrated when PSA levels reached >60 ng/ml. On average, after 2 weeks post castration, when serum PSA increased to pre-castrate levels, set as T=0, the mice were randomized into 4 treatment groups of vehicle, 25 mg/kg TAK-441 orally administered once daily, 15 mg/kg Docetaxel i.p. once a week for 3 weeks, and combination of TAK-441 with Docetaxel. This model closely approximates the progression of PCa from an AI cancer through castration and subsequent development of CRPC, while providing an ideal tool for assessing therapeutic approaches targeting advanced post CRPC progression of this disease. LNCaP tumour volume change was assessed compared to the volume at initial treatment (T=0). At 5 and 6 weeks post treatment, we observe a significant difference in the change in tumour volume between all cohorts except TAK-441 vs. vehicle or docetaxel. That being said, we believe that a more robust method of assessing xenograft tumour growth in these models is to examine the growth rate of the tumours. This more rigorous calculation is determined

by measuring the growth rate of each animal individually during the course of treatments through linear regression and using non-parametric multivariate statistical analysis to determine significance between each group. We observe an average growth rate of 75.4 ± 13.3 , 39.4 ± 9.7 , 23.4 ± 4.3 , and -2.5 ± 1.5 mm³/week for vehicle, TAK-441, Docetaxel, and combination respectively. Compared to vehicle neither TAK-441 nor Docetaxel were significant in a non-parametric Dunn's multiple comparison even with 1.9- and 3.2-fold reduction, respectively. The combination treatment was significantly lower than both TAK-441 and Docetaxel alone ($p < 0.01$ and $p < 0.5$ respectively) (Figure 3-6 A). A waterfall plot of tumour volume change for each mouse analysed in this study shows the distribution of treated animals and a regression in tumour volume in more than half the animals treated with combination therapy.

In this model, serum PSA levels are utilised as an indicator of tumour load as well as its time to doubling a marker of disease progression. As described above, we also assessed the PSA growth rate for each animal and observed an average rate of 46.8 ± 11.0 , 29.7 ± 5.2 , 13.1 ± 4.9 , and 1.0 ± 1.7 ng/ml/week for vehicle, TAK-441, Docetaxel, and combination respectively. When treating with Docetaxel, we observe a greater reduction of PSA level compared to that of tumour growth, which leads to a significant difference when comparing vehicle vs. Docetaxel but not with Docetaxel vs. combination treatments as seen above (Figure 3-6 B). The waterfall plot of PSA change at 6 weeks post treatment shows a similar pattern as observed above, with the combination treatment of TAK-441 and Docetaxel showing a decrease in PSA levels. In addition, when defining treatment failure in a PSA recurrent LNCaP CRPC model as a PSA doubling during the course of treatment, in the combination treatment cohort we observe no treatment failure. When performing a log-rank test on treatment failure-free survival, we observe a significant difference between both TAK-441 and Docetaxel vs. combination treatment ($p < 0.01$ and $p < 0.05$, respectively) (Figure

3-6 C). These experiments indicate that TAK-441 in combination with Docetaxel treatment significantly inhibits growth and progression of CRPC LNCaP xenografts.

To further examine the role of Hh inhibition in combination with taxane treatment in advanced PCa, we utilized another CRPC xenograft model using PC3 cells. PC3 cells were inoculated subcutaneously in athymic nude mice and the established tumours were regularly measured. When the tumours reached a volume of above 100 mm³, the mice were randomly assigned to 4 groups of vehicle, 25 mg/kg TAK-441 orally administrated once daily, 15 mg/kg Docetaxel intraperitoneal injection once a week for 3 weeks starting at T=0, and combination of TAK-441 and Docetaxel. As described above, tumour growth rates for each mouse was calculated and compared to other cohorts through non-parametric multivariate analysis. We observe an average growth rate of 155.7 ± 39.36 , 75.27 ± 29.44 , 69.04 ± 11.42 , and 19.59 ± 6.2 mm³/week for vehicle, TAK-441, Docetaxel, and combination, respectively. This represents a decrease in tumour growth rate of 2 fold when treated with TAK-441 and 2.25 fold when treated with docetaxel when comparing to vehicle. However, when using Dunn's multiple comparison test, the significant reduction in tumour growth rate is seen when comparing vehicle, TAK-441, and docetaxel versus combination treatment ($p < 0.001$, $p < 0.01$, and $p < 0.05$, respectively) (Figure 3-6 D). A waterfall plot of tumour volume change for each mouse analysed in this study demonstrated a clear reduction in PC3 tumour growth rate with a few animals showing regression of this aggressive tumour type when treated with TAK-441 in combination with docetaxel. These results are a comprehensive *in vivo* analysis of CRPC models, which demonstrate a clear role for targeting the Hh pathway in advance PCa, specially in combination with post androgen ablation treatments such as docetaxel.

3.4 Discussion

In this chapter we examine the mode of action as well as the role of Hh signalling in advanced PCa progression, focusing on metastatic potential and the utility of Hh pathway inhibitors as a therapeutic modality. In numerous investigations and as discussed in previous chapters, SHH signalling plays a pivotal role in PCa growth and progression to CRPC and has been recently shown to alter metastatic potential and drug resistance [213, 239, 241, 243, 264, 270, 312, 313]. Our results confirm that SHH, GLI1, and GLI2 levels are significantly elevated in patients with CRPC. As we have previously reported [242], SHH levels are relatively low in PCa specimens and show a drastic increase in CRPC as compared to benign, untreated, NHT, and recurrent PCa patient specimens. However, in the GLIs, we observe a gradual increase from benign to CRPC. Kim et al., has shown that increased SHH expression is an independent prognostic factor for biochemical recurrence [240]. In our results, by comparing low vs. high levels of each of these key Hh pathway proteins, we demonstrate for the first time that high levels of all three factors, SHH, GLI1, and GLI2, are representative of significant increase in PSA recurrence, which indicates a worsened prognostic outcome. These observations in our PCa clinical cohort elucidates a beneficial role for further studying the Hh pathway in a clinical setting and utilizing therapeutic targets to inhibit this pathway in CRPC patients.

To better understand the Hh pathway in advanced PCa, we utilized the hallmark CRPC cell line model, PC3s. We initially set out to compare the effect of SMO inhibition, by using cyclopamine and TAK-441, in PC3 cells versus the androgen-dependent LNCaP cell line, which we have characterized in chapter 2. In contrast to the previously described paracrine LNCaP cells, the PC3 cell line has an increased basal expression of SHH and GLI2 and responds to Hh inhibition in a monoculture setting as indicated by decreased expression of SMO and GLI2. In addition, Hh

pathway activity, as assessed by GLI luciferase reporter assay, was decreased when treated with TAK-441. Response to Hh pathway inhibition in PC3 cells in contrast to the previously described LNCaP line, as well as basally elevated downstream mediators of Hh transcriptional signalling such as GLI2, postulate that there may be a transition from a paracrine androgen-dependent PCa to a more self-sufficient autocrine CRPC model. This transitional model is in line with the previous IHC results by Tzelepi et al. [241], which could shed light on the disparity between early reports of autocrine Hh signalling in PCa [205, 213, 241, 280] versus those who demonstrated a more developmentally centric paracrine model [265, 268, 281, 314, 315].

As briefly discussed in chapter one, the Hh pathway induces cell migration as part of its role in patterning and axis formation in early development [204, 316], as well as wound healing in adults [198-200]. Furthermore, this pathway has been shown to enhance metastatic potential in a variety of cancers including breast, ovarian, and pancreatic [251, 317-321]. These findings, and the lack of a clear understanding into the role of the Hh pathway in altering the metastatic potential of CRPC, led to examining the effect of Hh modulation in migration, invasion, and metastatic potential of advanced PCa. Migration of PC3 cells in the presence of the SMO inhibitor, TAK-441, was significantly inhibited in a two-dimensional wound healing assay. Due to the limitations of this monolayer model, PC3 migration was also assessed in a more biologically relevant *in vitro* model, using three-dimensional Boyden chamber transwell-migration assay, which recapitulated the same inhibitory effect of TAK-441. To better gauge the effect of CRPC metastatic potential *in vitro*, TAK-441 significantly inhibited PC3 invasion in a transwell Boyden chamber Matrigel invasion assay. It is important to note that, although TAK-441 did not affect PC3 viability at above 100-fold its IC₅₀, it is imperative to inhibit cell proliferation in these experiments, which can be achieved by a short exposure to Mitomycin C, so not to confound the results [322].

There has been a great focus on studying the metastatic potential of PCa, which has led to a multitude of models in assessing the lethal aspect of this disease. This includes but is not limited to animal models with various loss of function and compound mutations, such as PTEN^{-/-} in conjunction with NKX3.1^{+/-} or Rb^{-/-} [323-326], selection for metastatic variants of PCa cell lines such as PC3M [327], and various techniques of experimental metastasis such as intrafemoral, heart, and tail vein injection [328, 329]. However, these and all subsequent iterations of these models have significant limitations and do not recapitulate the metastatic nature of the human disease itself. This led us to take a step back and establish a clear link between the Hh pathway modulation and the initial metastatic event, the extravasation of the PCa cells out of the lumen of the vasculature and into the surrounding stroma. By utilizing the CAM assay technique with distinct fluorescent tags and spinning disk microscopy, we were able to visualize both PC3 and PC3M cells as they transitioned through the endothelial lumen and out of the vasculature. By inhibiting the Hh pathway we were able to significantly hinder extravasation of both PC3 and the more metastatic PC3M cell lines. Our *in vitro* and *ex vivo* results demonstrate that disruption of the Hh pathway through inhibition of SMO hinders PCa cell migration, invasion and metastatic potential. These results, in addition to previous reports showing an elevated level of Hh gene expression in men who rapidly develop metastasis within the first 5 years post radical prostatectomy versus men who were metastasis-free in the same period [330], establish Hh targeted therapeutics as a viable candidate for advanced PCa treatment modality.

Hh inhibitors as monotherapy modalities in clinical settings has been effective in basal cell carcinoma and medulloblastoma, yet in other cancers including solid tumours it has not performed as well [303]. This has led to further investigations into better understating the mechanistic role of Hh signalling in solid tumours, as well as a more in depth look at Hh interactions with other

signalling pathways and treatment modalities. In recent findings, taxane treatment in breast cancer activated Hh signalling, which led to expansion of breast cancer lines and recurrence of the disease [331], while in refractory metastatic pancreatic cancer, combination of SMO inhibitors with chemotherapy in a clinical setting showed efficacy with a significant increase in mean overall survival [332]. However, similar studies in metastatic pancreatic cancer, using a combination of vismodegib and non-taxane chemotherapy, gemcitabine, did not demonstrate a significant clinical response [333]. In PCa, Domingo-Domenech et al. demonstrated over-expression of Hh signalling in the subpopulation of cells that have become docetaxel-resistant, while SMO inhibition depleted this population [334]. In our *in vivo* experiments, we utilized two xenograft models for CRPC, to examine the role of Hh inhibition through SMO targeting in combination with docetaxel treatment. The subcutaneous LNCaP CRPC xenograft model was used to assess the combinatory effect of TAK-441 and docetaxel post-castration, on PSA recurrence and CRPC progression. TAK-441 in combination with docetaxel significantly reduced tumour growth as compared to both docetaxel and TAK-441 alone. Furthermore, when examining PSA doubling as a marker of treatment failure, we observe no treatment failure in combinatory treatment, which provided a significantly improved outcome when compared to either treatment alone. In line with our *in vitro* studies, we also examined combinatory treatments of TAK-441 and docetaxel in subcutaneous PC3 CRPC xenograft model, which demonstrated a significant reduction of tumour growth in combination treatment compared to either TAK-441 or docetaxel alone. This result also recapitulated previous findings by Mimeault et al., using vismodegib in combination with docetaxel [335]. Our results provide substantial evidence for the use of Hh inhibitors in combination with taxanes as a viable therapeutic modality for advance PCa, especially metastatic CRPC.

Figure 3-1 A.

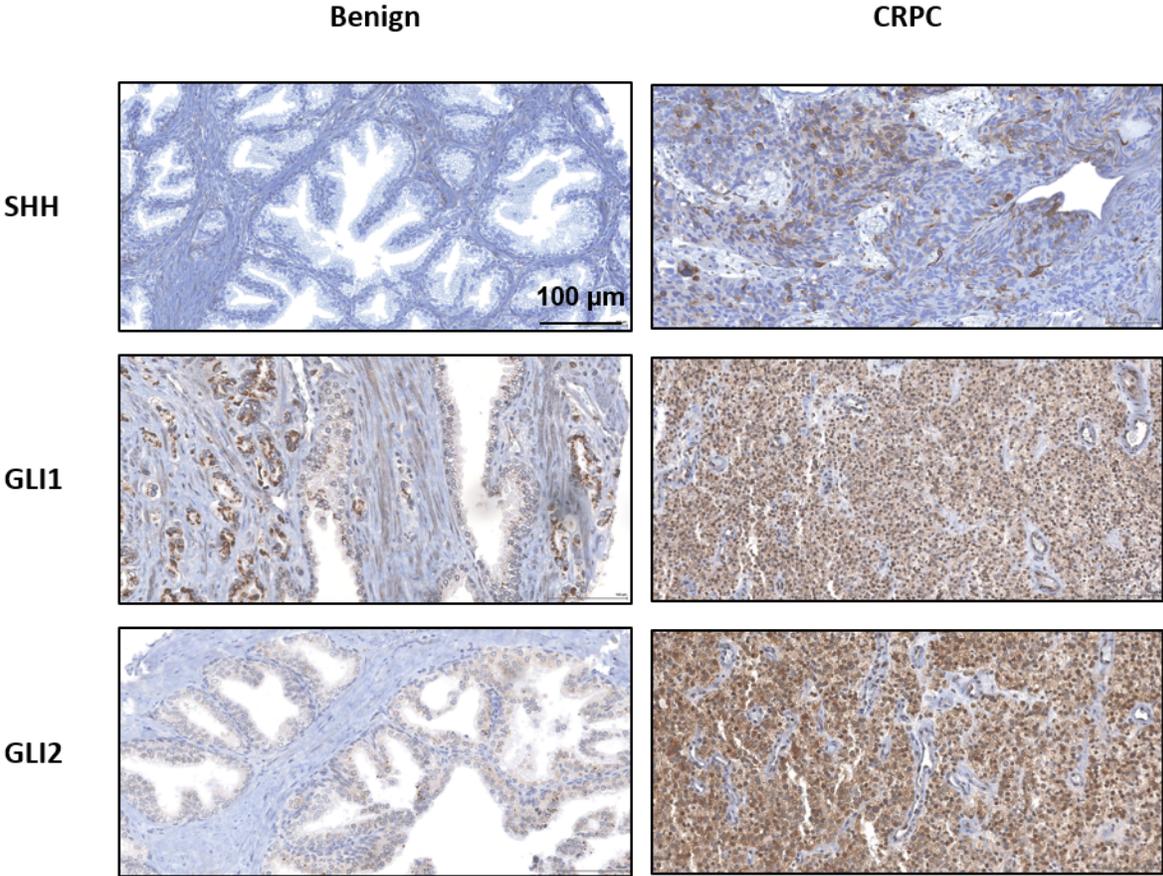


Figure 3-1 B.

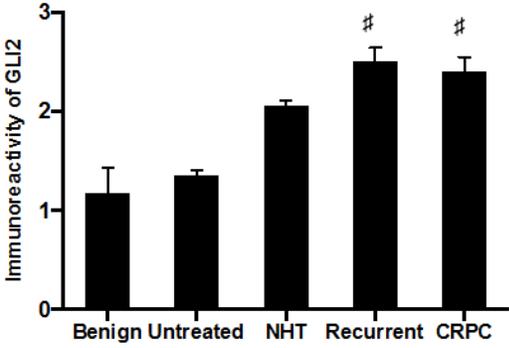
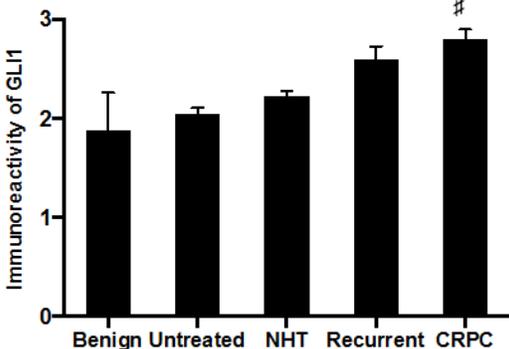
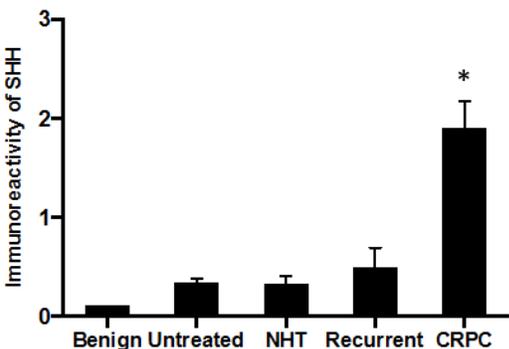


Figure 3-1 C.

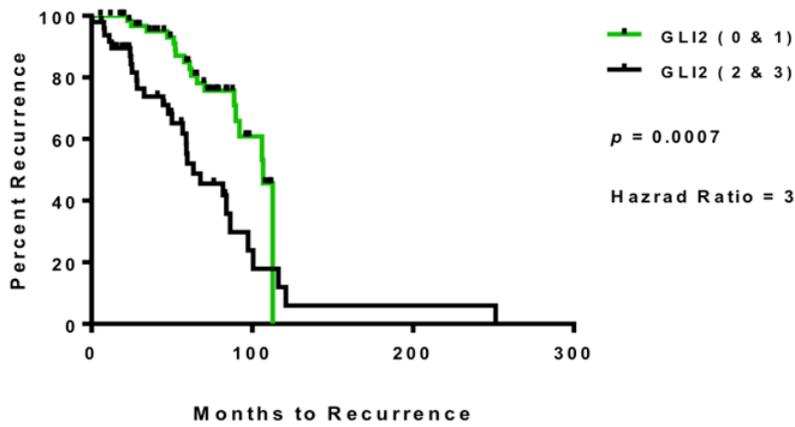
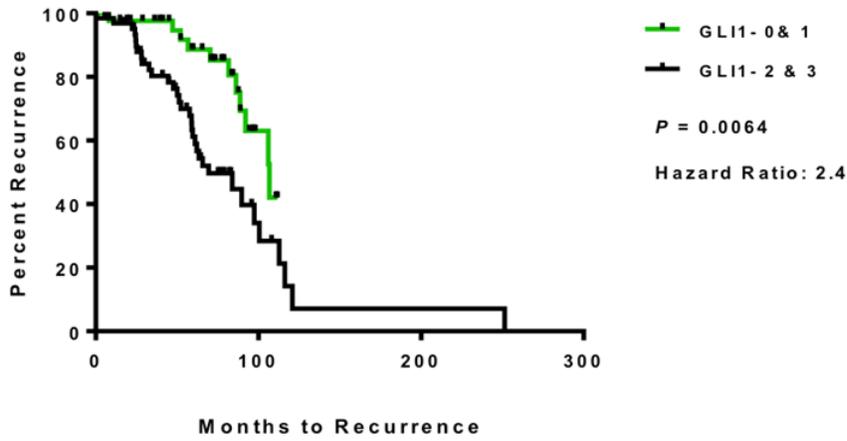
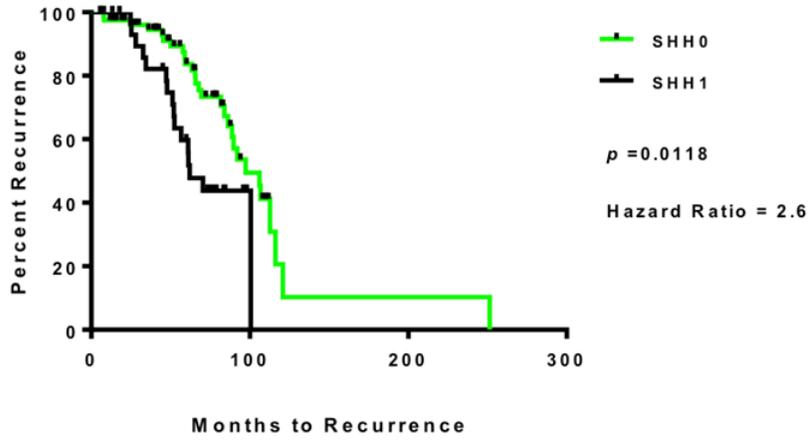


Figure 3-1 Immunohistochemical analysis of SHH, GLI1, and GLI2 shows elevated expression in advanced PCa and is correlated with time to biochemical PSA recurrence.

A. Representative IHC images are presented showing the extent and intensity of SHH, GLI1, and GLI2 immunoreactivity in benign and CRPC tissue (scale bar = 100 μ m). **B.** Intensity scores positive for SHH, DHH, IHH, GLI1, GLI2, and GLI3 expressed as the mean \pm SEM for tissue samples from benign, untreated, NHT, recurrent, and CRPC tissue samples. (Multi-variant analysis with $p < 0.05$ represented by * when compared to all treatment groups and # when compared to benign or untreated) **C.** Kaplan Meier analysis comparing low versus high expression of SHH, GLI1, and GLI2 based on pathologist scoring and time to PSA recurrence as defined by detectable PSA levels ≥ 0.2 ng/ml post-prostatectomy.

Figure 3-2 A.

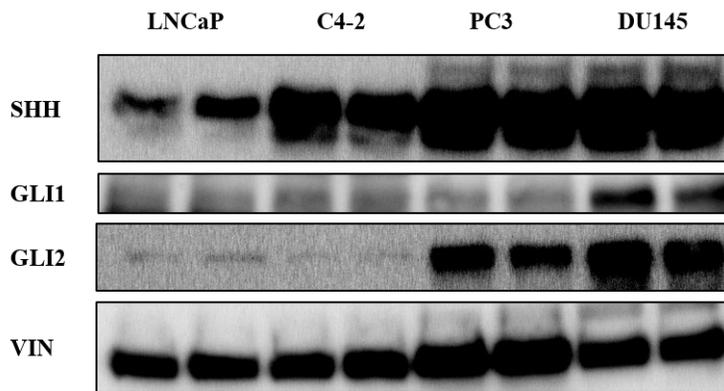


Figure 3-2 B.

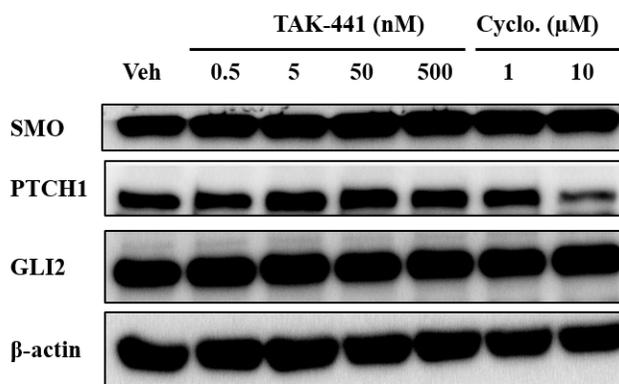


Figure 3-2 C.

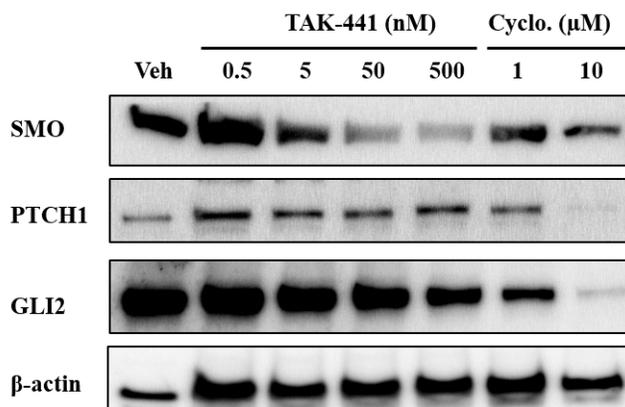


Figure 3-2 D.

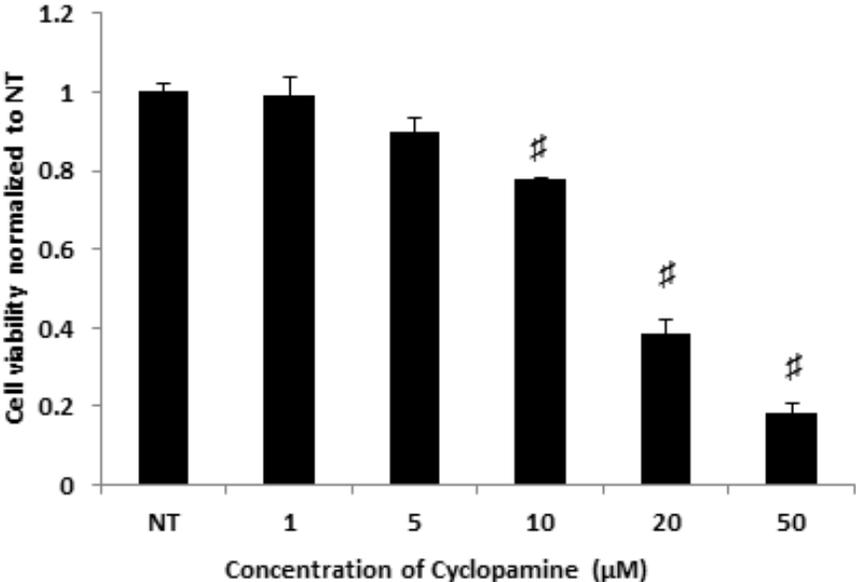


Figure 3-2 E.

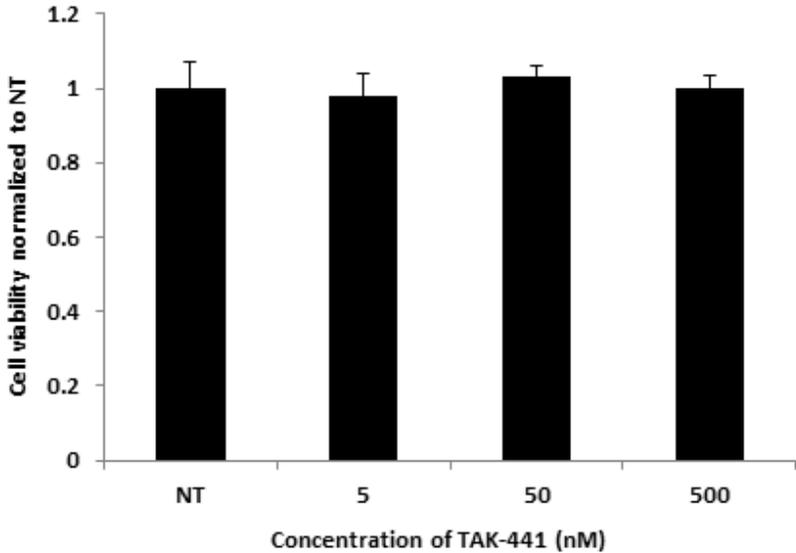


Figure 3-2 F.

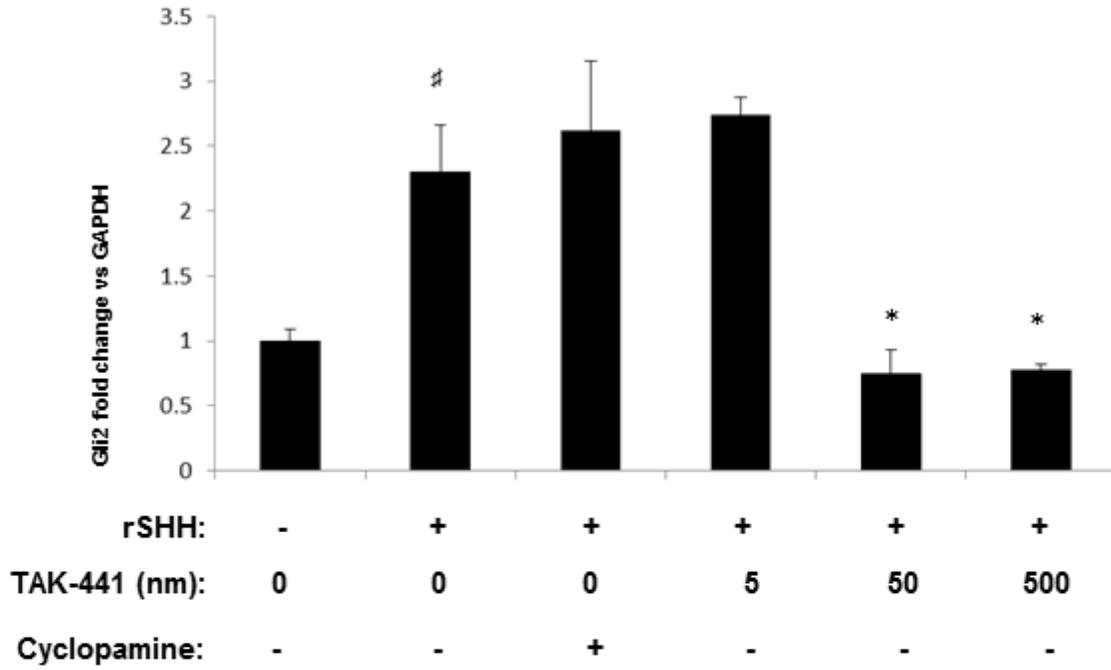


Figure 3-2 G.

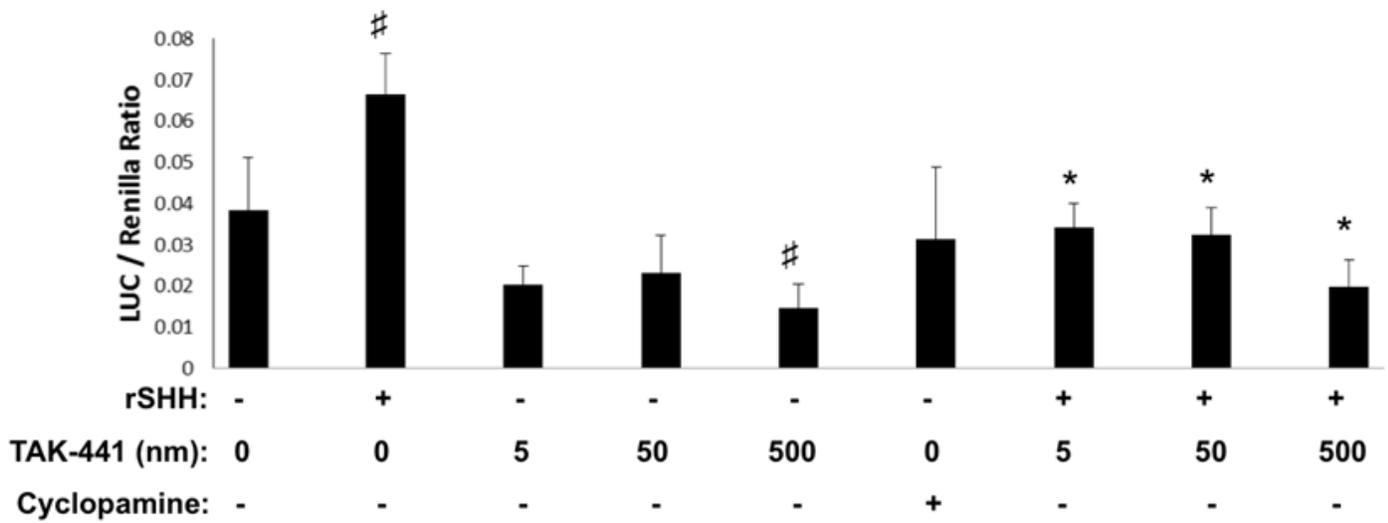


Figure 3-2. Expression of SHH, GLI1, and GLI2 in prostate cancer cell lines and effect of Hh pathway inhibition on expression of key Hh regulators, PC3 cell viability and GLI activity.

A. Western blot analysis of SHH, GLI1, and GLI2 expression in LNCaP, C4-2, DU145, and PC3 human PCa cell lines under standard conditions. Vinculin was used as a loading control. **B.** LNCap and **C.** PC3 cells were treated with indicated concentrations of TAK-441 or cyclopamine for 48 hrs. Western blot analysis of the expression of key Hh pathway modulators, SMO, PTCH1, and GLI2 was performed. β -actin was used as a loading control. MTS cell viability assay was performed on PC3 cells treated with **D.** cyclopamine (10 μ M) or **E.** indicated doses of TAK-441 (nM). Mean cell viability normalized to no treatment +/- SEM is represented. **F.** PC3 cells were cultured in FBS media and as indicated treated with 0 (-) or 200ng/ml (+) of rSHH plus 0, 5, 50, or 500 nM of TAK-441 or 10 μ M Cyclopamine. Real-time RT-PCR analysis of average mRNA fold change compared to GAPDH housekeeping gene is plotted +/- SEM. **G.** Dual luciferase assay was performed and the ratio of GLI-responsive Firefly luciferase to the constitutive Renilla luciferase expression vector was measured in PC3 cells treated with 0 or 200ng/ml of rSHH plus either 10 μ M of cyclopamine or indicated concentrations of TAK-441. (# $p < 0.05$ compared to vehicle, * $p < 0.05$ compared to rSHH).

Figure 3-3 A.

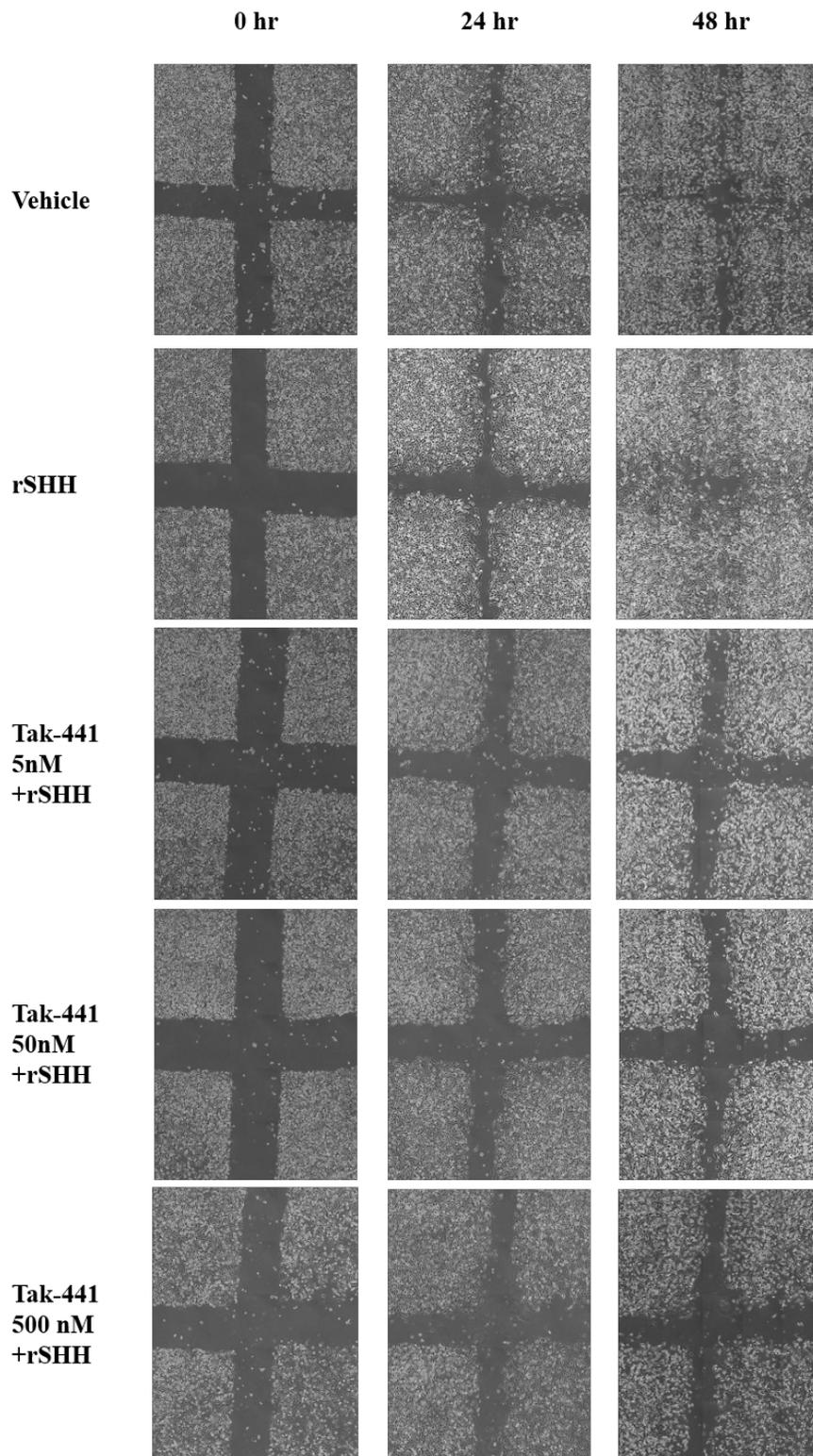


Figure 3-3 B.

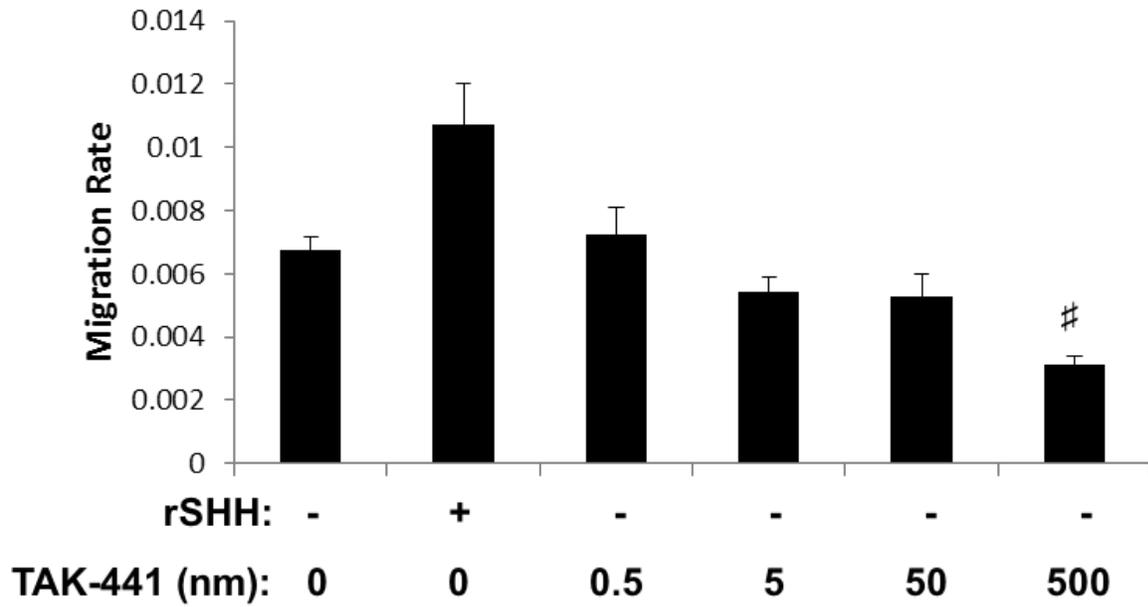


Figure 3-3 C.

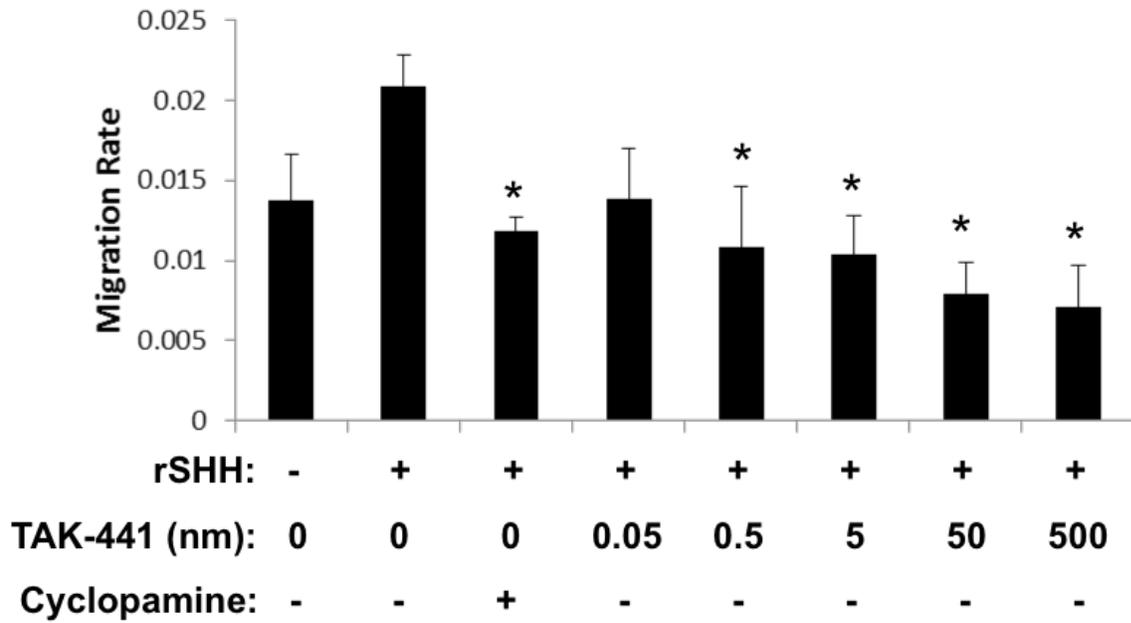


Figure 3-3. TAK-441 inhibits 2-D migration of PC3 cells by inhibiting the Hh pathway in a wound healing assay model.

PC3 cells were grown to confluence in full serum and treated with 30 $\mu\text{g/ml}$ mitomycin C for 2 hours prior to wounding. Cells were placed in serum free medium and treated with vehicle, or 200 ng/ml of rSHH plus either 10 μM of Cyclopamine or an escalating dose of TAK-441. **A**, representative panel of PC3 cells treated with rSHH and 5, 50, 500 nM of TAK-441 were imaged at 10x magnification in a 5x5 panel at 0, 24, and 48hrs post scratch and treatment. Average migration rate of PC3 cells were calculated, **B**, in the absence of rSHH and **C**, in the presence of rSHH. (# $p < 0.05$ compared to vehicle, * $p < 0.05$ compared to rSHH).

Figure 3-4 A.

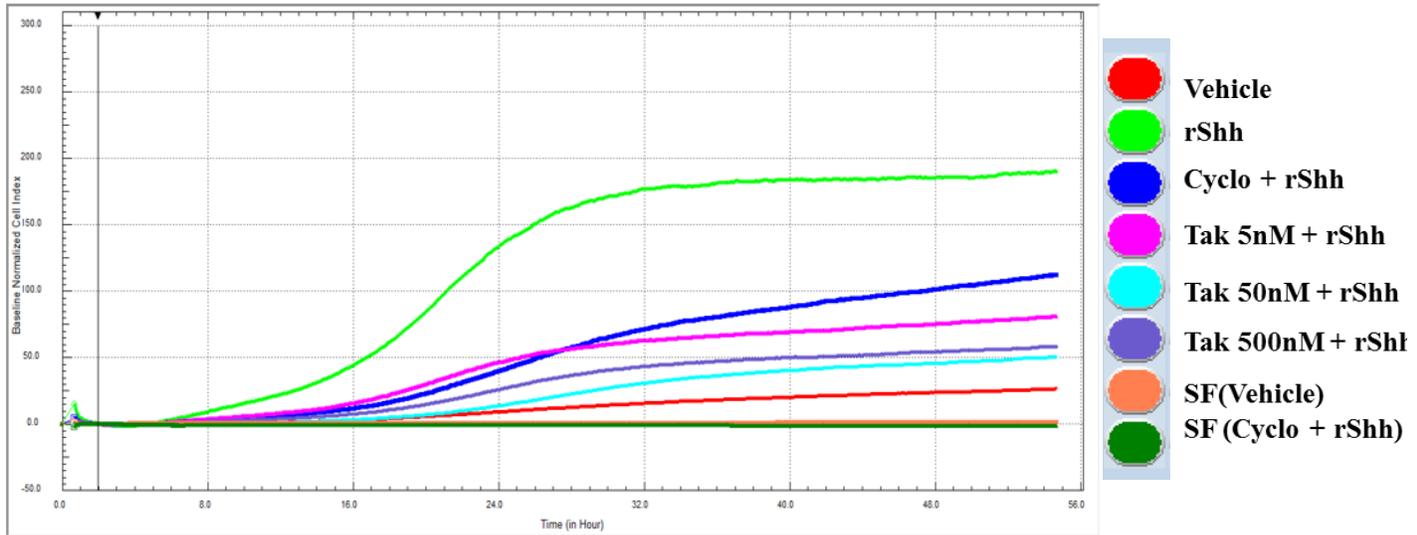


Figure 3-4 B.

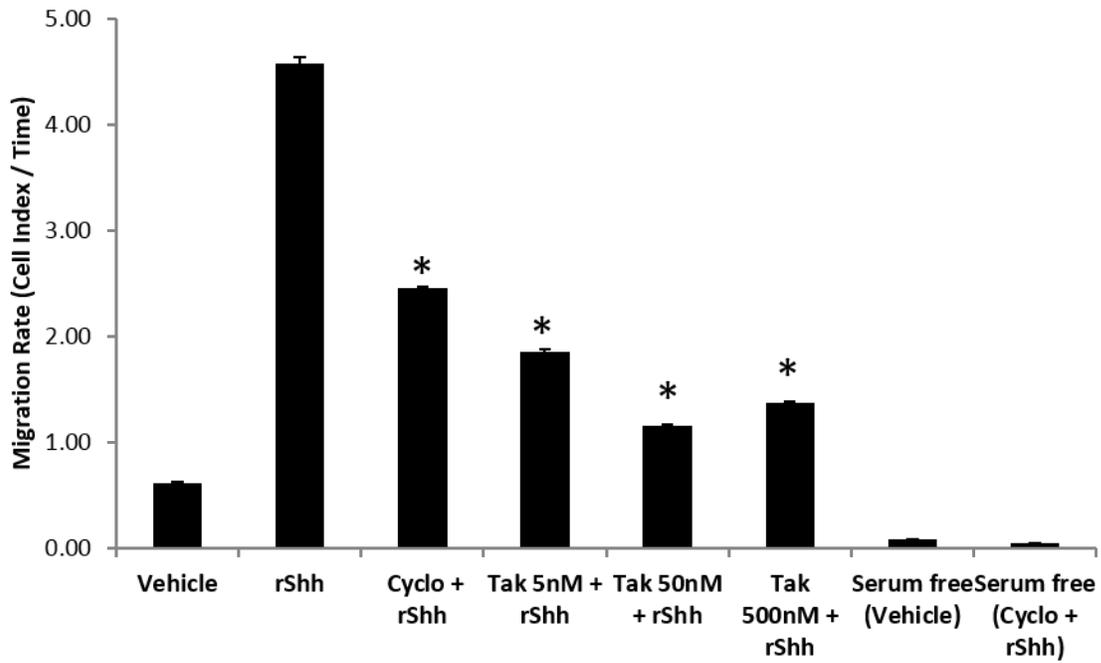


Figure 3-4 C.

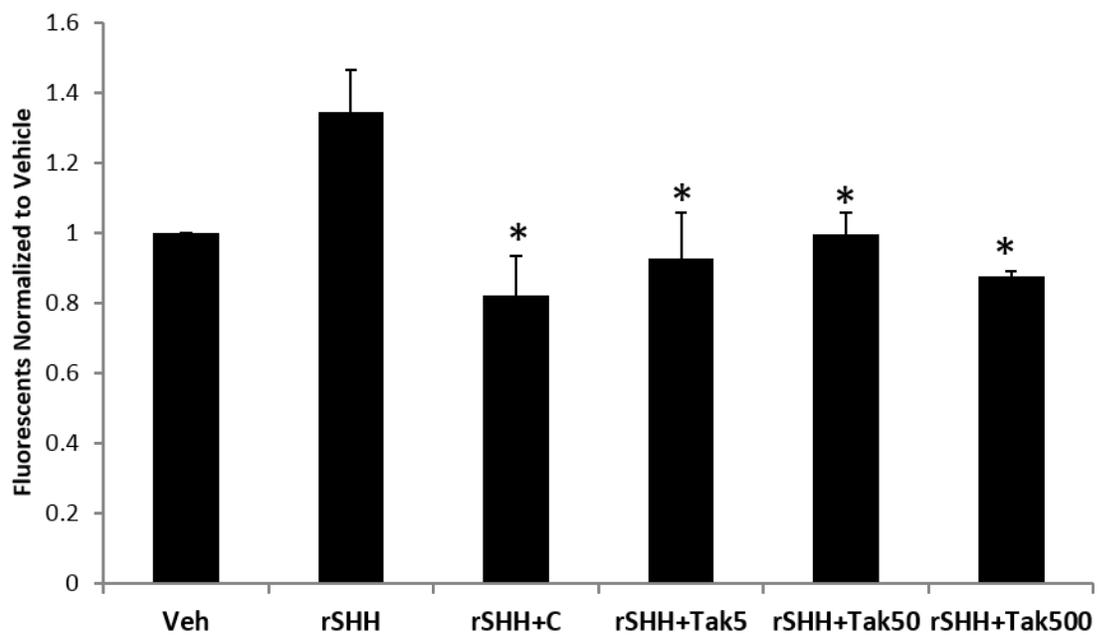


Figure 3-4. Inhibiting the Hh pathway decreases PC3M migration and invasion in a real-time XCELLigence trans-well migration assay and 3-D boyden chamber trans-well invasion assay.

A. 40,000 PC3M cells were seeded in the upper chamber of a CIM plate in a serum free medium, while 10% FBS was added to the lower chamber to create a gradient. Cells were treated with Vehicle or 200ng/ml of rSHH plus either an escalating dose of TAK-441 or 10 uM cyclopamine. Chambers without a FBS gradient also added as controls. The migration of these cells was recorded in real-time for 48hrs. **B.** The average migration rate based on cell index/time provided by the XCELLigence software +/- SEM was plotted for each treatment. **C.** 80,000 PC3M cells stained with 8 uM Calcein-AM for 45 minutes were seeded in the upper chamber of a 12-well trans-well invasion assay insert. The inserts were pre-coated with matrigel matrix and the cells were in a 1% FBS solution. The bottom chamber contained 10% FBS to create a gradient. The average fluorescence of the cells in the bottom chamber, normalized to vehicle +/- SEM were plotted. (* represents $p < 0.05$ compared to rSHH treatment).

Figure 3-5 A.

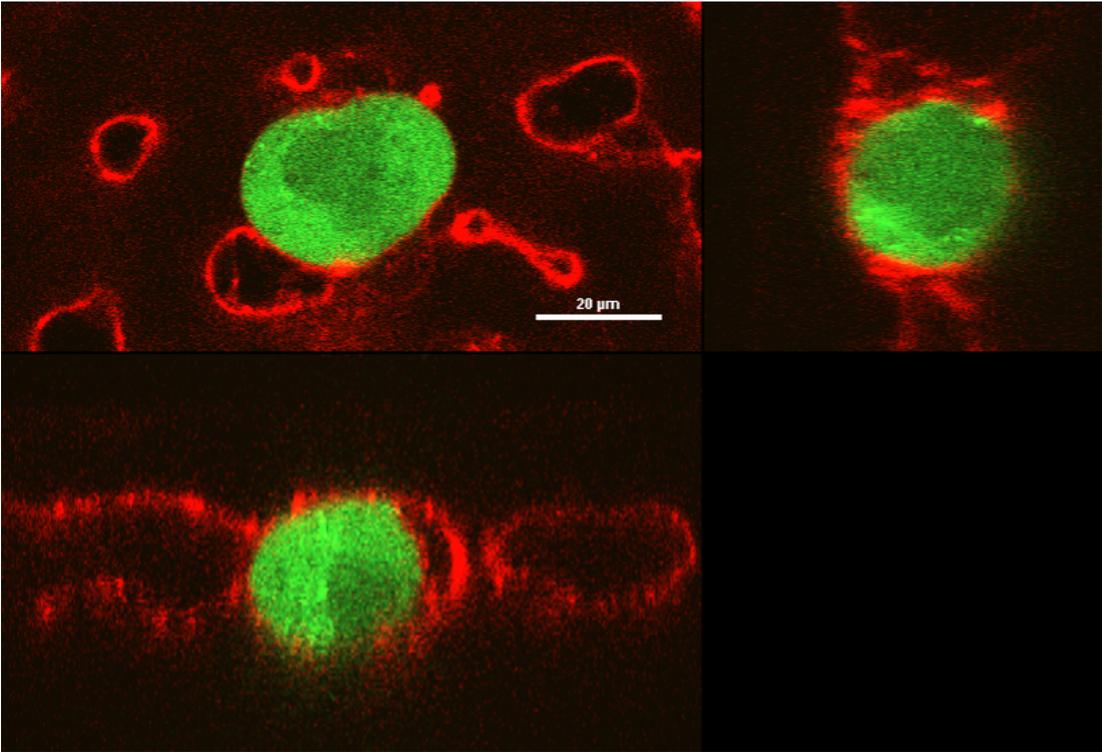


Figure 3-5 B.

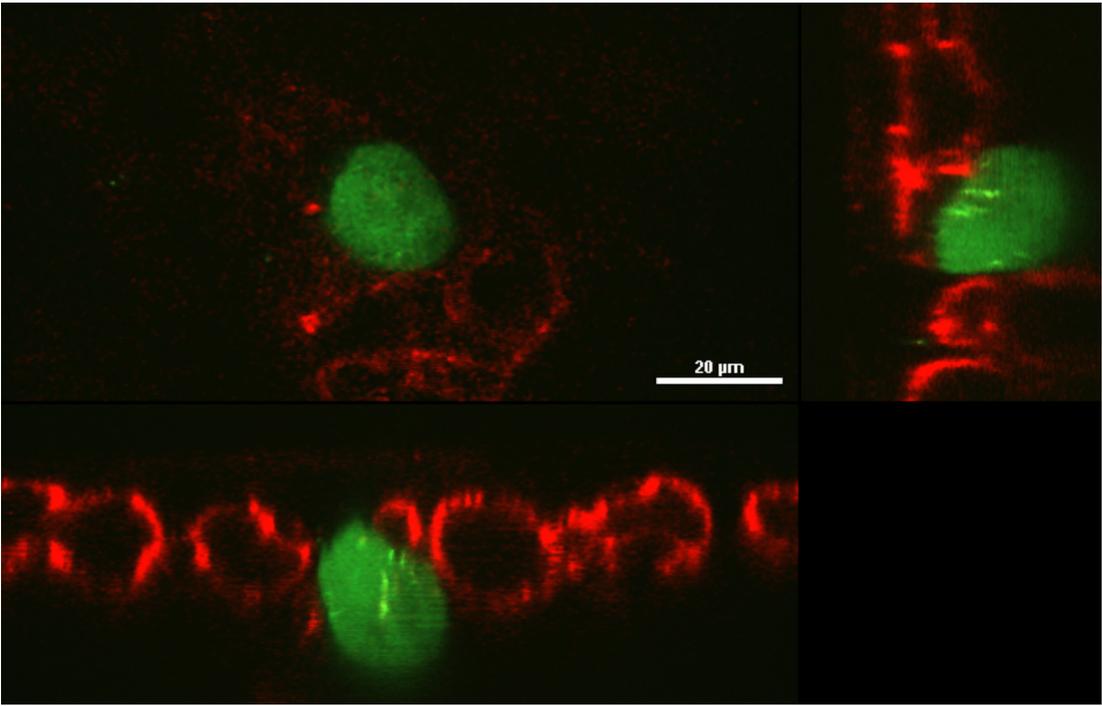


Figure 3-5 C.

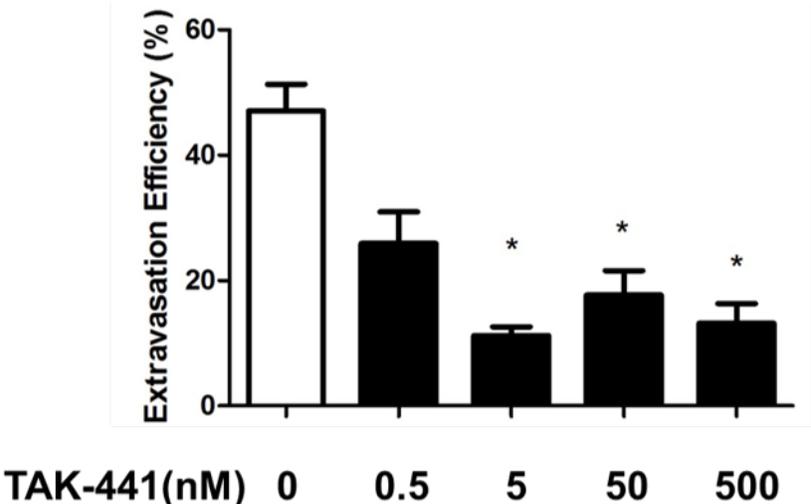


Figure 3-5 D.

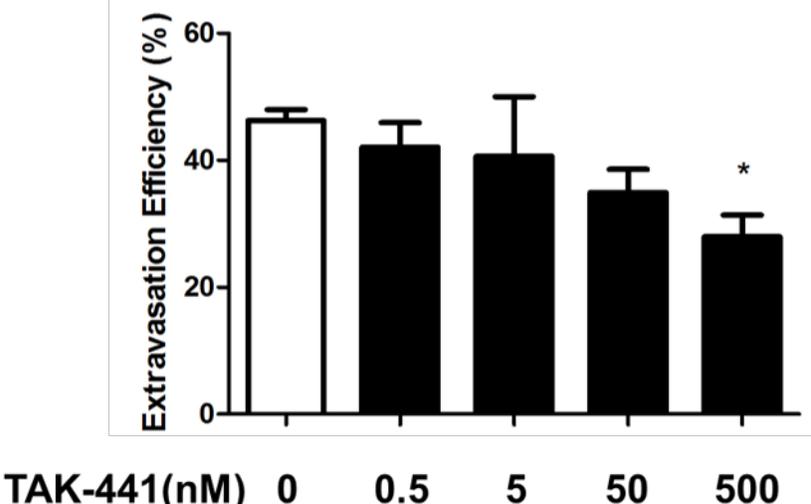
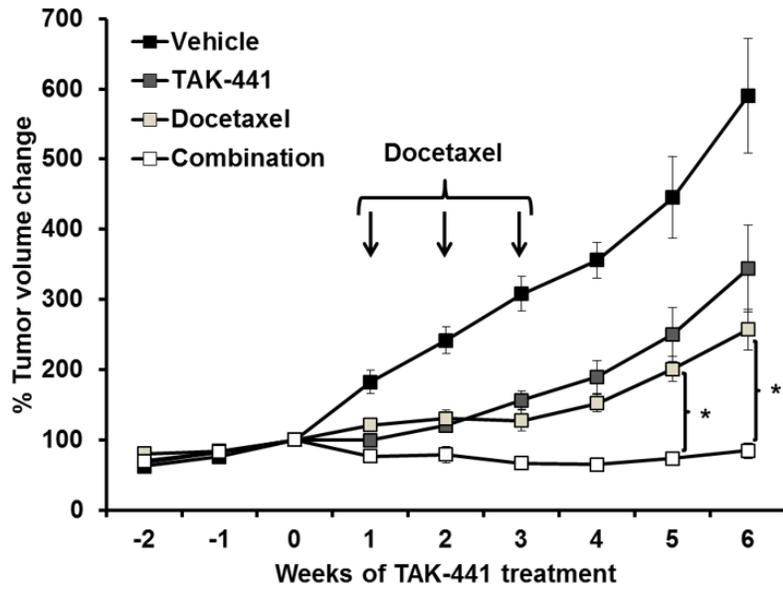


Figure 3-5. Quantitation of extravasation efficiency using chick chorioallantoic membrane assay demonstrates inhibitory effect of TAK-441 on PC3 and PC3M extravasation.

Day 13 embryos were IV injected with 500,000 GFP-labeled (Green) PC3 or PC3M cells. 24hrs later CAM vasculature was labeled with Rhodamine conjugated Lens Culinaris Agglutinin (Red). Representative images of **A.** intravascular cells treated with vehicle and **B.** extravasated cells treated with TAK-441, using spinning-disk confocal microscopy. Using fluorescence microscopy, the number of GFP-labelled cells that extravasate in a specific view were quantified immediately after injection and after 24hrs. The extravasation efficiency of **C.** PC3 cells and **D.** PC3M cells +/- SEM has been plotted. (* represents $p < 0.05$ based on multi-variant analysis comparing to vehicle).

Figure 3-6 A.



Kruskal-Wallis Test $p < 0.0001$

Dunn's multiple comparison test

Vehicle vs TAK-441:	$p > 0.05$
Vehicle vs Dotx:	$p < 0.05$
Vehicle vs Combo:	$p < 0.001$
TAK-441 vs. Dotx:	$p > 0.05$
TAK-441 vs. Combo:	$p < 0.01$
Dotx vs. Combo:	$p < 0.05$

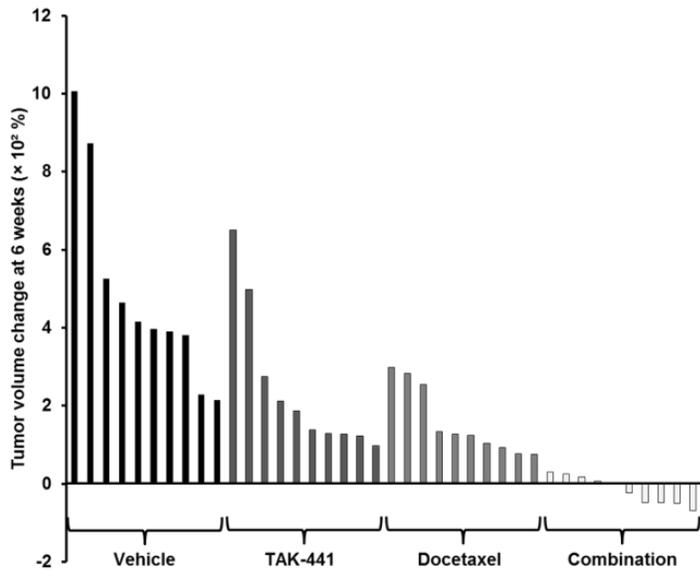


Figure 3-6 B.

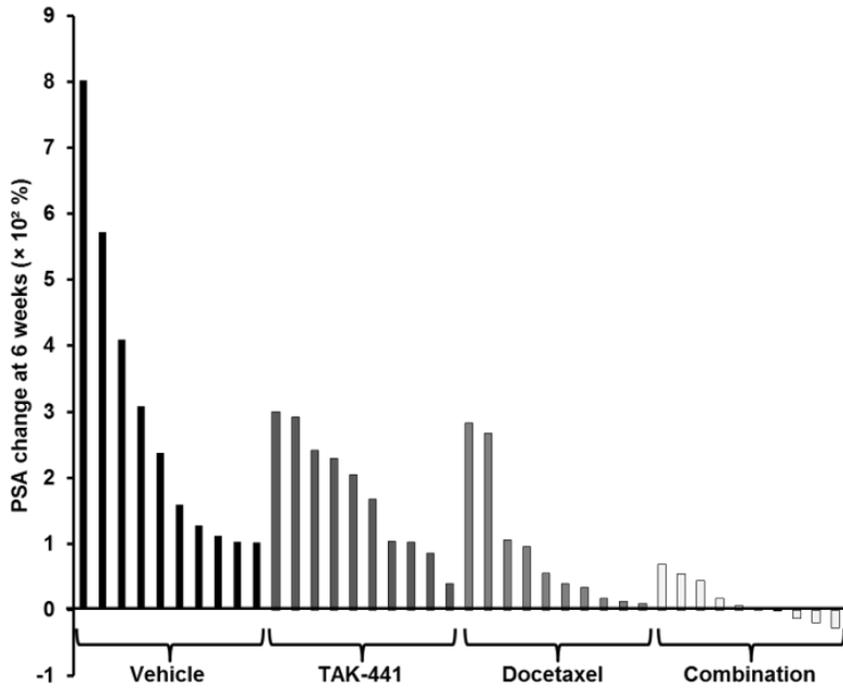
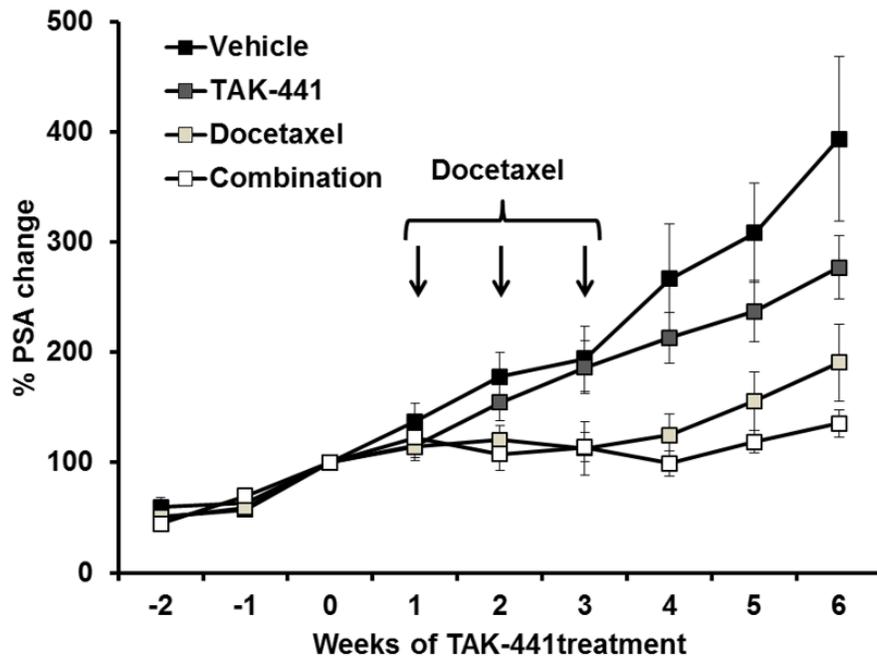
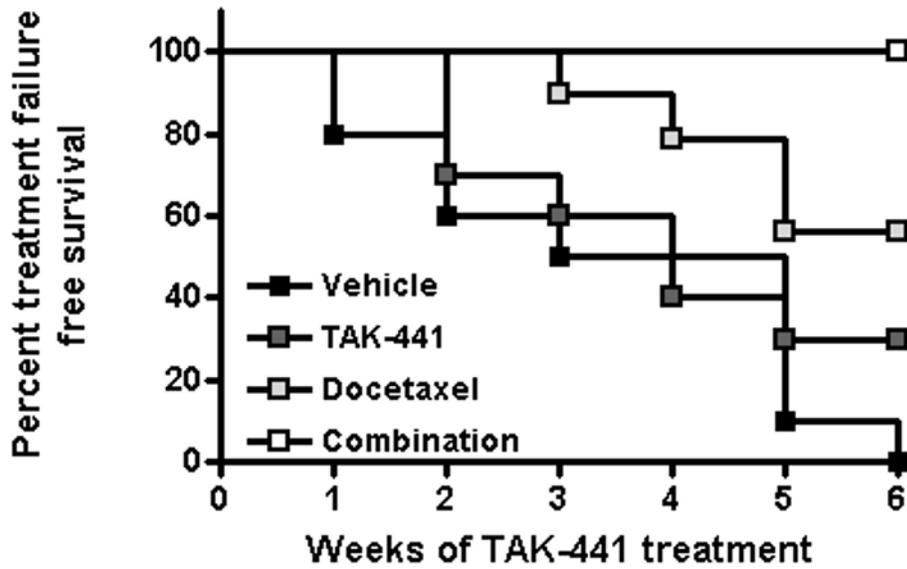


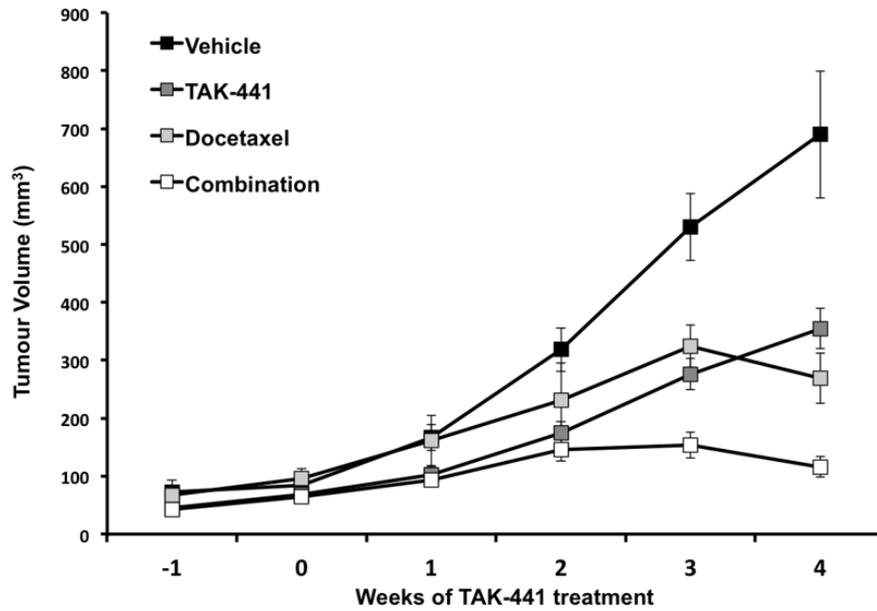
Figure 3-6 C.



•Logrank Test

Comparison	P value
Vehicle vs. TAK-441	ns P=0.2779
Vehicle vs. Docetaxel	** P=0.0069
Vehicle vs. Combination	*** P<0.001
TAK-441 vs. Docetaxel	ns P=0.1296
TAK-441 vs. Combination	** P=0.0012
Docetaxel vs. Combination	* P=0.0221

Figure 3-6 D.



Kruskal-Wallis Test $p < 0.0001$

Dunn's multiple comparison test

Vehicle vs TAK-441:	$p > 0.05$
Vehicle vs Dotx:	$p > 0.05$
Vehicle vs Combo:	$p < 0.001$
TAK-441 vs. Dotx:	$p > 0.05$
TAK-441 vs. Combo:	$p < 0.01$
Dotx vs. Combo:	$p < 0.05$

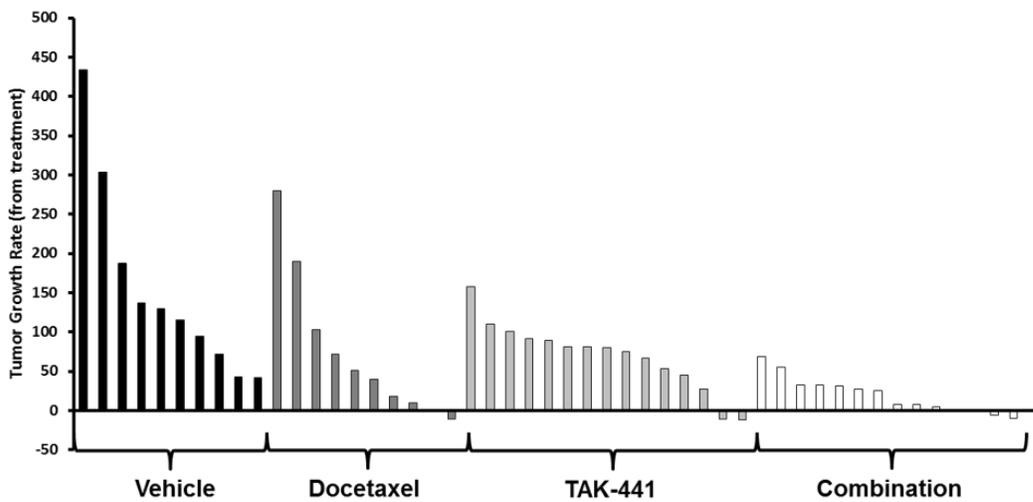


Figure 3-6. Smoothened antagonist in combination with Docetaxel delayed growth and PSA recurrence in LNCaP CRPC xenograft model and suppressed growth and progression of PC3 xenograft model.

A. LNCaP cells were inoculated subcutaneously and when tumour volume or PSA exceeded 200 mm³ or 60ng/ml respectively, mice were castrated. When PSA reached pre-castrate levels, the animals were randomly placed in 4 treatment groups of vehicle (0.5% methylcellulose/H₂O), 25 mg/kg TAK-441 orally treated once daily, 15 mg/kg Docetaxel IP once a week for 3 weeks, and combination of TAK-441 and Docetaxel. Treatments were initiated when PSA increased to pre-castration levels (CRPC model). Data points represent % tumour volume change compared to treatment initiation time-point +/- SEM. Kruskal-Wallis test results and waterfall plot showing percent change in each tumour volume at 6 weeks included. **B.** Serum samples were obtained from tail vein of mice once weekly to measure serum PSA. Data points represents % PSA change compared to start of treatment +/- SEM. Waterfall plot showing percent change of PSA for each tumour after 6 weeks. **C.** Kaplan Meier analysis in which treatment failure is determined by the time required for serum PSA levels to double post treatment. Logrank test results provide comparison between all treatment groups. **D.** PC3 cells were inoculated subcutaneously and when tumour volume exceeded 100 mm³, mice were randomly placed into 4 groups of vehicle, TAK-441, Docetaxel, and combination of TAK-441 and Docetaxel as previously specified. Data points represent mean tumour volume of each group of mice +/- SEM. Kruskal-Wallis test results and waterfall plot showing percent change in each tumour volume at 4 weeks included.

Chapter 4: Conclusion

4.1 Cancer therapeutics

In previous decades, the key aspects of cancer treatment have been the use of surgery, radiation, chemotherapy or combinations of these to attempt to control this deadly disease. Both chemotherapy and radiation target one of the inherent qualities of cancer, the aspect of uncontrolled growth, proving valuable tools in cancer treatment. But where one type of cancer responds well to a certain treatment, other types quickly develop resistance and develop into more aggressive subtypes. In PCa, about 30% of the cases recur and/or metastasize after primary therapies, where the disease is managed primarily by AR pathway inhibitors, aiming to reduce the circulating levels of androgens or block their binding to the AR [336]. The effectiveness of these therapies are known to be temporary due to emerging resistance mechanisms related to the AR, most commonly observed as progression to CRPC where AR-signalling is reestablished [337]. The majority of clinically used AR-targeted drugs, such as bicalutamide and enzalutamide [338], inhibit the AR by competing with androgens at the androgen binding site. This site is prone to mutations that convert anti-AR drugs from antagonists to agonists and in the case of AR mutation (F876L) can also drive resistance to AR antagonists such as enzalutamide [101, 339, 340], which are enriched in CRPC. Furthermore, many studies indicate that PCa cells can express constitutively active, ligand-independent AR splice isoforms, thus rendering most of the antiandrogens ineffective [341, 342]. In addition to the AR-related resistance mechanisms, treatment with AR pathway inhibitors comes with significant morbidities [343, 344]. Due to the ineffectiveness of current drugs, there is a pressing need for new therapeutics to treat metastatic and resistant PCa including CRPC.

As our understanding of cancer has increased there has been an intensified focus on targeting the signalling pathways that the cancer cells depend on for their aberrant abilities. These include some of the therapeutic breakthroughs and success stories in cancer treatment such as trastuzumab in HER2⁺ breast cancer and imatinib in BCR-ABL translocations in acute myeloid leukemia, as well as targeting the AR in PCa [345]. The discovery of the teratogenic compound cyclophosphamide offered an alternative strategy in targeting cancer, from treating uncontrolled growth, to targeting the abnormal signalling pathways that underlie the many properties of this evasive disease. There are increasing studies implicating a role for an aberrant activation of Hh signalling in the development and progression of PCa [244]. However, the exact role of Hh signalling and its mode of action are still to be discerned. The research summarized in this thesis has focused on addressing a controversy in the field on whether the mode of Hh action in PCa is paracrine, autocrine, a combination of both, or a progression of one to the other, as well as its role in PCa progression and its inhibition as a possible therapeutic modality in the advanced stages of this disease.

4.2 Hh mode of action

During prostate development, Hh activation occurs through epithelial-stromal signalling, in a paracrine fashion, leading us to believe that the same mechanism is recapitulated in PCa. Several reports confirm that Hh ligands secreted by tumour cells signal to the stroma, leading to tumour growth and survival [212, 265, 268, 281, 314, 315]. However, there are also studies demonstrating an autocrine requirement for Hh signalling in which the tumour cells both produce and respond to the ligand [205, 213, 241, 280]. In our study we observed a transition from a paracrine AR dependent model to an autocrine CRPC model. A clear understanding of this adaptive response and the mode of Hh action in different disease states allows this pathway to be optimally utilized as a therapeutic target during the progression of PCa. The switch to autocrine

signalling in CRPC, creates a self-sustaining Hh pathway activation, leading to an elevated level of downstream targets. Several studies report a positive correlation with high levels of Hh signalling and tumour grade and stage [205, 239, 240]. In our study, we examined the expression of Hh signalling in therapy naïve, NHT, and CRPC PCa patient specimens. SHH, GLI1, and GLI2 were significantly elevated in CRPC compared to benign and therapy naïve samples. More importantly, there have also been associations made between elevated Hh signalling and poor prognosis, including correlation between SHH, PTCH, SMO, and GLIs and Gleason score [240, 244]. In our patient cohort, we observed a significant correlation between high SHH, GLI1, and GLI2 levels and PSA recurrence when compared to low levels of the same markers. Understating this transition in Hh pathway signalling in PCa and the prognostic indicators it provides as the disease progresses to CRPC and metastatic PCa will be key in effective and impactful use of Hh pathway antagonists.

4.3 PCa models to study Hh signalling

A number of PCa models have been used to assess the role of Hh signalling in PCa. We show that the androgen responsive lines, LNCaP and C4-2 produce both SHH and DHH and that the androgen receptor-negative lines, DU145 and PC3 predominantly express SHH, indicating that these lines generally recapitulate the expression profile for these Hh ligands in cancer progression. As we moved forward, we mainly utilized the two well-established PCa cell lines, the LNCaP and PC3 models. The LNCaP model captures the early stages of AR-dependent PCa, which can be manipulated through AR deprivation to mimic the transition to CRPC. The PC3 model recapitulates the more advance stage of PCa, CRPC, and itself and its variants have shown to demonstrate some metastatic traits. These models provide great tools in ascertaining the role of Hh signalling in PCa progression and also examining its mode of action. As described previously,

there has been a clear disparity in the reported mode of action of Hh signalling in PCa, with early reports of autocrine Hh signalling in PCa [205, 213, 241, 280] versus those who demonstrated a more developmentally centric paracrine model [265, 268, 281, 314, 315]. There are confounding factors that undermine some of the reported findings. The use of cyclopamine as the only Hh inhibitor utilized, weakens some of the findings due to demonstrated non-specific effects and toxicity [238, 282]. In addition, while early studies conclude that cyclopamine decreased growth of several PCa cell lines [205, 213, 280], were suggestive of an autocrine mechanism, later reports demonstrated that this inhibition of PCa cell line proliferation is not accompanied by the expected changes in expression of the endogenous target genes, GLI1 and Ptch1 [265, 281]. Our results demonstrate that while cyclopamine suppressed LNCaP growth when cultured in FBS-containing media, viability was unaffected when cultured in CSS-containing media. This result is consistent with observations of Sirab et al. [280] indicating that cyclopamine-responsiveness may be regulated by androgen receptor activation. In our studies, we utilized a highly specific and selective SMO inhibitor, TAK-441 to antagonize the Hh pathway.

In our LNCaP model, we observed no change in cell viability or Hh expression when treated with TAK-441 *in vitro*. Although SHH levels were elevated under CSS culture conditions, no change was detected with TAK-441 treatment. In our LNCaP xenograft model, we observed significant reduction in tumour volume and PSA levels treated with TAK-441 post castration, demonstrating the first pre-clinical therapeutic effect of the selective SMO antagonist, TAK-441 on castrate resistant progression in PCa. LNCaP xenografts from TAK-441-treated mice exhibited down-regulation of murine Hh target genes, GLI1, GLI2 and Ptch1, but not of their human orthologues. These results indicate that LNCaP cells represent a paracrine model of Hh signalling in PCa, in which the selective impact of TAK-441 on LNCaP xenograft growth is due to up

regulated SHH expression by the tumours in response to castration and the subsequent activation of Hh signalling in tumour-associated murine stroma. Although these results set the basis for understanding the role of Hh pathway in AR dependent PCa, further investigation of the precise nature of the stromal-mediated events remain to be defined. A more in depth examination is required on the interaction of the Hh pathway with target genes such as angiogenic molecules (e.g., VEGF, ANG1-2), epithelial-mesenchymal transition regulators (e.g., SNAIL, MMP9), and other developmental signalling pathways (e.g., Wnt), as well as a more extensive understanding of non-canonical Hh signalling in PCa [177, 241, 244, 269]. Establishing robust models to study the paracrine signalling *in vitro* is a key limitation; from establishing three-dimensional culture models, which are significantly under-utilized in today's research, to finding the correct stromal components such as cancer associated fibroblasts and urogenital sinus mesenchymal cells, to reproducibly identifying secreted factors from the stromal compartment [65, 346-348]. Designing biologically accurate co-culture models is a constant struggle. The majority of the co-culture models used require separate compartments, which do not truly mimic the tumour-stromal interaction and introduces limitations. In addition, one of the issues we faced in our studies is the dilution of the secreted factors in an *in vitro* setting, which abrogates and disrupts the true impact of some of the signalling pathways studied. Recent use of micro-channel and nanotechnology as well as advances in imaging modalities can solve some of these limitations [283, 306]. In addition, there are some limitations in utilizing a subcutaneous xenograft model for this study when examining tumour-stromal interaction. Although LNCaP subcutaneous tumours are interestingly quite vascularized, subcutaneous tumours usually have a poor vascular supply, which may directly affect the tumour microenvironment as well as produce an exaggerated hypoxic state. This alongside the lack of proper stromal compartment, will require great care in interpreting findings

obtained. Although there is no ideal model for such a study, there may be few models in PCa that could address some of these limitations. One such model is prostate tissue recombinants engrafted under the renal capsule of mice to study the role of mesenchymal-epithelial interactions in both normal development and prostate tumorigenesis [349]. Hayward and colleagues generated tissue recombinants utilizing human prostatic fibroblasts or fetal rat UGM as the stromal component. On that note, The Rowley laboratories, developed a tissue recombination model utilizing normal human prostate stromal cells along with LNCaP cells, injected subcutaneously in mice. This model enhanced tumorigenesis and created tumors of malignant epithelial cells admixed with stromal cells with enhanced vascularity [350]. In these models, both cellular compartments can be manipulated, providing greater insight into the tumour stromal interaction. Another clinically relevant model is the patient derived PCa xenografts. Wang and colleagues, engrafted histologically intact human PCa tissue in SCID mice, with a graft recovery rate of 95% at the sub-renal capsule sites. This model becomes a valuable tool for investigating preclinical PCa therapeutics [351]. In addition, this group was able to propagate these grafts and generate several well-characterized transplantable xenograft lines, including a few lines derived from a metastatic PCa lesion that are invasive and metastasize to distant organs [352]. These alongside with other genetically engineered mouse models are ideal tools to study the role of Hh signaling in PCa.

In our model of advanced, AR negative PCa, the PC3 cell line, we observed a different Hh pathway modality. The SHH levels were constitutively high compared to the LNCaP model, which also led to an elevated GLI2 gene and protein expression. Hh pathway inhibition by TAK-441 down-regulated SMO and GLI2 protein expression and GLI2 gene expression. Hh pathway activation was also assessed through a luciferase reporter assay, wherein TAK-441 significantly decreased activation. From these results we can postulate that there is an adaptive response of the

disease where it transitions from paracrine AR dependent state, in which it relies on stromal signalling, to an autocrine CRPC state, in which it can produce the SHH ligand and activate the pathway without the need of stromal compartment. As discussed earlier, this transition to an autocrine mode of action creates a self-sufficient Hh signalling tumour, which significantly increases SHH ligand as well as GLI1 and GLI2, creating a positive feedback loop and in turn constitutively upregulates the pathway. This bypass pathway circumvents AR signalling and recapitulates AR growth and survival signals, leading to a more aggressive and potentially metastatic tumour and a worsened prognosis for the patient.

4.4 Role of Hh signalling in PCa migration, invasion, and metastatic potential

Hh signalling has been shown to be involved in cell motility as well as inducing metastatic potential. In normal adult physiology, Hh signalling has also been identified as an important factor in wound healing, which may be due to its effect in promoting angiogenesis as well as inducing motility to close the wound [198-200]. In addition, Hh signalling has been shown to mediate EMT, which facilitates cell motility and aids in the invasive properties of metastatic PCa. Recent reports demonstrated that, inhibition of Hh signalling decreased cell motility, invasion and migration of PCa cells through suppression of EMT [253]. Hh signalling may also contribute to PCa metastasis to bone, as bone marrow stromal cells are responsive to Hh ligands, which stimulate bone remodelling [254]. These findings led to further examining the role of the Hh pathway in advanced PCa and the effect of its modulation in migration, invasion, and metastatic potential. Tak-441 significantly inhibited PC3 migration in a wound-healing assay as well as a three-dimensional trans-well migration.

Although these migration studies provide insights in the role of Hh pathway in effecting motility, they are not as biologically relevant when assessing metastatic potential *in vitro*. In a trans-well matrigel invasion assay both cyclopamine and TAK-441 at a dose as low as 5 nM, significantly inhibited PC3M invasion. One of the major limitations in studying invasive and metastatic potential in PCa is the lack of reproducible and biologically relevant models *in vivo*, which recapitulate the metastatic nature of the disease itself. Various techniques are available for experimental metastasis such as intrafemoral, heart, and tail vein injection [328, 329], however, a clear understanding of their limitations is required for interpreting the results. None of these models truly captures metastasis, yet a snapshot of one of the key processes involved. Metastasis is a complex, multistep adaptive process involving cell motility and extracellular matrix breakdown, intravasation into the surrounding vasculature, extravasation, and proliferation in a novel niche [353]. Majority of findings in this study and the potential erroneous use of metastatic potential mainly refers to the initial step, cell motility. Cell motility is driven by a cyclic interplay of actin polymerization, cell adhesion, and acto-myosin contraction. Although this cycle can be well documented in a 2-D cell culture setting, the speed and character of cell motility *in vivo* is very different. The high speed of movement and the amoeboid motility cannot be observed in a culture setting [354]. Even the use of immune compromised animal models significantly alters this invasive characteristic, as the presence of immune cells have been shown to significantly alter cancer cell motility and invasive behaviours through creating a pro-metastatic microenvironment by producing cytokines and growth factors [355]. The next step is intravasation into blood or lymphatic vasculature which involves directional movement towards the vessels and production of factors such as VEGF to induce angiogenesis and in doing so, also create a “leaky” vessel [356]. This step is completely ignored in some of the *in vivo* models discussed above, as the cancer cells

as directly injected into the vasculature. In addition, it is postulated that the increased cortical actomyosin required in initial cell motility can withstand greater mechanical stress and protect the metastatic cells from the shear stress within the vessels, which will be lacking in cells that are injected from a culture dish into the vessel of an animal [357]. Majority of metastatic cells lodge in the capillaries and through the assistance of leukocytes as well as activation of local chemoattractants, extravasate into secondary tissue sites [358]. However, the majority of the protocols used for the current *in vivo* PCa metastatic studies require a large number of cells to be injected, which are trapped in capillary beds of the lung, liver, and bone depending on the injection site, causing congestion and leaky vessels, with minimal proof of extravasation through the endothelial cells of the vasculature. These limitations undermine the clinical relevance of some of the metastasis models currently utilized in PCa and create a need in developing novel and robust techniques to study metastasis both *in vitro* and *in vivo*. In the meanwhile, extra care needs to be taken in interpreting the findings from these studies.

In a pilot tail vein injection study using PC3M cells that constitutively expressed luciferase, we observed development of lung and liver mets in 10 of 10 control animal and 7 of 10 TAK441-treated animals. Since TAK did not completely suppress formation of these lesions, we considered whether we would be able to distinguish a 2 –fold difference in lesion formation. Using the variance of overall luminescence intensity of our control and TAK treated groups, a power analysis indicated that over 100 animals would be required to have an 80% confidence that we could measure a statistically significant change. We therefore assessed another animal-model of metastasis, the CAM assay. To establish a clear link between the Hh pathway modulation and the initial metastatic event, the extravasation of the PCa cells out of the lumen of the vasculature and into the surrounding stroma, we decided to use the *ex vivo* CAM assay technique with distinct

fluorescent tags and spinning disk confocal microscopy, wherein we were able to visualize both PC3 and PC3M cells as they transitioned through the endothelial lumen and out of the vasculature and inhibit this process with TAK-441. Interpreting the results of any of the aforementioned assays should be done with great care as they only highlight a subset of the metastatic characteristics of cancer. Development of biologically relevant models that mimic the metastasis of PCa to the bone will be a critical step in better understanding the most critical aspect of this disease. Our *in vitro* and *ex vivo* results demonstrate that disruption of the Hh pathway through inhibition of SMO hinders PCa cell migration, invasion and metastatic potential. These results shed light on the impact of Hh signalling in PCa and provide the insights in incorporating Hh targeted therapeutics as part of a treatment modality for advanced PCa patients.

4.5 Hh pathway combinatory treatment and clinical trials

Hh pathway modulation has been implicated in affecting PCa xenograft growth in a variety of models and has been used as a combination therapy with AR and MEK inhibitors [309, 310]. In addition, recent findings demonstrate the involvement of Hh pathway activation in acquired chemo-resistance [311, 312]. These along with our findings pertaining to CRPC progression led to examining the efficacy of Hh inhibition in combination with taxanes in a CRPC xenograft model. To further clarify the utility of Hedgehog pathway inhibitors as a therapeutic modality, we established two xenograft models of CRPC and treated the tumours with TAK-441 in combination with docetaxel. The LNCaP recurrent CRPC xenograft model, best mimics the biological progression of the disease from AR dependent PCa to CRPC, with the ability to monitor PSA levels, as is done in the clinic. The PC3 CRPC xenograft model is more aggressive, with a much faster rate of tumour growth as compared with the LNCaP model. In both models, TAK-441 in combination with docetaxel significantly inhibited tumour growth compared to either treatment

alone and in the LNCaP model it significantly increased PSA treatment failure free survival. By performing both these CRPC xenograft studies, we were able to provide substantial evidence for the role of Hh inhibitors in combination with docetaxel as a viable treatment approach. The limitations of such *in vivo* studies are their inherent nature in being a poor correspondence with clinical outcomes. These xenograft studies are incredible tools in studying a target, a pathway, or a drug in a complex system, yet they seldom provide a gauge in the efficacy of the treatments being tested in a large cohort clinical study.

There are currently no published data evaluating the combination of chemotherapy and Hh inhibition in patients with prostate cancer; however, a pilot trial of vismodegib in combination with gemcitabine in metastatic pancreatic cancer did not demonstrate an enhanced clinical response compared with gemcitabine alone despite achieving intratumoral Hh downmodulation [333]. Another drug of interest is itraconazole, an antifungal drug inhibiting SMO at a site distinct from other SMO antagonists [359]. The clinical experience with itraconazole in prostate cancer derives from a single randomized phase II study conducted in men with metastatic CRPC [290]. This study targeted patients with resistance to conventional hormonal therapies with none of the patients receiving prior cytotoxic chemotherapy. Although the low dose arm did not demonstrate a beneficial response, the high-dose arm was associated with clinical activity yielding a delayed PSA progression of 48% at 24 weeks. Given the potential clinical benefits seen with high-dose itraconazole in the CRPC setting and the relatively mild toxicity profiles in early-phase trials of more potent SMO inhibitors, there has been increased interest in evaluating the clinical utility of these inhibitors in men with prostate cancer. To this end, two trials, one in the neoadjuvant setting prior to radical prostatectomy (NCT02111187), and one in men with metastatic CRPC (NCT02115828), are currently accruing patients to determine the effect of small-molecule SMO

inhibitors on Hh pathway activity in the tumour and microenvironment directly [303]. Another trial, phase Ib, building on reported data supporting reversal of chemo-resistance with Hedgehog inhibition, investigating the safety and tolerability of combining sonidegib with docetaxel in men with docetaxel-resistant CRPC is planned (NCT02182622) [303].

There is great promise in the targeted inhibition of the Hh pathway as a therapeutic approach to PCa, yet due to the development of possible resistance as seen in medulloblastoma and basal cell carcinoma, combination with other therapies may be a better approach. In order to allow for the development and testing of rational combinations of Hh inhibitors with other agents, a better understanding of the interactions between the Hh pathway and other signalling pathways within prostate cancer cells is needed. Several strategies appear promising. For example, activation of Hedgehog signalling in hepatocellular carcinoma protected tumor cells against ionizing radiation, while Hh inhibition can radiosensitize tumours including non-small cell lung cancer and pancreatic cancer [360]. This potential synergy has not yet been evaluated in prostate cancer. Another pathway of interest is the PI3K-mTOR signalling pathway, which is altered in approximately half of primary prostate tumours and nearly all cases of metastatic CRPC [361]. In addition, the Hh pathway may be an integral treatment approach in multi-drug resistant stages of this disease.

To devise a robust treatment approach including combination with other modalities requires an even greater understanding of this pathway. The role of non-canonical Hh signalling in PCa is still unclear. The expression of primary cilia on PCa cells seems to be another controversial topic and the mode of activation of the pathway in the absence of these cilia is untouched. The stromal compartments involved and the secretory factors in the microenvironment that enhances Hh signalling needs to be ascertained. With all the unknowns, targeting the Hh pathway in CRPC

patients that demonstrate an elevated Hh signalling profile may control progression of the tumour and more importantly may hinder advancement to a metastatic disease.

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